

IgY technology: Methods for developing and evaluating avian immunoglobulins for the *in vitro* detection of biomolecules

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Abstract

The term “IgY technology” was introduced in the literature in the mid 1990s to describe a procedure involving immunization of avian species, mainly laying hens and consequent isolation of the polyclonal IgYs from the “immune” egg yolk (thus avoiding bleeding and animal stress). IgYs have been applied to various fields of medicine and biotechnology. The present article will deal with specific aspects of IgY technology, focusing on the currently reported methods for developing, isolating, evaluating and storing polyclonal IgYs. Other topics such as current information on isolation protocols or evaluation of IgYs from different avian species are also discussed. Specific advantages of IgY technology (*e.g.*, novel antibody specificities that may emerge *via* the avian immune system) will also be discussed. Recent *in vitro* applications of polyclonal egg yolk-derived IgYs to the field of disease diagnosis in human and veterinary medicine through *in vitro* immunodetection of target biomolecules will be presented. Moreover, ethical aspects associated with animal well-being as well as new promising approaches that are relevant to the original IgY technology (*e.g.*, development of monoclonal IgYs and IgY-like antibodies through the phage display technique or in transgenic chickens) and future prospects in the area will also be mentioned.

Key Words: Animal welfare; Polyclonal IgYs; Egg yolk; IgY technology; Relevant-to-IgY-technology approaches; *In vitro* immunodetection techniques

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Core Tip: IgY technology has been widely used during the last decades, especially as a means for the efficient *in vitro* immunodetection of biomolecules in various fields of research and disease diagnosis. Despite the very promising relevant new approaches,

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there is still space to further exploit the original IgY technology due to functional, practical, and ethical reasons/advantages associated with the unique features of IgYs, the highly efficient isolation of large amounts of IgYs from the immune egg yolk, and the avoidance of animal bleeding, respectively.

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INTRODUCTION

The term "IgY technology" was introduced in the 1990s to describe a procedure consisting of immunization of birds, especially laying hens, in order to produce polyclonal antibodies of the Y class (IgYs). IgYs can be isolated in large quantities from "immune" egg yolk (thus avoiding the animal bleeding procedure, which is stressful for an animal) and has been applied to various fields of biotechnology and biomedicine[1-3]. To date, IgYs developed in poultry and isolated from the egg yolk as aforementioned have been and are still being used as specific laboratory tools, especially for detecting biomolecules in biological specimens through various *in vitro* techniques (and also as *in vivo* immunotherapeutic agents).

The origins of the IgY technology can be traced back many years, *i.e.* at the end of the 19th century, when Klemperer observed that immunized hens (*Gallus domesticus*) generated antibodies that were present in the egg yolk[2-4]. Subsequently, a new type of immunoglobulin was found in the blood and egg yolk of birds (also in lungfish, amphibians and reptiles), which was called IgY[3,5]. Actually, birds, which do not produce colostrum like mammalian organisms do, use the yolk of their eggs as a very effective source of antibodies through which they can transfer humoral immunity to their offspring, until the latter develops fully mature immune system[6]. Transfer/accumulation of IgY from blood to/in the egg yolk, which is realized by a selective transport mechanism in avian mature oocytes and mediated by specific receptor(s)[7-9], enables the non-invasive isolation of antibodies and eliminates the need to bleed the animal. Isolation and subsequent application of egg yolk-derived antibodies minimize animal suffering and this meets at least one of the three main requirements for animal welfare, *i.e.* "Reduction," "Replacement," "Refinement," as they have been summarized in the "3Rs principle"[10]. As a consequence, in 1996 the European Centre for the Validation of Alternative Methods to animal testing (ECVAM) strongly recommended avian antibodies as alternative to mammalian ones[1]. In parallel, in the mid 1990s the term "IgY technology" was introduced in the literature, as already mentioned; in 1999, the IgY technology was approved as an alternative method for supporting animal welfare by the Veterinary Office of the Swiss Government[3].

Egg yolk is composed mainly of water, which accounts for approximately 50% of its weight, and contains many important nutrients and preservatives, since it serves the role of a protective chamber for the hen embryo. The dry weight of egg yolk is composed mostly by lipids (67%) and also proteins (33%). Egg yolk proteins are distributed between granules and plasma, in which granules are suspended. Granule proteins are divided into α - and β -lipovitellins (70%), phosvitin (16%), and low-density lipoproteins (12%), whereas plasma proteins include α -, β - and γ -livetins and low-density proteins[11]. A precursor of the major egg yolk proteins is vitellogenin, consisting of vitellogenin I (molecular weight [MW]: 260 kDa), vitellogenin II or major vitellogenin (MW: 246 kDa), and vitellogenin III (MW: 210 kDa)[12-14]. IgYs, which are the main constituent of γ -livetins, are among the most important and most abundant egg yolk proteins[11].

IgY is considered to be the functional equivalent and evolutionary precursor of mammalian IgG and probably of mammalian IgE[15]. Due to this functional and evolutionary relationship, some researchers use the term (avian) IgG instead of IgY; however, the first articles in the field have put emphasis on the distinct differences between IgG and IgY and strongly suggested use of the term IgY[5]. In addition to IgYs, there are two more avian immunoglobulin classes, avian IgM and IgA, which are

similar to mammalian IgM and IgA. Mammalian equivalents of IgE and IgD have not been found in hens[16].

Like mammalian IgG, IgY is composed of two heavy (H) and two light (L) polypeptide chains, which are organized in the Y-shaped characteristic “unit,” and contains two identical binding sites for the antigen. However, the structure of IgY is actually different than that of IgG and this results in distinct properties, as well. The nucleotide sequence corresponding to the hen epsilon (“ ν ”) heavy chain has revealed that the molecule contains four constant and one variable Ig heavy chain domains; the additional domain (C ν 2) has been conserved in mammalian IgE, but “transformed” into the flexible hinge region in mammalian IgG. As a consequence, the IgY molecule has higher molecular mass (approximately 180 kDa), than mammalian IgG (approximately 160 kDa). Moreover, the Fc part of IgY has a different carbohydrate content compared to the Fc part of IgG. An intact Fc part is necessary for the transfer of IgY from blood serum to egg yolk. In ducks an alternatively spliced form of IgY, the so-called IgY Δ Fc, is also present. This variant lacks the Fc region and is mainly found in the blood serum. Hen as well as ostrich and pigeon express only the full-length version of IgY. In some birds, including hen, duck, zebra finch and ostrich, only a single κ light-chain locus has been found. The bursa of fabricius is the site in which immature B-cells are differentiated into mature and competent B-cells, while the spleen is the organ in which plasma cells, *i.e.* the antibody-producing cells, proliferate and memory cells are located. IgY’s heavy and light chain loci consist of single functional V, D, and J genes; in addition to the single functional V genes, there are several pseudo-V genes that lack the usual transcription-regulatory and signal-recognition sequences and are not functional. The antibody diversity in avian organisms is mainly achieved by the so-called gene conversion, through which 10 to more than 120 base pairs from not functional pseudo-genes are transferred to the functional V gene[3,16,17].

The distinct structural features of IgY offer several functional advantages to this unique immunoglobulin type, rendering IgY a versatile and invaluable *in vitro* tool in biotechnology research and in disease diagnostics. Moreover, many reports have suggested *in vivo* application of IgYs in various fields of immunotherapy. The advantages of IgYs include: high potential for developing specific IgYs against conserved mammalian proteins due to the evolutionary distance between mammals and birds, avoidance of activating the mammalian (including human) complement system and reaction with mammalian Fc receptors, ability to isolate substantial amounts of IgYs from immune egg yolks, and avoidance of animal bleeding, which fulfills the “refinement” ethical requirement, as already mentioned[3,18,19].

In the last several decades, more complicated technologies associated with the original IgY technology have emerged, such as the development of avian monoclonal antibodies *via* hybridoma and recombinant techniques, mainly through the phage display technique[20]. Although the above antibodies are IgYs (or IgY-like) immunoglobulins and therefore have all (or part of) the consequent advantages, they are isolated from the supernatant of suitable cell cultures and are not egg yolk-derived. Thus, strictly speaking and at least in our opinion, the techniques leading to the development of monoclonal IgYs cannot be classified as a part of the original IgY technology. On the other hand, transgenic chickens[21] have been used for the production of recombinant proteins, including recombinant antibodies (mostly human/humanized ones), which can be isolated mainly from egg white and are recommended especially for *in vivo* therapeutic applications. Though the aforementioned antibodies have not gained wide application yet and their development and evaluation are considered outside the main scope of the present article, they are considered very promising and will be briefly presented.

The present review article will focus on specific aspects of the original IgY technology, such as immunization of laying hens, isolation of the IgYs developed from the immune egg yolk and consequent immunochemical evaluation. Various recent applications of polyclonal IgYs to the *in vitro* immunodetection of various biomolecules will be also presented and discussed.

DEVELOPMENT AND EVALUATION OF EGG YOLK-DERIVED POLYCLONAL IGYS

General aspects

IgY technology has produced a large number of valuable immunochemical tools for biotechnology and medicine since the 1990s. Various parameters that are associated

with and can affect the results of the IgY technology have been reported in the literature such as housing and breeding conditions, line, age, and stage of development of the immunized birds[2,3,18,22]. Laying hens are the avian organisms of choice (*e.g.*, White Leghorn and Rhode Island Red hens) and are used for immunization to produce polyclonal IgYs throughout their egg-laying period. Other types of poultry such as duck, goose, ostrich, and quail have been referred to in the literature, though to a lesser extent[23-26]. Normal hen lines and conventional housing, *e.g.*, in suitable cages[27], are usually adequate to produce IgYs for research purposes; however, when the IgYs are to be applied as human therapeutics, the use of specific pathogen-free hens is considered necessary[1,3]. Administration of specific food supplements during hens' breeding, *e.g.*, carnitine, has been proposed in the literature as a means to improve overall yield of IgY production, but the results are often contradictory[28].

Immunizing protocols

Parameters that may influence the immune response include antigen nature and dose, use of adjuvants, route of administration, and overall immunization schedule[3].

Both, complex antigens, *e.g.*, whole viruses, bacteria and parasites[29-33] and individual biomolecules, *e.g.*, large proteins[34,35], or small peptides conjugated to a suitable carrier protein, such as keyhole limpet hemocyanin (KLH)[36,37], have been used to stimulate development of specific IgYs in hens. Our team tried to develop IgYs against various antigens, including a recombinant protein of high molecular mass, *i.e.* human kallikrein-related peptidase 6[38] as well as peptides of the alpha- and beta-thymosin families isolated from mammalian tissues or synthetically prepared, either conjugated to KLH or not[39-41]. Moreover, we successfully developed IgYs against the olive fruit fly pheromone by using a KLH-conjugate of the synthetic hapten (\pm)- β -[3-(1,7-dioxaspiro[5.5]undecane)] propionic acid[27].

The antigen dose may be also critical, since too much or too little antigen can lead to an undesirable immune response[2]. Different antigen doses have been reported in the literature. In an early study, a good immune response in hens immunized with bovine serum albumin at doses as low as 0.1-1.0 μ g was reported[3]; however, higher doses ranging from 10 to 1000 μ g (most often 50-100 μ g) have been also used. Information on the doses administered to immunize hens has been presented in a recent review[18].

The outcome of immunization is commonly enhanced by the addition of adjuvants, though successful immunization of hens without any adjuvant has been reported in the literature[3]. Among the adjuvant preparations that have been described till now, Freund's complete adjuvant (FCA) is still considered the gold standard for generating high levels of antibodies in animals, including birds. FCA is a suspension of heat-killed and dried mycobacteria (*Mycobacterium spp.*) in mineral oil, which forms a depot at the injection site and slows down release of the antigen in the host organism, so that long-lasting exposure and a non-specific immune stimulation is achieved. The main problem of FCA is the severe tissue damage it causes at the injection sites, which is usually attributed to the mycobacteria it contains. Although a few studies have reported that hens can better tolerate FCA, in comparison with mammals, other studies have reported contradictory data. For this reason, Freund's incomplete adjuvant, *i.e.* Freund's adjuvant without mycobacteria, is commonly used for booster injections as an alternative to FCA, which is used only in the first immunization[18]. Use of other adjuvants has been also reported in IgY technology, such as the so-called, mineral-oil based Montanide adjuvant, along with oligodeoxynucleotides containing C-phosphate guanosine motifs, which are promising immunoenhancing agents[28]. Research in the area of developing new adjuvants, both highly efficient and animal welfare-friendly, is being continued.

