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Review paper

IgY: A promising antibody for use in immunodiagnostic and in immunotherapy

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ABSTRACT

Immunoglobulin IgY is the major antibody produced by chickens (*Gallus domesticus*). After their V-C gene is rearranged in B cells, IgY is continually synthesized, excreted into the blood and transferred to the egg yolk, where it is accumulated. IgY is produced by hens to provide their offspring with an effective humoral immunity against the commonest avian pathogens until full maturation of their own immune system. In this review we aim to give an overview about the generation, structure, properties of IgY, as well as the advantages of chicken antibodies use over mammalian antibodies in immunodiagnostics and immunotherapy.

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1. Introduction

B lymphocytes and their mature counterpart, plasmocytes, are highly specialized secretor type of cells whose exclusive products are glycoproteins, the immunoglobulins (Igs) or antibodies. These cells are found in several

animal groups including mammals and birds. According to the “clonal selection theory” proposed by F. MacFarlane Burnet in the 1950s, which has now been demonstrated to be correct, an enormous number of antibody-producing cells are constantly differentiated and each one expresses on its surface an Ig specific for a unique antigen.

In the human immunoglobulin molecule, the protein prototype has two identical 25 kDa light (L) chains and also two identical 55–77 kDa heavy (H) chains. Two structurally distinct regions are found in both chains: in light

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chains, V_L (110 amino acids) and C_L (110 amino acids) and in heavy chains, V_H (110 amino acids) and C_H (330–440 amino acids). Therefore, the whole immunoglobulin molecule contains 1320–1540 amino acid residues (Edelman, 1991). The L and H polypeptides are positioned in such way that V domains and C domains of L chains are in parallel with their counterpart of the H chain. Each HL monomer contains one antigen-binding site. Inter-chain disulfide bonds and non-covalent interactions associate each L chain with one H chain and the two H chains with the each other (Padlan, 1994; Ramsland and Farrugia, 2002).

The amino acid sequences in L and H chains, rather than being straight, are folded into regular segments by intra-chain disulfide bonds forming globular regions, the molecular domains. L chains contain two domains, V_L and C_L , whereas the H chains have four to five domains, V_H1 , C_H1 , C_H2 , C_H3 (or C_H4). In the tetrameric Ig molecule, H_2L_2 , there are a total of 12–14 domains: 4V domains and 8–10C domains. Pairing of one entire L chain (L_VL_C) with the first domain of the H chain (H_VH_{C1}) results in (L_VL_C) (H_VH_{C1}), called Fab, while pairing C_H2 and C_H3 from the two H chains results in the formation of (C_H2 C_H3) (C_H2 C_H3), called Fc portion (Padlan, 1994; Ramsland and Farrugia, 2002). In most Igs there are, therefore, two Fabs and one Fc. The two Fab regions join the Fc region to form the Y-like structure at the *hinge* region. This region is rich in proline residues, providing flexibility to this region (Saphire et al., 2002). Sites of carbohydrate attachments and regions for interactions with the complement component, C1, are located within the C_H2 domain of Igs with three C_H domains, or in the C_H3 domain of the Igs with four C_H domains (Davies and Metzger, 1983; Bengten et al., 2000) (Fig. 1A).

Immunoglobulin IgY is the major antibody produced by chickens (*Gallus domesticus*). After their V-C gene is

rearranged in B cells, IgY is continually synthesized, secreted into the blood and transferred to the egg yolk, where it is accumulated (Warr et al., 1995). IgY is produced by hens to provide their offspring with an effective humoral immunity against the commonest avian pathogens until full maturation of their own immune system. In chickens, only three immunoglobulins classes have been well identified, IgM, IgA and IgY, and suggested but not yet proven are IgD and IgE (Chen et al., 1982; Burns and Maxwell, 1981). IgY (167,250 kDa), although prevalent in circulating blood (5–7 mg/ml of serum) and in egg yolk (100 mg/egg yolk), is also found in duodenal contents, tracheal secretions and in seminal fluid (Leslie and Clem, 1969).

IgY, like its mammalian counterpart IgG, is composed of two L and two H chains. Their L (18,660 kDa) and H (designated as upsilon, ν , 65,105 kDa) chains are also composed of V and C regions (Sun et al., 2001). The L chain contains one V and one C domain, and the ν chain contains one V domain and four C domains ($C\nu1$, $C\nu2$, $C\nu3$ and $C\nu4$) (Zhao et al., 2000; Carlander, 2002). The Fab regions, similar to mammalian immunoglobulins, contain the antigen-binding sites, whereas the Fc region contains domains responsible for complement activation, opsonization and mast cell sensitization for anaphylactic reactions (Fig. 1B).

By the use of a recombinant fragment of IgY-Fc consisting of a dimer of the $C\nu3/C\nu4$ domains, the putative common origin for mammalian and avian immunoglobulins was investigated. The $Fc\nu3-4$ was expressed, crystallized, and its X-ray structure determined. The presence of common features to both IgG-Fc and IgE-Fc in the obtained $Fc\nu3-4$ crystals reinforced such structural ancient commonality (Alexander et al., 2009). Although such common origin, marked structural differences distinguish mammalian IgG and avian IgY.