Regarding the route of administration, several approaches have been tested. The most recommended one is the intramuscular injection (i.m.) into the breast tissues[3, 29,34,42] in multiple sites; i.m. administration in the thigh muscle has been also used but according to some reports it may cause lameness and has to be avoided[18]. Subcutaneous (s.c.) immunization in the neck has also been used by several research teams including our team[27,38,39]. As reported, i.m. immunization in breast muscle is most suited especially for young hens[18]. The intravenous (i.v.) route has been very rarely used, without adjuvants and at a very slow injection rate. The intraperitoneal (i.p.) route, which Klempner has followed in his pioneer work, is hardly used these days. Efforts to immunize hens orally have been also reported[3,30,43].

The interval between the first and second (*i.e.* first booster) immunization is considered a critical parameter in hen immunization protocols. Age of hens when first immunized might also be an issue. However, literature information on these specific parameters substantially varies. A general recommendation is to administer a booster immunization when the IgY titer reaches a plateau or begins to decrease[44]. If a

substantial decrease in the antibody titer has been observed, further immunizations can be performed during the entire laying period, which lasts about 72 wk[22], to keep the antibody titer adequately high for as long as possible, in many cases for more than 150 d[18]. As presented in a previous review[3], some immunization protocols have recommended antigen administration at days 0, 14 and 28, or once a week for 7 consecutive weeks, or at day 0, week 10, and week 15. Other protocols propose hen immunization at 10-d intervals, but in most cases, the interval between the first and second immunization is at least 4 wk, while another protocol has reported achievement of a high antibody titer by prolonging the boost interval from 14 to 42 d. Intervals among booster injections also vary, averaging 2 wk[3]. Our team has mainly used 3-mo-old hens for immunization; the first booster was administered 2 wk after first immunization, while several further injections were given, mostly at 4 wk-intervals[27].

In general, eggs are collected weekly, starting 1 wk prior to the first immunization (pre-immune eggs), eggshells are washed or sanitized with 70% ethanol, and stored at 4°C until further processed for IgY isolation. Lyophilization of egg yolk has also been reported, resulting in an easy-to-mix egg yolk powder with an extended shelf-life[45].

Immunization with plasmids: “DNA-designed” IgYs

Apart from the conventional administration of antigen along with adjuvant, the so-called genetic immunization has also been applied to the production of polyclonal IgYs in avian species[46]. In this context, avian organisms have been immunized with plasmid vectors encoding target eukaryotic antigens, *e.g.*, bovine interferon gamma protein[47], prokaryotic antigens, *e.g.*, *Botulinum* toxin A1[48], as well as viral ones, *e.g.*, antigens from Andes virus[23]; in almost all cases, antibodies Y of desired immunochemical characteristics have been developed. A great deal of effort has been put forth to improve DNA-vaccine delivery, and consequently, immunogenicity. The “gene gun” method has garnered much attention, since low doses of DNA applied *via* a gene gun can efficiently induce high antibody titers against the antigen encoded[49]. Although DNA immunization is a promising approach, which prevents costly and tedious preparation of purified antigens or presence of adjuvants in the immunization mixture, it has not yet gained wide application.

Isolation of IgYs from the egg yolk

Hen eggs are an excellent source of high amounts of antibodies[19]. An average hen can lay roughly 325 eggs a year. Given that according to the literature an egg can produce 60-150 mg[50], or 40-80 mg total IgY per egg yolk depending on the hen’s age [22], one hen can roughly produce 20-40 g of antibodies a year, with 1%-2% up to 10% of the antibodies being antigen-specific[18,51], which is much higher than that obtained from mammalian sources[11].

Isolation of IgYs from the “immune” egg yolk in pure form is a challenging task. Several protocols have been described, with different characteristics in terms of total yield, purity, duration, convenience, and cost[42]. IgYs account for about 3%-5% of the egg yolk proteins, which are dispersed in a lipid emulsion combined with lipoproteins and glycoproteins. Consequently, in most cases, IgY isolation involves, first, removal of lipids to form a water-soluble fraction (“de-lipidation” step), and then precipitation of the antibodies that are present in the water-soluble fraction with various approaches[3,18].

The most commonly used de-lipidation technique is the “acidified water dilution method”[52], using 6- to 10-fold dilution of egg yolk in water at pH ~5, incubation for several hours at 4°C and then centrifugation, at the end of which the lipid portion is precipitated and the water-soluble portion is collected in the supernatant. Alternatively, lipid removal has been successfully performed by means of organic solvents (chloroform, acetone, isopropanol)[53,54], acids (caprylic acid, trichloroacetic acid)[55] or natural gums (polyanionic polysaccharides, *e.g.*, xanthans)[56]. A de-lipidation solution containing polysaccharides (such as pectin, λ -carrageenan, carboxymethylcellulose, methylcellulose, and dextran sulfate) has been also reported[57].

After de-lipidation, various IgY extraction methods that can be applied either to laboratory- or to large-scale production have appeared in the literature; these methods can be divided into three main groups, *i.e.* precipitation, chromatographic and filtration methods.

Precipitation methods, involving precipitation of IgYs with saturated salt solutions, such as ammonium sulfate, sodium sulfate or sodium chloride[58,59], polyethylene glycol (PEG)[60], caprylic acid[61,62] and carrageenan[63]. PEG precipitation usually involves, first, dilution of egg yolk in phosphate-buffered saline (PBS) containing PEG 6000 at low concentration (3.5%), to facilitate de-lipidation. After centrifugation, the

supernatant is treated with 8.5% and then with 12% PEG 6000 to precipitate IgYs[30]. Among the above methods, ammonium sulfate precipitation is considered one of the best choices for the scale-up purification of IgY[11], with most suitable concentration of ammonium sulfate being 20%[55]. Extracted IgY samples usually undergo a final dialysis step, usually against PBS, to eliminate residual salts from the extraction procedure.

Chromatographic methods include low-pressure chromatography[30], ion exchange chromatography[52,59], high-resolution chromatography through multicolumn systems[64] and affinity chromatography[65]. Conventional affinity chromatographic methods using protein A or protein G columns cannot be performed for IgY purification, since IgYs, contrary to IgGs, do not bind to protein A or G[66]. Other types of ligands are therefore required, such as the elastin-like polypeptide-tagged immunoglobulin-binding domain of streptococcal protein G[67]. Still other ligands, such as IgY-binding peptides screened from a random peptide library, have been also proposed as a means of IgY purification[68]. IgY can also be purified with thiophilic adsorption chromatography, usually through commercially available IgY-extraction columns[18,69]. However, chromatographic techniques are generally expensive and impractical for the large-scale production of antibodies, while they have not proven to substantially increase purity of the final product when compared with simple precipitation methods, such as ammonium sulfate precipitation.

Filtration methods, such as ultrafiltration[52,70], have also been used as IgY extraction methods.

As reported, a combination of the aforementioned methods, *e.g.*, a combination of PEG precipitation with affinity chromatography[22] or ammonium sulfate precipitation with ion exchange chromatography[59], can further increase the purity of the IgY preparation. Moreover, sequential precipitation with 31% ammonium sulfate and 12% PEG resulted in IgY antibodies of more than 95% purity without any loss in immunoreactivity[64].

Despite the numerous protocols described in the literature, the most popular isolation strategy of IgYs from immune eggs involves a de-lipidation step, in which IgY is extracted in the supernatant after treating the egg yolk with 10 volumes of acidic water and a subsequent precipitation step, in which IgY precipitates with ammonium sulfate or PEG, at suitable concentrations[30].

Storage

According to the literature, after their isolation, IgYs can be stored for long periods (from a few months to a few years), preferably at -20 °C[22,71], since they are considered reasonably stable biomolecules, like mammalian IgGs[72]. IgY is stable at pH 4-9 and up to 65 °C in aqueous solutions. The addition of stabilizing reagents or high concentrations of salts can further increase resistance of the IgY molecule; *e.g.*, heat stability could be increased up to 70 °C by the addition of sugars, such as 30% sucrose, trehalose or lactose[3]. Useful information concerning earlier findings on the stability and storage conditions of IgYs has appeared in recent review articles[73]. Freeze-drying has been used to facilitate long storage of IgYs[74], though some researchers have reported that freeze-drying may lead to some loss of antigen-binding activity of IgY[45]. Lyophilization of proteins, including IgYs, induces freezing and dehydration stresses, which may result in protein structural changes or even unfolding [75]. Therefore, the addition of cryoprotectants and lyoprotectants has been recommended to protect IgYs during lyophilization[45]. Our team has recently evaluated IgYs that were developed against a KLH-conjugate of the polypeptide prothymosin alpha many years ago and kept as lyophilized powder at -30 °C. As revealed, the IgYs have kept immunoreactivity and were successfully applied to a specific enzyme-linked immunosorbent assay (ELISA) for prothymosin alpha[76].

Evaluation of egg yolk IgYs

Protein concentration: Determination of protein concentration in IgY extracts is usually performed before proceeding to further IgY evaluation. Total protein concentration in IgY extracts has been determined mainly with the Bradford method (indicative references[30,34,35,38,42]), the Lowry method[58] and the bicinchoninic acid protein assay[77]. In addition, protein concentration was assessed with ultraviolet absorption at 280 nm, according to the Lambert-Beer law (indicative references[29,32,33,57,76]).

Purity: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is considered the gold standard technique and has been widely used to assess the purity of the egg yolk-isolated IgYs (indicative references[30,33,34,62,78]). SDS-PAGE

separation under non-reducing or reducing conditions would reveal one or two protein bands, the latter corresponding to heavy and light IgY chains.

Western blotting has been used complementarily with SDS-PAGE to confirm the presence and assess purity of IgYs isolated from immune egg yolks (indicative references[31,33,62,78]). Visualization of the specific protein bands is performed mainly through a color or chemiluminescence development.

In a few cases, additional analytical methods such as high-performance liquid chromatography[57] have also been used to evaluate the purity of IgYs.

Immunoreactivity: The immunoreactivity of egg yolk-derived IgYs is evaluated with well-established immunochemical methods such as dot-blot and ELISA. Dot-blot can be actually considered as a simplified form of ELISA offering mostly qualitative results. Nevertheless, it is a fast, easy, and low-cost technique that may provide useful information and has, therefore, been used by several researchers to evaluate immunoreactivity of IgYs[32,34,36,39,76]. In most cases, however, evaluation of IgY immunoreactivity involves determination of titer against the target antigen through non-competitive ELISAs (indicative references[32,35,45,76,78]). Moreover, other immunochemical characteristics of the isolated IgYs are assessed, such as putative cross-reactivity with various substances through competitive ELISAs (indicative references[31,36,39]). It should be noted that till now and despite the numerous new technologies introduced in the field, ELISA remains the gold standard method for evaluating the basic immunological characteristics of any antibody developed, independently of the antibody class or the production method.

IN VITRO APPLICATION OF EGG YOLK-DERIVED POLYCLONAL IGYS TO THE DETECTION OF BIOMOLECULES

IgY is considered an excellent tool especially for developing *in vitro* methods to detect biomolecules of interest in biological specimens for a series of reasons. First, the evolutionary distance between mammals and birds may facilitate generation of specific IgYs against conserved mammalian proteins, since avian organisms possess a different antibody repertoire than that of mammals and the epitope spectrum of avian antibodies is potentially larger/different than that of mammalian IgGs including novel specificities[19,64]. Second, IgY does not activate the mammalian (including human) complement system and does not react with mammalian Fc receptors; this feature has rendered IgYs an ideal *in vitro* reagent, especially for immunoassays designed to detect biomolecules in human blood serum[64]. Third, substantial amounts of IgY can be isolated from egg yolks; as already mentioned (isolation of IgYs from the egg yolks), one hen can produce 20-40 g of IgY in 1 year, 1%-10% of which is antigen-specific. This advantage of egg yolk IgY is accompanied by other practical superiorities, such as low animal care cost, ease of isolation of antibodies from the egg yolk with simple biochemical methods and overall low production cost[73]. These advantages along with the large-scale facilities currently available render production of egg yolk-derived IgYs, a technically feasible and efficient procedure at industrial level. Some other positive characteristics of IgYs have been reported in the literature, *e.g.*, they can be developed even when hens are immunized with very small amounts of the corresponding antigens[64,71] or that they show higher specificity, binding affinity, and avidity for their targets in comparison with mammalian IgGs[38,73], although other reports have shown controversial data[3]. Last but not least, in the list of IgY advantages is that use of egg yolk IgYs is especially desirable from an ethical aspect of view, concerning refinement of animal experimentation, as already mentioned. Some recent indicative applications of IgYs to the *in vitro* detection of biomolecules (as well as whole viruses/microorganisms) have been summarized and presented in Table 1. Lately, specific IgYs have been developed and used for the immunodiagnosis of pandemic coronavirus disease-2019 (COVID-19)[79], while non-specific IgY has been used to form/visualize the “control line” in point-of-care *in vitro* tests that detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigens[80].

Table 1 *In vitro* applications of polyclonal IgYs

Target biomolecule(s)	<i>In vitro</i> immunochemical technique	Proposed field of application	Ref.
Major surface antigen of <i>Toxoplasma gondii</i> (SAG1)	Latex agglutination assay	Diagnosis of Toxoplasmosis	Cakir-Koc <i>et al</i> [132], 2020
Protein A of <i>Staphylococcus aureus</i>	Immunocapture PCR assay	Detection of <i>Staphylococcus aureus</i> in food samples, skin and nasal swabs	Kota <i>et al</i> [133], 2020
Peptides/proteins present in detoxified western Russell's viper venom	Paper-based microfluidic immunochromatographic test	Differential diagnosis of Russell's viper envenomation	Lin <i>et al</i> [134], 2020
SARS-CoV-2 antigen	Fluorescence immunochromatographic rapid-antigen test	Diagnosis of COVID-19	Porte <i>et al</i> [79], 2020
Antigens present in total saline extract of <i>Taenia crassiceps</i> metacestodes	ELISA	Detection of neurocysticercosis	daSilva <i>et al</i> [32], 2020
Antigens present in total saline extract of <i>Ancylostoma ceylanicum</i>	ELISA	Diagnosis of Hookworm infection	Souza <i>et al</i> [135], 2020
Non-glycosylated synthetic oligopeptides of <i>Dermatophagoides</i> group I allergens	Immuno-dot blot assay (with the use of IgY-colloidal gold nanoparticles conjugates)	Detection of indoor dust mite allergens	Egea <i>et al</i> [136], 2019
Antigens present in whole bacterial suspension of formalin- and heat-inactivated <i>Salmonella typhimurium</i> and <i>Salmonella enteritidis</i>	<i>In vitro</i> immunochemical techniques	Diagnosis of infection with <i>Salmonella typhimurium</i> and <i>Salmonella enteritidis</i>	Esmailnejad <i>et al</i> [26], 2019
Antigenic extracts of <i>Strongyloides venezuelensis</i> infectious filariform larvae and parthenogenetic females	ELISA	Diagnosis of human strongyloidiasis	deFaria <i>et al</i> [33], 2019
Antigens present in total saline extract of <i>Ascaris suum</i> adult life forms	Tissue indirect immunofluorescence assay & ELISA	Diagnosis of human ascariasis	Lopes <i>et al</i> [31], 2019
Free prostate specific antigen	ELISA	Diagnosis of human prostate cancer	Lupicka-Słowic <i>et al</i> [137], 2019
Antigens (capsid proteins VP2 & VP3) present in beta-propiolactone-inactivated enterovirus 71	Fluorescence sensor assay	Diagnosis of hand-foot-and-mouth disease caused by enterovirus 71 infection	Nie <i>et al</i> [138], 2019
<i>Fusarium verticillioides</i> 97K exoantigen	ELISA	Detection of <i>Fusarium verticillioides</i> (and prediction of fumonisin contamination) in poultry feed	Omori <i>et al</i> [139], 2019
Recombinant purified catalytic domain of Karilysin	ELISA	Evaluation of karilysin (<i>i.e.</i> an enzyme secreted by the periodontopathogen <i>Tannerella forsythia</i>) as a biomarker for the diagnosis of periodontitis	Skottrup <i>et al</i> [34], 2019
Fumonisin B1	Lateral flow immunoassay	Detection of fumonisin B1 and fumonisin B2 in maize	Tran <i>et al</i> [140], 2019
Synthetic extracellular peptide of matrix-2 protein of influenza A virus, conserved in all strains	Latex agglutination assay	Diagnosis of infection with Influenza A virus	Budama-Kılınc <i>et al</i> [141], 2018
Sulfamethazine (SMZ)	ELISA, FPIA	Detection of veterinary drug residues (SMZ) in milk	Liang <i>et al</i> [142], 2018
Native calf adenosine deaminase (ADA)	ELISA	Evaluation of ADA as a cancer biomarker	Lupicka-Słowic <i>et al</i> [143], 2018
Nucleoprotein of influenza A virus	Immunocytochemistry, Immunohistochemistry	Diagnosis of infection with influenza A virus	da Silva <i>et al</i> [144], 2018

ADA: Adenosine deaminase; COVID-19: Coronavirus disease-2019; ELISA: Enzyme-linked immunosorbent assay; FPIA: Fluorescence polarization immunoassay; PCR: Polymerase chain reaction; SAG1: Surface antigen 1 of *Toxoplasma gondii*; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SMZ: Sulfamethazine.

RELEVANT APPROACHES AND FUTURE PROSPECTS

Monoclonal IgYs

Since the late 1980s many efforts have been directed toward development and use of avian monoclonal antibodies (mAbs) for research, diagnostic, and therapeutic purposes, because avian mAbs may combine the advantages of avian immuno-

globulins with those of monoclonality, *i.e.* precise characterization and continuous production. Initially, several technical difficulties have emerged; even after technical problems have been addressed and avian mAbs have been produced by hybridomas [81,82], the hybridoma technology has not gained wide application, because it is considered a complex, time-consuming and low-yield process by many researchers. By contrast, antibody-engineering methods proved to be the most frequently techniques used for the production of chicken mAbs. Actually, chicken provides an ideal basis for generating large immune antibody fragment libraries as compared to most mammalian species. In chickens, the large and diverse antibody repertoire is generated by gene conversion, in which segments from non-functional V pseudogenes located upstream are inserted into the rearranged gene, and somatic hypermutation. Since gene conversion has not been observed at the 5'- and 3'-ends of the rearranged gene, it is possible to perform real-time reverse transcription polymerase chain reaction (PCR) of the V-region repertoire with a single pair of primers[20,72]. Of the various recombinant antibody fragments, the full-length single-chain variable fragment (scFv) is the most commonly used. For construction of the scFv antibody library, total RNA is isolated from the spleen cells of immunized or non-immunized chicken and reverse-transcribed into cDNA. Then the variable heavy and light chain domain genes of immunoglobulin antibody cDNA are amplified by PCR and properly assembled to form the full-length scFv fragments, which resemble a functional Fv region. Then the scFv genes are cloned into suitable vectors to construct an antibody-expressing library [83]. Currently, phage display systems are the most often applied recombinant methods for generation and isolation of chicken mAbs[83,84]. In phage display methods, genetically-engineered phages that are capable of displaying recombinant fragments of antibodies on their coat surface can undergo several rounds of biopanning and re-propagation in *Escherichia coli* to enrich for clones exhibiting specific binding. Many IgY-scFv were produced with the phage display method combined with *in vitro* selection technologies, either by research groups[84-87] or companies that provide custom services for the development of monoclonal antibodies Y[88,89]. Among recent technologies reported for producing and isolating monoclonal IgYs is the gel encapsulated microenvironment assay, which is capable of "cross-examining" the entire population of splenic B cells from immunized chickens[90]. In an effort to produce mAbs suitable for *in vivo* administration in immunotherapy, the highly immunogenic constant region of chicken IgYs has been replaced with that of human to generate chicken-human chimeric antibodies[91]. Moreover, humanization of chicken scFvs has been successfully performed using the complementarity-determining region (CDR)-grafting strategy, which replaces human CDRs with chicken CDRs while retaining the human framework region residues, and followed by further optimization when necessary[92,93]. On the other hand, chimeric chicken-mouse or mouse-chicken recombinant mAbs have been produced and their characteristics have been studied[94,95].

Antibodies produced by genetically modified chickens (transgenic chickens)

Over the last decades, significant progress was made in generating recombinant proteins, including mAbs for therapeutic applications, in genetically modified chickens[21,96]. Difficulties in generating modified chickens are mainly attributed to the complex structure of the chicken zygote and the different organization of the chick embryo in comparison to mammals. To successfully generate genetically modified chickens, different methods have been used to achieve stable genomic integration of transgenes and the highest efficiency of germline transmission[97], including direct DNA microinjection into the chicken zygote[98] and use of viral vectors for gene transfer, which is the first applied and considered one of the most successful methods. Thus, the first genetically modified chicken was generated by the insertion of retroviral foreign DNA delivered by avian leukosis virus successfully integrated to the germline[99]. Since then, various viral vectors have been used to generate transgenic chickens for the production of recombinant proteins[100-102] including mAbs[103]. Among these, lentiviral vectors have been reported to offer specific advantages, including ability to transduce dividing and non-dividing cells, a relatively large transgene capacity and the apparent resistance of transduced cells to gene silencing [104]. Lentiviral vectors have been used to introduce transgene constructs comprising suitable sequences from the ovalbumin gene to direct synthesis of associated proteins to oviduct[105]. Despite the fact that the use of viral vectors improves germline transmission, the size limitation of the transgene and the lack of possibility of precise edits remain as drawbacks. One of the most effective approaches to produce transgenic chickens is the *in vitro* transfection of avian cell lines, such as primordial germ cells (PGCs) and embryonic stem cells (ES), the clonal selection and reinsertion

into the embryo leading to fully transgenic progeny in the next generation[106-108]. Following this approach, production of human mAbs in the egg white of chimeric transgenic chickens with the use of genetically modified ES cells carrying ovalbumin expression vectors was successfully performed for the first time; however, although a high amount of functional mAb was produced in the egg white, no transgenic offspring were initially obtained[107]. Heritable transgenic chickens capable of producing mAbs in their egg whites were generated using transfected PGCs with a gene construct designed to express the mAb in chicken oviduct magnum[108]. Specific gene editing of PGCs could be improved using genome-editing tools, such as transcriptional activator-like effector nucleases[109] and the clustered regularly interspaced short palindromic repeats-associated protein 9 system (CRISPR/Cas9 system)[110,111]. CRISPR/Cas9 has been used to generate transgenic chickens for the production of recombinant proteins in the white egg[111], including mAbs[112], or exhibiting resistance to pathogens[113]. Another recent promising approach is the replacement of the chicken immunoglobulin variable regions by human V regions and use of synthetic pseudogene arrays in order to produce affinity matured antibodies in transgenic chickens, called OmniChickens; OmniChicken can thus generate antibodies of basically human sequence, which retain the epitope repertoire of chicken immunoglobulins[114].

DISCUSSION

IgY technology has produced a great number of valuable immunochemical tools for biotechnology and medicine since 1990's. Various parameters that are associated with and can affect the results of the IgY technology have been reported in the literature, such as the immunization procedure. One of the most important parameters is the extraction/purification protocol used for isolating the IgYs from the egg yolk. Several methods of isolation and purification of IgYs from "immune" egg yolks have been reported, as already mentioned; the choice of a specific method depends on several criteria, such as desired yield, purity and final application of the IgYs along with cost and scale of extraction. The most popular isolation strategy consists in a de-lipidation step, in which IgY is extracted in the supernatant after treating the egg yolk with 10 volumes of acidic water, and a subsequent precipitation step, in which IgY precipitates with ammonium sulfate or with PEG, at suitable concentrations[30].

Our team have used the acidified water dilution method followed by precipitation with 19% sodium sulfate[39] or with 8.5% and 12% PEG 6000[27] for the isolation of IgYs from immune egg yolks. SDS-PAGE and western blot analysis of IgYs isolated with sodium sulfate precipitation has revealed a protein impurity with MW of ~35 kDa, which underwent liquid chromatography tandem mass spectrometry analysis and was proposed to be identical with the C-terminal fragment of vitellogenin II precursor protein[39]. The same impurity was also observed by other researchers, who had followed a different isolation protocol involving precipitation with PEG 6000[22]. As later shown[115,116], IgY from hen egg yolk occurs as a complex with peptides, named yolkin, which exhibit immunoregulatory and other biological activity. Yolkin contains several peptides with an apparent molecular weight ranging between 1 and 35 kDa. As reported, purified yolkin constituents are homologous with some fragments of the C-terminal region of vitellogenin II; more specifically, yolkin fractions of MW > 16 kDa are glycoproteins corresponding to the amino acid sequence of vitellogenin II starting at position 1572 aa[12,117]. In our hands, presence of the above impurity did not seem to interfere with the efficiency of IgYs as specific *in vitro* immune reagents.

As already mentioned, egg yolk IgYs have been thought to be superior to mammalian IgGs for *in vitro* applications. The *in vitro* efficiency of IgYs may be questioned only under rare conditions, *e.g.*, due to the putative presence of anti-hen antibodies in biological samples of specific individuals who have been sensitized to hen egg yolk[72]; however, to what extent IgY-specific antibodies may occur in human individuals remains to be clarified. Exempt from the aforementioned few concerns, IgYs are considered ideal and are being continuously developed and used as invaluable *in vitro* laboratory tools up to now (Table 1).

One of the great advantages of the IgY technology is the enhanced probability of generating specific IgYs against conserved mammalian proteins, since hens may exhibit a different antibody repertoire than that of mammalian organisms. With this in mind, our team has immunized hens against the poorly immunogenic, highly conserved polypeptide prothymosin alpha (ProT α , MW: approximately 12 kDa,

isolated from bovine thymus). The anti-ProT α antibodies Y were isolated from the egg yolk and evaluated through dot-blot and ELISA experiments in parallel with antibodies G isolated from the antiserum of rabbits immunized against the same immunogen. As revealed, not only antibodies G, but also antibodies Y showed hardly detectable titer/affinity for ProT α [39]. The above negative result may be attributed to the fact that ProT α is thought to be highly conserved during evolution and ProT α -homologues have been reported in non-mammalian organisms as well[76,118]. Similarly, hens were immunized against the highly conserved polypeptide thymosin beta4 (T β 4, MW: ~5 kDa, synthetic), either conjugated to KLH (T β 4/KLH) or non-conjugated, leading to IgYs of either relatively high titer or, on the contrary, not-detectable titer, respectively[41]. Interestingly, antibodies Y that we developed against a KLH-conjugate of ProT α (anti-ProT α /KLH IgYs) showed high titer and practically no cross-reactivity with a series of ProT α -fragments, including the N-terminal fragment ProT α [1-28] (also known as T α 1), being therefore highly specific for whole-length ProT α , while the corresponding anti-ProT α /KLH rabbit IgGs did cross-react with T α 1[76]. Moreover, when various synthetic fragments of ProT α or T β 4 were conjugated to KLH and used for immunizing hens and rabbits, the results revealed that specific antibodies Y of hardly detectable titer were obtained; on the contrary, rabbit immunization with the same immunogens led to high-titer antibodies G, specific for ProT α or T β 4, respectively[39,41]. The above results support the assumption that novel antibody specificities may emerge *via* the avian immune system and can be obtained through the IgY technology.

Although IgYs for research applications are mainly produced in hens, other birds have also served this purpose, as already mentioned, including duck[23,119], goose [24], quail[26] and ostrich[25], following immunization and isolation protocols similar to those used for hens[18]. Quail, ostrich and other avian species may provide further advantages in the field of IgY technology, such as convenient housing and breeding conditions (quail[26]) or exceptionally high amounts of IgYs obtained (ostrich[25]). Previously, our team has isolated immunoglobulins Y from the egg yolk of several avian species, including ostrich (*Struthio camelus*) and quail (*Coturnix japonica*); the isolation protocol has been developed in-house and based on the acidified water dilution and the PEG precipitation method. Ostrich and quail immunoglobulins Y were characterized in terms of their molecular weight (SDS-PAGE and western-blotting) and their ability to recognize and bind to a commercially available horseradish peroxidase (HRP)-labeled rabbit anti-hen IgY antibody in an ELISA system[120]. As revealed, the ostrich IgYs could be hardly recognized by the HRP-labeled anti-hen antibody we used, though other researchers reported successful use of commercially available secondary anti-hen antibodies to assess the immunochemical efficiency of specific ostrich IgYs[121]. On the other hand, HRP-labeled secondary anti-ostrich-IgY antibodies have been specially developed and used to evaluate ostrich IgYs with ELISA[25]. According to experimental results of ours[120] and others'[26,122], the quail IgYs could be recognized by the HRP-labeled secondary anti-hen antibody, which indicates that quail and hen IgYs may share at least some homology in immunochemically important structural features[123,124]. Wide availability of secondary antibodies for IgYs originated from avian species other than hens will support further expansion of the IgY technology.

In addition to their unequivocal usefulness as *in vitro* immunodetection reagents, IgYs have been proposed as promising *in vivo* therapeutics, *e.g.*, as an alternative to antibiotics treatment against multi-drug resistant or difficult-to-treat pathogens, since they exhibit *in vivo* pathogen-neutralizing activity, especially in mouth, throat, the respiratory tract and lungs[73]. Moreover, since IgYs are not absorbed by the gastrointestinal tube, they have been proposed as perorally administered immunotherapeutics against various viral, bacterial, and fungal infections of the gastro-intestinal tract, especially in veterinary medicine and fish-cultivation[3]; a limitation in wide therapeutic application of perorally administered IgYs is their reduced stability at low pH[72] and several efforts have been made to address this shortcoming. IgYs have been also proposed as locally administered immunotherapeutics for treating skin and other local infections[3]. Lately, specific IgYs have been developed and used for treatment of the pandemic COVID-19[35,64,66,125]. Overall, despite the new promising technologies emerged, literature on the IgY technology continues to expand, encompassing various applications ranging from *in vitro* immunodetection of biomolecules and *in vitro* immunodiagnostics to *in vivo* immunotherapeutics[18,126].

Though development of monoclonal IgYs cannot be considered as a part of the original "IgY technology", it seems very attractive and will probably be the next big step in the area, since it combines the advantages of mAbs with those of avian IgYs. At the initial phase, production of chicken mAbs had to overcome several technical

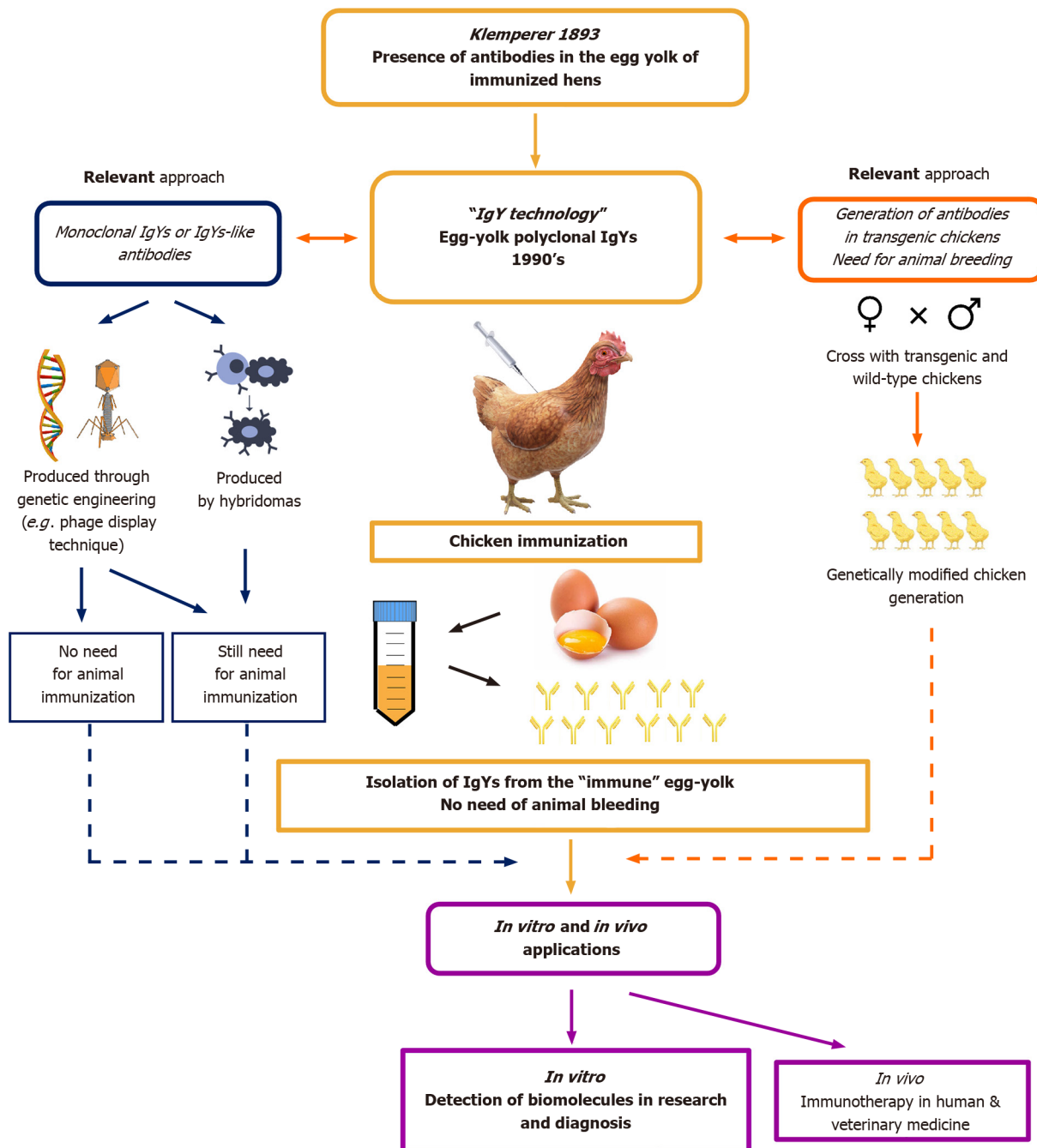


Figure 1 Schematic representation of the main parts comprising the original immunoglobulin Y technology (central axis); promising relevant approaches are also shown (periphery, left and right).

difficulties, including lack of appropriate fusion partners and loss of antibody secreting ability by the hybridoma cells over time[81]; this has been successfully addressed when monoclonal IgYs were generated through combinatorial antibody libraries *via* the phage display methodology[127]. Thus, over the past years, avian libraries have been constructed and several reports on the isolation of avian-derived antibody fragments have been published[20]. The different spectrum of epitopes recognized by the avian immune system may facilitate the development of novel diagnostics, *e.g.*, through targeting highly conserved mammalian proteins, while monoclonality may especially facilitate the development of novel therapeutics for human use, provided that the technology of chimeric avian/human fusions could be fully exploited. One should also keep in mind that recombinant technologies can lead to the generation of monoclonal IgY or IgY-like antibodies circumventing the need for animal immunization[72,83], which is desirable from an ethical point of view concerning the animal welfare.

It is important to remind that the IgY technology was introduced in 1990's as an alternative that could at least partly fulfil the ethics requirements set by the 3Rs principle[1,3]. Recently, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) has recommended that “animals should not be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications any longer”, taking into account the Opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) on the scientific validity of replacements for animal-derived antibodies[128]. As referred to by the ESAC, the 2018 Nobel Prize in Chemistry was awarded “for the phage display of peptides and antibodies”[129,130], which, according to the Committee, proves maturity and supports wide application and full exploitation of the phage display technology in the area of antibody production. The EURL ECVAM recommendation may accelerate transformation/switch of the original IgY technology toward development of monoclonal IgYs through phage display techniques that totally avoid the animal immunization step. Total avoidance of animal immunization will further minimize the risk of zoonotic diseases, which is very low but still present when antibodies are produced in chickens, both wild and transgenic.

HIGHLIGHTS

The avian polyclonal antibodies/IgYs have unique and highly desirable functional features.

The term “IgY technology” describes the procedure involving immunization of avian species, consequent isolation of the polyclonal IgYs from the “immune” egg yolk (thus avoiding bleeding and animal stress) and application of the IgYs to various areas of medicine and biotechnology.

During the last decades the IgY technology has been widely used, especially as a means for the efficient *in vitro* immunodetection of biomolecules in many fields of research and disease diagnosis.

Despite the very promising relevant new approaches, there is still space for further exploiting the original IgY technology, due to specific functional, practical and ethical reasons and/or advantages.

CONCLUSION

Until now, development of polyclonal IgYs through the IgY technology has been widely used as a low cost and highly efficient tool, offering a lot of advantages and thus gaining wide application mainly in the *in vitro* immunodetection of biomolecules in biological specimens. Since polyclonal antibodies exhibit some unique functional qualities[131], there is still space for performing research to improve different aspects of the IgY technology. On the other hand, the original IgY technology may “merge” with relevant highly promising approaches, eventually leading, *e.g.*, to worldwide application of non-animal-derived recombinant IgYs or IgY-like immunoglobulins, which, among other benefits, will fulfil strict ethical requirements concerning animal welfare (Figure 1). However, until the practical problems associated with the above-mentioned approaches, *e.g.*, high-cost and/or limited availability of necessary reagents and protocols, have been fully addressed, the original IgY technology still remains a feasible, well-established procedure, in particular for low- and middle-income countries and research laboratories and especially in the field of *in vitro* immunodetection of biomolecules.

REFERENCES

- 1 Schade R, Staak C, Hendriksen C, Erhard M, Hugl H, Koch G, Larsson A, Pollmann W, van Regenmortel M, Rijke E, Spielmann H, Steinbusch H, Straughan D. The production of avian (egg yolk) antibodies: IgY. The report and recommendations of ECVAM workshop 21. *Altern Lab Anim* 1996; 24: 925-934 [DOI: [10.1177/026119299602400607](https://doi.org/10.1177/026119299602400607)]
- 2 Schade R, Behn I, Erhard M, Hlinak A, C Staak C. Chicken egg yolk antibodies, production and application – IgY technology. Berlin: Springer Verlag, 2001
- 3 Schade R, Calzado EG, Sarmiento R, Chacana PA, Porankiewicz-Asplund J, Terzolo HR. Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and

- human and veterinary medicine. *Altern Lab Anim* 2005; **33**: 129-154 [PMID: 16180988 DOI: 10.1177/026119290503300208]
- 4 **Klemperer F.** Über natürliche Immunität und ihre Verwerthung für die Immunisierungstherapie. *Arch Exp Pathol Pharmacol* 1893; **31**: 356-382
 - 5 **Leslie GA, Clem LW.** Phylogen of immunoglobulin structure and function. 3. Immunoglobulins of the chicken. *J Exp Med* 1969; **130**: 1337-1352 [PMID: 5352783 DOI: 10.1084/jem.130.6.1337]
 - 6 **Ulmer-Franco AM, Cherian G, Quezada N, Fassenko GM, McMullen LM.** Hatching egg and newly hatched chick yolk sac total IgY content at 3 broiler breeder flock ages. *Poult Sci* 2012; **91**: 758-764 [PMID: 22334753 DOI: 10.3382/ps.2011-01757]
 - 7 **Murai A, Hamano T, Kakiuchi M, Kobayashi M, Horio F.** Evaluation of a receptor gene responsible for maternal blood IgY transfer into egg yolks using bursectomized IgY-depleted chickens. *Poult Sci* 2020; **99**: 1914-1920 [PMID: 32241471 DOI: 10.1016/j.psj.2019.11.045]
 - 8 **Murai A, Kakiuchi M, Hamano T, Kobayashi M, Tsudzuki M, Nakano M, Matsuda Y, Horio F.** An ELISA for quantifying quail IgY and characterizing maternal IgY transfer to egg yolk in several quail strains. *Vet Immunol Immunopathol* 2016; **175**: 16-23 [PMID: 27269788 DOI: 10.1016/j.vetimm.2016.04.013]
 - 9 **Hamal KR, Burgess SC, Pevzner IY, Erf GF.** Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poult Sci* 2006; **85**: 1364-1372 [PMID: 16903465 DOI: 10.1093/ps/85.8.1364]
 - 10 **Russell WMS, Burch RL.** The principles of humane experimental technique. London: Methuen & Co., 1959
 - 11 **Huang X, Ahn DU.** How Can the Value and Use of Egg Yolk Be Increased? *J Food Sci* 2019; **84**: 205-212 [PMID: 30620779 DOI: 10.1111/1750-3841.14430]
 - 12 **Kazana W, Mitkiewicz M, Ochnik M, Sochocka M, Zambrowicz A, Piechowiak G, Macała J, Miernikiewicz P, Zabłocka A.** Yolkin Isolated from Hen Egg Yolk as a Natural Immunoregulator, Activating Innate Immune Response in BMDM Macrophages. *Oxid Med Cell Longev* 2020; **2020**: 5731021 [PMID: 32509146 DOI: 10.1155/2020/5731021]
 - 13 **Zambrowicz A, Dąbrowska A, Bobak Ł, Szołtysik M.** Egg yolk proteins and peptides with biological activity. *Postepy Hig Med Dosw (Online)* 2014; **68**: 1524-1529 [PMID: 25834095 DOI: 10.5604/17322693.1133600]
 - 14 **Yamamura J, Adachi T, Aoki N, Nakajima H, Nakamura R, Matsuda T.** Precursor-product relationship between chicken vitellogenin and the yolk proteins: the 40 kDa yolk plasma glycoprotein is derived from the C-terminal cysteine-rich domain of vitellogenin II. *Biochim Biophys Acta* 1995; **1244**: 384-394 [PMID: 7599159 DOI: 10.1016/0304-4165(95)00033-8]
 - 15 **Taylor AL, Fabiane SM, Sutton BJ, Calvert RA.** The crystal structure of an avian IgY-Fc fragment reveals conservation with both mammalian IgG and IgE. *Biochemistry* 2009; **48**: 558-562 [PMID: 19115948 DOI: 10.1021/bi8019993]
 - 16 **Härtle S, Magor KE, Göbel TW, Davison F, Kaspers B.** Structure and evolution of avian immunoglobulins. In: Schat KA, Kaspers B, Kaiser P, editors. Avian immunology. 2nd ed. Boston: Academic Press, 2014: 103-120
 - 17 **Sun Y, Wei Z, Li N, Zhao Y.** A comparative overview of immunoglobulin genes and the generation of their diversity in tetrapods. *Dev Comp Immunol* 2013; **39**: 103-109 [PMID: 22366185 DOI: 10.1016/j.dci.2012.02.008]
 - 18 **Pereira EPV, van Tilburg MF, Florean EO, Guedes MIF.** Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review. *Int Immunopharmacol* 2019; **73**: 293-303 [PMID: 31128529 DOI: 10.1016/j.intimp.2019.05.015]
 - 19 **Larsson A, Sjöquist J.** Chicken IgY: utilizing the evolutionary difference. *Comp Immunol Microbiol Infect Dis* 1990; **13**: 199-201 [PMID: 2076606 DOI: 10.1016/0147-9571(90)90088-B]
 - 20 **Lee W, Syed Atif A, Tan SC, Leow CH.** Insights into the chicken IgY with emphasis on the generation and applications of chicken recombinant monoclonal antibodies. *J Immunol Methods* 2017; **447**: 71-85 [PMID: 28502720 DOI: 10.1016/j.jim.2017.05.001]
 - 21 **Bahrani S, Amiri-Yekta A, Daneshpour A, Jazayeri SH, Mozdziak PE, Sanati MH, Gourabi H.** Designing A Transgenic Chicken: Applying New Approaches toward A Promising Bioreactor. *Cell J* 2020; **22**: 133-139 [PMID: 31721526 DOI: 10.22074/cellj.2020.6738]
 - 22 **Pauly D, Chacana PA, Calzado EG, Brembs B, Schade R.** IgY technology: extraction of chicken antibodies from egg yolk by polyethylene glycol (PEG) precipitation. *J Vis Exp* 2011 [PMID: 21559009 DOI: 10.3791/3084]
 - 23 **Brocato R, Josleyn M, Ballantyne J, Vial P, Hooper JW.** DNA vaccine-generated duck polyclonal antibodies as a postexposure prophylactic to prevent hantavirus pulmonary syndrome (HPS). *PLoS One* 2012; **7**: e35996 [PMID: 22558299 DOI: 10.1371/journal.pone.0035996]
 - 24 **Haese N, Brocato RL, Henderson T, Nilles ML, Kwilas SA, Josleyn MD, Hammerbeck CD, Schiltz J, Royals M, Ballantyne J, Hooper JW, Bradley DS.** Antiviral Biologic Produced in DNA Vaccine/Goose Platform Protects Hamsters Against Hantavirus Pulmonary Syndrome When Administered Post-exposure. *PLoS Negl Trop Dis* 2015; **9**: e0003803 [PMID: 26046641 DOI: 10.1371/journal.pntd.0003803]
 - 25 **Adachi K, Handharyani E, Sari DK, Takama K, Fukuda K, Endo I, Yamamoto R, Sawa M, Tanaka M, Konishi I, Tsukamoto Y.** Development of neutralization antibodies against highly pathogenic H5N1 avian influenza virus using ostrich (*Struthio camelus*) yolk. *Mol Med Rep* 2008; **1**: 203-209 [PMID: 21479398 DOI: 10.3892/mmr.1.2.203]

- 26 **Esmailnejad A**, Abdi-Hachesoo B, Hosseini Nasab E, Shakoory M. Production, purification, and evaluation of quail immunoglobulin Y against *Salmonella typhimurium* and *Salmonella enteritidis*. *Mol Immunol* 2019; **107**: 79-83 [PMID: 30665061 DOI: 10.1016/j.molimm.2019.01.012]
- 27 **Neokosmidi A**, Ragoussis V, Zikos C, Paravatou-Petsotas M, Livaniou E, Ragoussis N, Evangelatos G. Determination of natural olive fruit fly pheromone in insect samples by enzyme linked immunoassays. *Talanta* 2008; **74**: 539-546 [PMID: 18371673 DOI: 10.1016/j.talanta.2007.06.015]
- 28 **Marcq C**, Marlier D, Beckers Y. Improving adjuvant systems for polyclonal egg yolk antibody (IgY) production in laying hens in terms of productivity and animal welfare. *Vet Immunol Immunopathol* 2015; **165**: 54-63 [PMID: 25813905 DOI: 10.1016/j.vetimm.2015.02.012]
- 29 **Grando TH**, Baldissera MD, de Sá MF, do Carmo GM, Porto BCZ, Aguirre GSV, Azevedo MI, de Jesus FPK, Santurio JM, Sagrillo MR, Stefani LM, Monteiro SG. Avian antibodies (IgY) against *Trypanosoma cruzi*: Purification and characterization studies. *J Immunol Methods* 2017; **449**: 56-61 [PMID: 28697990 DOI: 10.1016/j.jim.2017.07.002]
- 30 **Amro WA**, Al-Qaisi W, Al-Razem F. Production and purification of IgY antibodies from chicken egg yolk. *J Genet Eng Biotechnol* 2018; **16**: 99-103 [PMID: 30647711 DOI: 10.1016/j.jgeb.2017.10.003]
- 31 **Lopes CA**, de Faria LS, de Sousa JEN, Borges IP, Ribeiro RP, Bueno LL, Rodrigues Ávila VM, Ferreira Júnior Á, Costa-Cruz JM. Anti-*Ascaris suum* immunoglobulin Y as a novel biotechnological tool for the diagnosis of human ascariasis. *J Helminthol* 2019; **94**: e71 [PMID: 31409433 DOI: 10.1017/S0022149X19000701]
- 32 **Silva GBD**, Faria LSD, Lopes CA, Nunes DS, Ribeiro VS, de Sousa JEN, Paiva GCM, Gonçalves-Pires MRF, Borges IP, Santos MM, Ávila VMR, Júnior ÁF, Costa-Cruz JM. Egg yolk immunoglobulin Y as a promising tool to detect immune complexes in neurocysticercosis serum samples. *Trans R Soc Trop Med Hyg* 2020; **114**: 585-592 [PMID: 32484880 DOI: 10.1093/trstmh/traa028]
- 33 **de Faria LS**, de Souza DLN, Ribeiro RP, de Sousa JEN, Borges IP, Ávila VMR, Ferreira-Júnior Á, Goulart LR, Costa-Cruz JM. Highly specific and sensitive anti-*Strongyloides venezuelensis* IgY antibodies applied to the human strongyloidiasis immunodiagnosis. *Parasitol Int* 2019; **72**: 101933 [PMID: 31128257 DOI: 10.1016/j.parint.2019.101933]
- 34 **Skottrup PD**, López R, Ksiazek M, Højrup P, Baelum V, Potempa J, Kaczmarek JZ. An IgY-based immunoassay to evaluate the biomarker potential of the *Tannerella forsythia* virulence factor karilysin in human saliva. *J Immunol Methods* 2019; **469**: 26-32 [PMID: 30880264 DOI: 10.1016/j.jim.2019.03.003]
- 35 **Lu Y**, Wang Y, Zhang Z, Huang J, Yao M, Huang G, Ge Y, Zhang P, Huang H, Li H, Wang W. Generation of Chicken IgY against SARS-COV-2 Spike Protein and Epitope Mapping. *J Immunol Res* 2020; **2020**: 9465398 [PMID: 33134398 DOI: 10.1155/2020/9465398]
- 36 **Grzywa R**, Lupicka-Słowik A, Walczak M, Idzi M, Bobrek K, Boivin S, Gawel A, Stefaniak T, Oleksyszyn J, Sieńczyk M. Highly sensitive detection of cancer antigen 15-3 using novel avian IgY antibodies. *ALTEX* 2014; **31**: 43-52 [PMID: 24270753 DOI: 10.14573/altex.1309181]
- 37 **Lupicka-Słowik A**, Walczak M, Grzywa R, Bobrek K, Łęcka M, Boivin S, Gawel A, Stefaniak T, Oleksyszyn J, Sieńczyk M. Generation and application of polyclonal IgY antibodies specific for full-length and nicked prostate-specific antigen. *Bioanalysis* 2014; **6**: 3197-3213 [PMID: 25529887 DOI: 10.4155/bio.14.172]
- 38 **Sotiropoulou G**, Pampalakis G, Prosnikli E, Evangelatos GP, Livaniou E. Development and immunochemical evaluation of a novel chicken IgY antibody specific for KLK6. *Chem Cent J* 2012; **6**: 148 [PMID: 23216878 DOI: 10.1186/1752-153X-6-148]
- 39 **Klimentzou P**, Paravatou-Petsotas M, Zikos C, Beck A, Skopeliti M, Czarnecki J, Tsitsilonis O, Voelter W, Livaniou E, Evangelatos GP. Development and immunochemical evaluation of antibodies Y for the poorly immunogenic polypeptide prothymosin alpha. *Peptides* 2006; **27**: 183-193 [PMID: 16150512 DOI: 10.1016/j.peptides.2005.07.002]
- 40 **Klimentzou P**, Drougou A, Fehrenbacher B, Schaller M, Voelter W, Barbatis C, Paravatou-Petsotas M, Livaniou E. Immunocytological and preliminary immunohistochemical studies of prothymosin alpha, a human cancer-associated polypeptide, with a well-characterized polyclonal antibody. *J Histochem Cytochem* 2008; **56**: 1023-1031 [PMID: 18711212 DOI: 10.1369/jhc.2008.950956]
- 41 **Livaniou E**, Paravatou-Petsotas M, Bourkoula A, Klimentzou P, Zikos C, Evangelatos GP. Development of anti-thymosin beta-4 IgY-antibodies and their preliminary application to the immunostaining of human breast cancer cells. In: Flegel M, Fridkin M, Gilon C, Slaninova J, editors. Proceedings of the 28th European Peptide Symposium; 2004 Sep 5-10; Prague, Czech Republic. Geneva: Kenes International, 2005: 1125-1126
- 42 **Ren H**, Yang W, Thirumalai D, Zhang X, Schade R. A comparative evaluation of six principal IgY antibody extraction methods. *Altern Lab Anim* 2016; **44**: 11-20 [PMID: 27031600 DOI: 10.1177/026119291604400111]
- 43 **Thibodeau A**, Fravallo P, Perron A, Lewandowski SL, Letellier A. Production and characterization of anti-*Campylobacter jejuni* IgY derived from egg yolks. *Acta Vet Scand* 2017; **59**: 80 [PMID: 29208016 DOI: 10.1186/s13028-017-0346-4]
- 44 **Leenaars M**, Hendriksen CF. Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. *ILAR J* 2005; **46**: 269-279 [PMID: 15953834 DOI: 10.1093/ilar.46.3.269]
- 45 **Vandeputte J**, Martel A, Antonissen G, Verlinden M, De Zutter L, Heyndrickx M, Haesebrouck F,

- Pasmans F, Garmyn A. Research Note: Lyophilization of hyperimmune egg yolk: effect on antibody titer and protection of broilers against *Campylobacter* colonization. *Poult Sci* 2020; **99**: 2157-2161 [PMID: 32241501 DOI: 10.1016/j.psj.2019.11.054]
- 46 Cova L. DNA-designed avian IgY antibodies: novel tools for research, diagnostics and therapy. *J Clin Virol* 2005; **34** Suppl 1: S70-S74 [PMID: 16461227 DOI: 10.1016/s1386-6532(05)80013-7]
- 47 Nikbakht Brujeni G, Jalali SA, Koohi MK. Development of DNA-designed avian IgY antibodies for quantitative determination of bovine interferon-gamma. *Appl Biochem Biotechnol* 2011; **163**: 338-345 [PMID: 20652441 DOI: 10.1007/s12010-010-9042-9]
- 48 Niederstadt L, Hohn O, Dorner BG, Schade R, Bannert N. Stimulation of IgY responses in gene gun immunized laying hens by combined administration of vector DNA coding for the target antigen Botulinum toxin A1 and for avian cytokine adjuvants. *J Immunol Methods* 2012; **382**: 58-67 [PMID: 22580181 DOI: 10.1016/j.jim.2012.05.005]
- 49 Witkowski PT, Bourquain DR, Hohn O, Schade R, Nitsche A. Gene gun-supported DNA immunisation of chicken for straightforward production of poxvirus-specific IgY antibodies. *J Immunol Methods* 2009; **341**: 146-153 [PMID: 19100269 DOI: 10.1016/j.jim.2008.11.008]
- 50 Pauly D, Dorner M, Zhang X, Hlinak A, Dorner B, Schade R. Monitoring of laying capacity, immunoglobulin Y concentration, and antibody titer development in chickens immunized with ricin and botulinum toxins over a two-year period. *Poult Sci* 2009; **88**: 281-290 [PMID: 19151341 DOI: 10.3382/ps.2008-00323]
- 51 Tini M, Jewell UR, Camenisch G, Chilov D, Gassmann M. Generation and application of chicken egg-yolk antibodies. *Comp Biochem Physiol A Mol Integr Physiol* 2002; **131**: 569-574 [PMID: 11867282 DOI: 10.1016/s1095-6433(01)00508-6]
- 52 Akita EM, Nakai S. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. *J Immunol Methods* 1993; **160**: 207-214 [PMID: 8459107 DOI: 10.1016/0022-1759(93)90179-b]
- 53 Bade H, Stegemann H. Rapid method of extraction of antibodies from hen egg yolk. *J Immunol Methods* 1984; **72**: 421-426 [PMID: 6432912 DOI: 10.1016/0022-1759(84)90010-3]
- 54 Bizhanov G, Vyshniauskis G. A comparison of three methods for extracting IgY from the egg yolk of hens immunized with Sendai virus. *Vet Res Commun* 2000; **24**: 103-113 [PMID: 10720096 DOI: 10.1023/a:1006460506303]
- 55 Araújo AS, Lobato ZI, Chávez-Olórtegui C, Velarde DT. Brazilian IgY-Bothrops antivenom: Studies on the development of a process in chicken egg yolk. *Toxicon* 2010; **55**: 739-744 [PMID: 19925817 DOI: 10.1016/j.toxicon.2009.11.004]
- 56 Hatta H, Kim M, Yamamoto T. A novel isolation method for hen egg yolk antibody, "IgY". *Agric Biol Chem* 1990; **54**: 2531-2535 [PMID: 1368596 DOI: 10.1271/bbb1961.54.2531]
- 57 Tong C, Geng F, He Z, Cai Z, Ma M. A simple method for isolating chicken egg yolk immunoglobulin using effective delipidation solution and ammonium sulfate. *Poult Sci* 2015; **94**: 104-110 [PMID: 25542196 DOI: 10.3382/ps/peu005]
- 58 Lee HY, Abeyrathne ED, Choi I, Suh JW, Ahn DU. Sequential separation of immunoglobulin Y and phosphatidylcholine from chicken egg yolk without using organic solvents. *Poult Sci* 2014; **93**: 2668-2677 [PMID: 25085938 DOI: 10.3382/ps.2014-04093]
- 59 Ko KY, Ahn DU. Preparation of immunoglobulin Y from egg yolk using ammonium sulfate precipitation and ion exchange chromatography. *Poult Sci* 2007; **86**: 400-407 [PMID: 17234857 DOI: 10.1093/ps/86.2.400]
- 60 Polson A, Coetzer T, Kruger J, von Maltzahn E, van der Merwe KJ. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol Invest* 1985; **14**: 323-327 [PMID: 4065934 DOI: 10.3109/08820138509022667]
- 61 McLaren RD, Prosser CG, Grieve RC, Borissenko M. The use of caprylic acid for the extraction of the immunoglobulin fraction from egg yolk of chickens immunised with ovine alpha-lactalbumin. *J Immunol Methods* 1994; **177**: 175-184 [PMID: 7822824 DOI: 10.1016/0022-1759(94)90154-6]
- 62 Redwan EM, Aljadawi AA, Uversky VN. Simple and efficient protocol for immunoglobulin Y purification from chicken egg yolk. *Poult Sci* 2021; **100**: 100956 [PMID: 33652537 DOI: 10.1016/j.psj.2020.12.053]
- 63 Tan SH, Mohamedali A, Kapur A, Lukjanenko L, Baker MS. A novel, cost-effective and efficient chicken egg IgY purification procedure. *J Immunol Methods* 2012; **380**: 73-76 [PMID: 22484081 DOI: 10.1016/j.jim.2012.03.003]
- 64 Constantin C, Neagu M, Diana Supeanu T, Chiurciu V, A Spandidos D. IgY - turning the page toward passive immunization in COVID-19 infection (Review). *Exp Ther Med* 2020; **20**: 151-158 [PMID: 32536989 DOI: 10.3892/etm.2020.8704]
- 65 Ntakarutimana V, Demedts P, van Sande M, Scharpé S. A simple and economical strategy for downstream processing of specific antibodies to human transferrin from egg yolk. *J Immunol Methods* 1992; **153**: 133-140 [PMID: 1517583 DOI: 10.1016/0022-1759(92)90315-k]
- 66 Pérez de la Lastra JM, Baca-González V, Asensio-Calavia P, González-Acosta S, Morales-delaNuez A. Can Immunization of Hens Provide Oral-Based Therapeutics against COVID-19? *Vaccines (Basel)* 2020; **8** [PMID: 32872186 DOI: 10.3390/vaccines8030486]
- 67 Xia W, Lu H, Li Y, Cao J, Zhou X, Zhang X, Xia X, Sun H. Purification of chicken IgY by binding capture using elastin-like polypeptide-tagged immunoglobulin-binding domain of streptococcal protein G. *Vet Immunol Immunopathol* 2017; **192**: 13-19 [PMID: 29042010 DOI: 10.1016/j.vetimm.2017.09.002]

- 68 **Khan KH**, Himeno A, Kosugi S, Nakashima Y, Rafique A, Imamura A, Hatanaka T, Kato DI, Ito Y. IgY-binding peptide screened from a random peptide library as a ligand for IgY purification. *J Pept Sci* 2017; **23**: 790-797 [PMID: 28758361 DOI: 10.1002/psc.3027]
- 69 **Hansen P**, Scoble JA, Hanson B, Hoogenraad NJ. Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatography. *J Immunol Methods* 1998; **215**: 1-7 [PMID: 9744742 DOI: 10.1016/s0022-1759(98)00050-7]
- 70 **Hernández-Campos FJ**, Brito-De la Fuente E, Torrestiana-Sánchez B. Purification of egg yolk immunoglobulin (IgY) by ultrafiltration: effect of pH, ionic strength, and membrane properties. *J Agric Food Chem* 2010; **58**: 187-193 [PMID: 19994898 DOI: 10.1021/jf902964s]
- 71 **Larsson A**, Bälöw RM, Lindahl TL, Forsberg PO. Chicken antibodies: taking advantage of evolution--a review. *Poult Sci* 1993; **72**: 1807-1812 [PMID: 8415358 DOI: 10.3382/ps.0721807]
- 72 **Spillner E**, Braren I, Greunke K, Seismann H, Blank S, du Plessis D. Avian IgY antibodies and their recombinant equivalents in research, diagnostics and therapy. *Biologicals* 2012; **40**: 313-322 [PMID: 22748514 DOI: 10.1016/j.biologicals.2012.05.003]
- 73 **Abbas AT**, El-Kafrawy SA, Sohrab SS, Azhar EIA. IgY antibodies for the immunoprophylaxis and therapy of respiratory infections. *Hum Vaccin Immunother* 2019; **15**: 264-275 [PMID: 30230944 DOI: 10.1080/21645515.2018.1514224]
- 74 **Fu CY**, Huang H, Wang XM, Liu YG, Wang ZG, Cui SJ, Gao HL, Li Z, Li JP, Kong XG. Preparation and evaluation of anti-SARS coronavirus IgY from yolks of immunized SPF chickens. *J Virol Methods* 2006; **133**: 112-115 [PMID: 16325277 DOI: 10.1016/j.jviromet.2005.10.027]
- 75 **Emami F**, Vatanara A, Park EJ, Na DH. Drying Technologies for the Stability and Bioavailability of Biopharmaceuticals. *Pharmaceutics* 2018; **10** [PMID: 30126135 DOI: 10.3390/pharmaceutics10030131]
- 76 **Karachaliou CE**, Kostopoulos IV, Vassilakopoulou V, Klimentzou P, Paravatou-Petsotas M, Voelter W, Kalbacher H, Zikos C, Tsitsilonis O, Livanou E. Development of a specific IgY-based ELISA for prothymosin alpha, a bioactive polypeptide with diagnostic and therapeutic potential. *Heliyon* 2019; **5**: e02616 [PMID: 31720448 DOI: 10.1016/j.heliyon.2019.e02616]
- 77 **Yi L**, Qin Z, Lin H, Zhou Y, Li J, Xu Z, Babu V S, Lin L. Features of chicken egg yolk immunoglobulin (IgY) against the infection of red-spotted grouper nervous necrosis virus. *Fish Shellfish Immunol* 2018; **80**: 534-539 [PMID: 29906624 DOI: 10.1016/j.fsi.2018.06.024]
- 78 **Zhu Y**, Ma Y, Lu M, Zhang Y, Li A, Liang X, Li J. Efficient Production of Human Norovirus-Specific IgY in Egg Yolks by Vaccination of Hens with a Recombinant Vesicular Stomatitis Virus Expressing VP1 Protein. *Viruses* 2019; **11** [PMID: 31100802 DOI: 10.3390/v11050444]
- 79 **Porte L**, Legarraga P, Vollrath V, Aguilera X, Munita JM, Araos R, Pizarro G, Vial P, Iruretagoyena M, Dittrich S, Weitzel T. Evaluation of a novel antigen-based rapid detection test for the diagnosis of SARS-CoV-2 in respiratory samples. *Int J Infect Dis* 2020; **99**: 328-333 [PMID: 32497809 DOI: 10.1016/j.ijid.2020.05.098]
- 80 **Grant BD**, Anderson CE, Williford JR, Alonzo LF, Glukhova VA, Boyle DS, Weigl BH, Nichols KP. SARS-CoV-2 Coronavirus Nucleocapsid Antigen-Detecting Half-Strip Lateral Flow Assay Toward the Development of Point of Care Tests Using Commercially Available Reagents. *Anal Chem* 2020; **92**: 11305-11309 [PMID: 32605363 DOI: 10.1021/acs.analchem.0c01975]
- 81 **Nishinaka S**, Suzuki T, Matsuda H, Murata M. A new cell line for the production of chicken monoclonal antibody by hybridoma technology. *J Immunol Methods* 1991; **139**: 217-222 [PMID: 2045661 DOI: 10.1016/0022-1759(91)90191-h]
- 82 **Matsuda H**, Mitsuda H, Nakamura N, Furusawa S, Mohri S, Kitamoto T. A chicken monoclonal antibody with specificity for the N-terminal of human prion protein. *FEMS Immunol Med Microbiol* 1999; **23**: 189-194 [PMID: 10219590 DOI: 10.1111/j.1574-695X.1999.tb01238.x]
- 83 **Davies EL**, Smith JS, Birkett CR, Manser JM, Anderson-Dear DV, Young JR. Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *J Immunol Methods* 1995; **186**: 125-135 [PMID: 7561141 DOI: 10.1016/0022-1759(95)00143-x]
- 84 **Nakamura N**, Shimokawa M, Miyamoto K, Hojyo S, Horiuchi H, Furusawa S, Matsuda H. Two expression vectors for the phage-displayed chicken monoclonal antibody. *J Immunol Methods* 2003; **280**: 157-164 [PMID: 12972196 DOI: 10.1016/s0022-1759(03)00204-7]
- 85 **van Wyngaardt W**, Malatji T, Mashau C, Fehrnsen J, Jordaan F, Miltiadou D, du Plessis DH. A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnol* 2004; **4**: 6 [PMID: 15059288 DOI: 10.1186/1472-6750-4-6]
- 86 **Ge S**, Xu L, Li B, Zhong F, Liu X, Zhang X. Canine Parvovirus is diagnosed and neutralized by chicken IgY-scFv generated against the virus capsid protein. *Vet Res* 2020; **51**: 110 [PMID: 32883344 DOI: 10.1186/s13567-020-00832-7]
- 87 **Lee YC**, Leu SJ, Hung HC, Wu HH, Huang IJ, Hsieh WS, Chiu WT, Hsieh MS, Cheng TF, Yang YY. A dominant antigenic epitope on SARS-CoV spike protein identified by an avian single-chain variable fragment (scFv)-expressing phage. *Vet Immunol Immunopathol* 2007; **117**: 75-85 [PMID: 17360045 DOI: 10.1016/j.vetimm.2007.02.001]
- 88 **Genwaybiotech**. IgY Monoclonal Antibodies. [cited 4 February 2021]. Available from: <https://www.genwaybio.com/technologies/igy-monoclonal-antibodies>
- 89 **Creative-biolabs**. Chicken IgY Antibody Production. [cited 4 February 2021]. Available from: <https://www.creative-biolabs.com/igy-antibody-generation.html>
- 90 **Mettler Izquierdo S**, Varela S, Park M, Collarini EJ, Lu D, Pramanick S, Rucker J, Lopalco L,

- Etches R, Harriman W. High-efficiency antibody discovery achieved with multiplexed microscopy. *Microscopy (Oxf)* 2016; **65**: 341-352 [PMID: 27107009 DOI: 10.1093/jmicro/dfw014]
- 91 **Nishibori N**, Shimamoto T, Nakamura N, Shimokawa M, Horiuchi H, Furusawa S, Matsuda H. Expression vectors for chicken-human chimeric antibodies. *Biologicals* 2004; **32**: 213-218 [PMID: 15572103 DOI: 10.1016/j.biologicals.2004.09.002]
- 92 **Tsurushita N**, Park M, Pakabunto K, Ong K, Avdalovic A, Fu H, Jia A, Vásquez M, Kumar S. Humanization of a chicken anti-IL-12 monoclonal antibody. *J Immunol Methods* 2004; **295**: 9-19 [PMID: 15627607 DOI: 10.1016/j.jim.2004.08.018]
- 93 **Nishibori N**, Horiuchi H, Furusawa S, Matsuda H. Humanization of chicken monoclonal antibody using phage-display system. *Mol Immunol* 2006; **43**: 634-642 [PMID: 16360012 DOI: 10.1016/j.molimm.2005.04.002]
- 94 **Tateishi Y**, Nishimichi N, Horiuchi H, Furusawa S, Matsuda H. Construction of chicken-mouse chimeric antibody and immunogenicity in mice. *J Vet Med Sci* 2008; **70**: 397-400 [PMID: 18460836 DOI: 10.1292/jvms.70.397]
- 95 **Choi J**, Kim M, Lee J, Seo Y, Ham Y, Kim JK, Kwon MH. Antigen-binding affinity and thermostability of chimeric mouse-chicken IgY and mouse-human IgG antibodies with identical variable domains. *Sci Rep* 2019; **9**: 19242 [PMID: 31848417 DOI: 10.1038/s41598-019-55805-4]
- 96 **Park JS**, Lee KY, Han JY. Precise Genome Editing in Poultry and Its Application to Industries. *Genes (Basel)* 2020; **11** [PMID: 33053652 DOI: 10.3390/genes11101182]
- 97 **Sid H**, Schusser B. Applications of Gene Editing in Chickens: A New Era Is on the Horizon. *Front Genet* 2018; **9**: 456 [PMID: 30356667 DOI: 10.3389/fgene.2018.00456]
- 98 **Love J**, Gribbin C, Mather C, Sang H. Transgenic birds by DNA microinjection. *Biotechnology (N Y)* 1994; **12**: 60-63 [PMID: 7764327 DOI: 10.1038/nbt0194-60]
- 99 **Salter DW**, Smith EJ, Hughes SH, Wright SE, Crittenden LB. Transgenic chickens: insertion of retroviral genes into the chicken germ line. *Virology* 1987; **157**: 236-240 [PMID: 3029962 DOI: 10.1016/0042-6822(87)90334-5]
- 100 **Salter DW**, Crittenden LB. Artificial insertion of a dominant gene for resistance to avian leukosis virus into the germ line of the chicken. *Theor Appl Genet* 1989; **77**: 457-461 [PMID: 24232709 DOI: 10.1007/BF00274263]
- 101 **Kamihira M**, Ono K, Esaka K, Nishijima K, Kigaku R, Komatsu H, Yamashita T, Kyogoku K, Iijima S. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. *J Virol* 2005; **79**: 10864-10874 [PMID: 16103139 DOI: 10.1128/jvi.79.17.10864-10874.2005]
- 102 **Harvey AJ**, Speksnijder G, Baugh LR, Morris JA, Ivarie R. Consistent production of transgenic chickens using replication-deficient retroviral vectors and high-throughput screening procedures. *Poult Sci* 2002; **81**: 202-212 [PMID: 11873828 DOI: 10.1093/ps/81.2.202]
- 103 **Kamihira M**, Kawabe Y, Shindo T, Ono K, Esaka K, Yamashita T, Nishijima K, Iijima S. Production of chimeric monoclonal antibodies by genetically manipulated chickens. *J Biotechnol* 2009; **141**: 18-25 [PMID: 19428726 DOI: 10.1016/j.jbiotec.2009.02.022]
- 104 **McGrew MJ**, Sherman A, Ellard FM, Lilloco SG, Gilhooley HJ, Kingsman AJ, Mitrophanous KA, Sang H. Efficient production of germline transgenic chickens using lentiviral vectors. *EMBO Rep* 2004; **5**: 728-733 [PMID: 15192698 DOI: 10.1038/sj.embor.7400171]
- 105 **Lilloco SG**, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous KA, Elliot EA, Sang HM. Oviduct-specific expression of two therapeutic proteins in transgenic hens. *Proc Natl Acad Sci U S A* 2007; **104**: 1771-1776 [PMID: 17259305 DOI: 10.1073/pnas.0610401104]
- 106 **van de Lavoie MC**, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME, Etches RJ. Germline transmission of genetically modified primordial germ cells. *Nature* 2006; **441**: 766-769 [PMID: 16760981 DOI: 10.1038/nature04831]
- 107 **Zhu L**, van de Lavoie MC, Albanese J, Beenhouwer DO, Cardarelli PM, Cuisson S, Deng DF, Deshpande S, Diamond JH, Green L, Halk EL, Heyer BS, Kay RM, Kerchner A, Leighton PA, Mather CM, Morrison SL, Nikolov ZL, Passmore DB, Pradas-Monne A, Preston BT, Rangan VS, Shi M, Srinivasan M, White SG, Winters-Digiacinto P, Wong S, Zhou W, Etches RJ. Production of human monoclonal antibody in eggs of chimeric chickens. *Nat Biotechnol* 2005; **23**: 1159-1169 [PMID: 16127450 DOI: 10.1038/nbt1132]
- 108 **Kim YM**, Park JS, Kim SK, Jung KM, Hwang YS, Han M, Lee HJ, Seo HW, Suh JY, Han BK, Han JY. The transgenic chicken derived anti-CD20 monoclonal antibodies exhibits greater anti-cancer therapeutic potential with enhanced Fc effector functions. *Biomaterials* 2018; **167**: 58-68 [PMID: 29554481 DOI: 10.1016/j.biomaterials.2018.03.021]
- 109 **Taylor L**, Carlson DF, Nandi S, Sherman A, Fahrenkrug SC, McGrew MJ. Efficient TALEN-mediated gene targeting of chicken primordial germ cells. *Development* 2017; **144**: 928-934 [PMID: 28174243 DOI: 10.1242/dev.145367]
- 110 **Dimitrov L**, Pedersen D, Ching KH, Yi H, Collarini EJ, Izquierdo S, van de Lavoie MC, Leighton PA. Germline Gene Editing in Chickens by Efficient CRISPR-Mediated Homologous Recombination in Primordial Germ Cells. *PLoS One* 2016; **11**: e0154303 [PMID: 27099923 DOI: 10.1371/journal.pone.0154303]
- 111 **Oishi I**, Yoshii K, Miyahara D, Kagami H, Tagami T. Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Sci Rep* 2016; **6**: 23980 [PMID: 27050479 DOI: 10.1038/srep23980]

- 112 **Mukae T**, Okumura S, Watanobe T, Yoshii K, Tagami T, Oishi I. Production of Recombinant Monoclonal Antibodies in the Egg White of Gene-Targeted Transgenic Chickens. *Genes (Basel)* 2020; **12** [PMID: 33396657 DOI: 10.3390/genes12010038]
- 113 **Lee HJ**, Lee KY, Jung KM, Park KJ, Lee KO, Suh JY, Yao Y, Nair V, Han JY. Precise gene editing of chicken Na⁺/H⁺ exchange type 1 (chNHE1) confers resistance to avian leukosis virus subgroup J (ALV-J). *Dev Comp Immunol* 2017; **77**: 340-349 [PMID: 28899753 DOI: 10.1016/j.dci.2017.09.006]
- 114 **Ching KH**, Collarini EJ, Abdiche YN, Bedinger D, Pedersen D, Izquierdo S, Harriman R, Zhu L, Etches RJ, van de Lavoie MC, Harriman WD, Leighton PA. Chickens with humanized immunoglobulin genes generate antibodies with high affinity and broad epitope coverage to conserved targets. *Mabs* 2018; **10**: 71-80 [PMID: 29035625 DOI: 10.1080/19420862.2017.1386825]
- 115 **Polanowski A**, Zabłocka A, Sosnowska A, Janusz M, Trziszka T. Immunomodulatory activity accompanying chicken egg yolk immunoglobulin Y. *Poult Sci* 2012; **91**: 3091-3096 [PMID: 23155018 DOI: 10.3382/ps.2012-02546]
- 116 **Polanowski A**, Sosnowska A, Zabłocka A, Janusz M, Trziszka T. Immunologically active peptides that accompany hen egg yolk immunoglobulin Y: separation and identification. *Biol Chem* 2013; **394**: 879-887 [PMID: 23492558 DOI: 10.1515/hsz-2012-0337]
- 117 **Zambrowicz A**, Zabłocka A, Bobak Ł, Macała J, Janusz M, Polanowski A, Trziszka T. A simple and rapid method of isolation of active polypeptide complex, yolkin, from chicken egg yolk. *Food Chem* 2017; **230**: 705-711 [PMID: 28407970 DOI: 10.1016/j.foodchem.2017.03.101]
- 118 **Hannappel E**, Huff T. The thymosins. Prothymosin alpha, parathymosin, and beta-thymosins: structure and function. *Vitam Horm* 2003; **66**: 257-296 [PMID: 12852257 DOI: 10.1016/s0083-6729(03)01007-0]
- 119 **Chiou VY**. The development of IgY(DeltaFc) antibody based neuro toxin antivenoms and the study on their neutralization efficacies. *Clin Toxicol (Phila)* 2008; **46**: 539-544 [PMID: 18584367 DOI: 10.1080/15563650701771973]
- 120 **Gouliaris K**. Preliminary immunochemical evaluation of immunoglobulins Y isolated from twelve avian species. M.Sc. Thesis, NCSR "Demokritos" & Birmingham University, 2003
- 121 **Tobias FL**, Garcia LN, Kanashiro MM, Medina-Acosta E, Brom-de-Luna JG, de Almeida CM, Azevedo Junior RR, Lemos M, Vieira-da-Motta O. Growth inhibition of *Staphylococcus aureus* and *Escherichia coli* strains by neutralizing IgY antibodies from ostrich egg yolk. *Braz J Microbiol* 2012; **43**: 544-551 [PMID: 24031862 DOI: 10.1590/S1517-83822012000200015]
- 122 **Najdi S**, Nikbakht Brujeni G, Sheikhi N, Chakhkar S. Development of anti-*Helicobacter pylori* immunoglobulins Y (IgYs) in quail. *Iran J Vet Res* 2016; **17**: 106-110 [PMID: 27822235]
- 123 **Bae HD**, Kitaguchi K, Horio F, Murai A. Higher incorporation of heterologous chicken immunoglobulin Y compared with homologous quail immunoglobulin Y into egg yolks of Japanese quail (*Coturnix japonica*). *Poult Sci* 2009; **88**: 1703-1711 [PMID: 19590086 DOI: 10.3382/ps.2008-00238]
- 124 **Losonczy S**, Szabó C, Kiss Z, Bárdos L. Application of an anti-HQIgY antibody for the measurement of IgY concentrations of hen's and quail's serum and yolk. *Acta Physiol Hung* 1999; **86**: 253-258 [PMID: 10943656]
- 125 **IGY Life Sciences**. IgY antibodies—sustainable and efficacious therapeutics for human and animal health. [cited 4 February 2021]. Available from: <https://www.nature.com/articles/d43747-020-01049-5>
- 126 **Leiva CL**, Gallardo MJ, Casanova N, Terzolo H, Chacana P. IgY-technology (egg yolk antibodies) in human medicine: A review of patents and clinical trials. *Int Immunopharmacol* 2020; **81**: 106269 [PMID: 32036273 DOI: 10.1016/j.intimp.2020.106269]
- 127 **McCafferty J**, Griffiths AD, Winter G, Chiswell DJ. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 1990; **348**: 552-554 [PMID: 2247164 DOI: 10.1038/348552a0]
- 128 **JRC Publications Repository**. EURL ECVAM Recommendation on Non-Animal-Derived Antibodies. [cited 4 February 2021]. Available from: <https://ec.europa.eu/jrc/en/publication/euro-scientific-and-technical-research-reports/eurl-ecvam-recommendation-non-animal-derived-antibodies>
- 129 **Nature**. Nobel Prize in Chemistry 2018. [cited 4 February 2021]. Available from: <https://www.nature.com/collections/mnphfjdrdf>
- 130 **Barderas R**, Benito-Peña E. The 2018 Nobel Prize in Chemistry: phage display of peptides and antibodies. *Anal Bioanal Chem* 2019; **411**: 2475-2479 [PMID: 30888467 DOI: 10.1007/s00216-019-01714-4]
- 131 **Ascoli CA**, Aggeler B. Overlooked benefits of using polyclonal antibodies. *Biotechniques* 2018; **65**: 127-136 [PMID: 30089399 DOI: 10.2144/btn-2018-0065]
- 132 **Cakir-Koc R**, Budama-Kilinc Y, Ustun E, Babur C. Conjugation and Characterization of Latex Particles with *Toxoplasma gondii*-specific Immunoglobulin Y Antibodies for Diagnostic Aim and Evaluation Efficiency in In Vitro Culture. *J Equine Vet Sci* 2020; **92**: 103145 [PMID: 32797775 DOI: 10.1016/j.jevs.2020.103145]
- 133 **Kota RK**, Reddy PN, Sreerama K. Application of IgY antibodies against staphylococcal protein A (SpA) of *Staphylococcus aureus* for detection and prophylactic functions. *Appl Microbiol Biotechnol* 2020; **104**: 9387-9398 [PMID: 32960294 DOI: 10.1007/s00253-020-10912-5]

- 134 **Lin JH**, Lo CM, Chuang SH, Chiang CH, Wang SD, Lin TY, Liao JW, Hung DZ. Collocation of avian and mammal antibodies to develop a rapid and sensitive diagnostic tool for Russell's Vipers Snakebite. *PLoS Negl Trop Dis* 2020; **14**: e0008701 [PMID: 32956365 DOI: [10.1371/journal.pntd.0008701](https://doi.org/10.1371/journal.pntd.0008701)]
- 135 **Souza DC**, de Faria LS, Sousa JEN, Lopes CA, Ribeiro VDS, da Silva VJ, Ribeiro RP, Rabelo ÉML, Rodrigues Ávila VM, Ferreira Júnior Á, Costa-Cruz JM. Use of polyclonal IgY antibodies to detect serum immune complexes in patients with active hookworm infection. *Parasitology* 2020; **147**: 715-720 [PMID: 32051048 DOI: [10.1017/S0031182020000220](https://doi.org/10.1017/S0031182020000220)]
- 136 **Egea E**, Mendoza D, Garavito G, Saavedra S, Gómez H, Sanjuan M. Nanogold - IgY antibodies. An immunoconjugated for the detection of house dust mite (Dermatophagoides) allergens. *J Immunol Methods* 2019; **464**: 15-21 [PMID: 30165063 DOI: [10.1016/j.jim.2018.08.013](https://doi.org/10.1016/j.jim.2018.08.013)]
- 137 **Lupicka-Słowik A**, Grzywa R, Leporowska E, Procyk D, Oleksyszyn J, Sieńczyk M. Development and Evaluation of an Immunoglobulin Y-Based ELISA for Measuring Prostate Specific Antigen in Human Serum. *Ann Lab Med* 2019; **39**: 373-380 [PMID: 30809983 DOI: [10.3343/alm.2019.39.4.373](https://doi.org/10.3343/alm.2019.39.4.373)]
- 138 **Nie W**, Zhao C, Guo X, Sun L, Meng T, Liu Y, Song X, Xu K, Wang J, Li J. Preparation and identification of chicken egg yolk immunoglobulins against human enterovirus 71 for diagnosis of hand-foot-and-mouth disease. *Anal Biochem* 2019; **573**: 44-50 [PMID: 30831098 DOI: [10.1016/j.ab.2019.02.029](https://doi.org/10.1016/j.ab.2019.02.029)]
- 139 **Omori AM**, Ono EYS, Hirozawa MT, de Souza Sugiura IM, Hirooka EY, Pelegrinelli Fungaro MH, Ono MA. Development of Indirect Competitive Enzyme-Linked Immunosorbent Assay to Detect *Fusarium verticillioides* in Poultry Feed Samples. *Toxins (Basel)* 2019; **11** [PMID: 30658385 DOI: [10.3390/toxins11010048](https://doi.org/10.3390/toxins11010048)]
- 140 **Tran TV**, Do BN, Nguyen TPT, Tran TT, Tran SC, Nguyen BV, Nguyen CV, Le HQ. Development of an IgY-based lateral flow immunoassay for detection of fumonisin B in maize. *FI000Res* 2019; **8**: 1042 [PMID: 31956398 DOI: [10.12688/fi000research.19643.2](https://doi.org/10.12688/fi000research.19643.2)]
- 141 **Budama-Kilinc Y**, Cakir-Koc R, Ozdemir B, Kaya Z, Badur S. Production and characterization of a conserved M2e peptide-based specific IgY antibody: evaluation of the diagnostic potential via conjugation with latex nanoparticles. *Prep Biochem Biotechnol* 2018; **48**: 930-939 [PMID: 30388960 DOI: [10.1080/10826068.2018.1525564](https://doi.org/10.1080/10826068.2018.1525564)]
- 142 **Liang X**, Sheng Y, Yu W, Zhao S, Shan H, Zhang Q, Wang Z. Comparison of chicken IgY and mammalian IgG in three immunoassays for detection of sulfamethazine in milk. *Food Anal Methods* 2018; **11**: 3452-3463 [DOI: [10.1007/s12161-018-1316-9](https://doi.org/10.1007/s12161-018-1316-9)]
- 143 **Lupicka-Słowik A**, Psurski M, Grzywa R, Bobrek K, Smok P, Walczak M, Gawel A, Stefaniak T, Oleksyszyn J, Sieńczyk M. Development of Adenosine Deaminase-Specific IgY Antibodies: Diagnostic and Inhibitory Application. *Appl Biochem Biotechnol* 2018; **184**: 1358-1374 [PMID: 29043661 DOI: [10.1007/s12010-017-2626-x](https://doi.org/10.1007/s12010-017-2626-x)]
- 144 **da Silva MC**, Schaefer R, Gava D, Souza CK, da Silva Vaz I Jr, Bastos AP, Venancio EJ. Production and application of anti-nucleoprotein IgY antibodies for influenza A virus detection in swine. *J Immunol Methods* 2018; **461**: 100-105 [PMID: 30158073 DOI: [10.1016/j.jim.2018.06.023](https://doi.org/10.1016/j.jim.2018.06.023)]



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