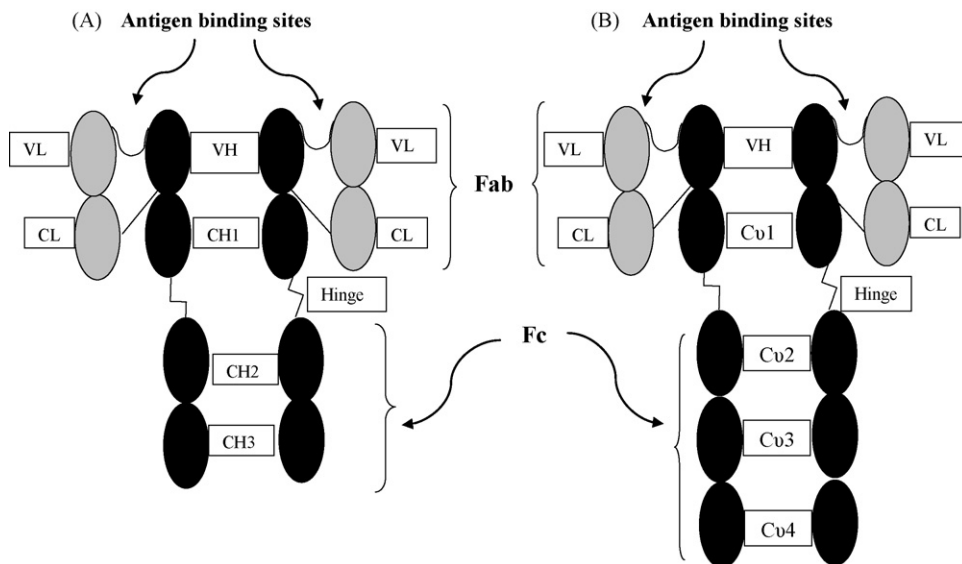


Fig. 1. The structural organization of the immunoglobulins. (A) Human IgG: VH, variable domain of heavy chain; VL, variable domain of light chain; CL, constant domain of light chain; CH1, CH2 and CH3, constant domains of heavy chain. (B) Chicken IgY: VH, variable domain of heavy chain; VL, variable domain of light chain; CL, constant domain of light chain; Cν1, Cν2, Cν3 and Cν4, constant domains of heavy chain.

2. Immunoglobulin diversity

The estimated diversity in antibody specificity is close to 10^{11} and it is enough to cover the antigen repertoire. Effective production of antibodies for the existent antigenic repertoire requires rearrangement of the different immunoglobulin protein segments (Tonegawa, 1981; Alt et al., 1987). In mammalian species, such as mouse and human, the presence in the genome of multiple functional V (variable), D (diversity) and J (joining) segments ensures rearrangement to form VDJ_H and VJ_L complexes, which encodes V_H and V_L domains, respectively for the heavy (H) and light (L) chains of the Ig molecule. For a Ig rearranged functional antibody molecule, some V segments are randomly selected to form the V_LJ and $V_HJ(D)$ DNA segments (Fig. 2). The estimated rearranging segment numbers in mammalian Ig loci are:

Mouse: V_H , 100–200; V_L kappa, 250–300; V_L lambda, 3; D, 27 exclusively for heavy chain; J, 4 for both chains.
Human: V_H , 130; V_L kappa, 75; V_L lambda 75; D, 15 exclusively for heavy chain; J, 6 for heavy chain; 5 and 7 for the light kappa and lambda chains, respectively.

An additional mechanism that generates new diversity, throughout the already assembled Ig locus, is known as somatic hypermutation. Although somatic mutations

occur in low frequency in all dividing cells, their frequency is higher in B cells at a rate of 10^3 mutations per nucleotide pair per cell division. This high rate of point mutation, approximately one million times higher than in other cells, is restricted to the rearranged Ig locus. Point mutations, besides assure such enormous increase in the antibody repertoire is responsible for the emergence of immunoglobulins with high affinity for their specific antigens. In mammalian the antibody diversity can be also generated through a “multi-V(D)J” mechanism. In sheep in which the Ig locus contains a limited number of different VJ_L rearrangements, diversity is further created by the introduction of multiple point mutations into the rearranged segments (Reynaud et al., 1991; Weill et al., 1995) (Fig. 2).

In chickens, both H and L chain gene loci consist of a single V gene (Reynaud et al., 1985; Weill and Reynaud, 1987). This intrinsic deficiency in the capacity to generate antibody diversity by germ line rearrangement of V locus DNA segments is over passed by the gene hyper conversion (Weill and Reynaud, 1987; Reynaud et al., 1987), V-J flexible joining (McCormick et al., 1989) and somatic point mutation (Parvari et al., 1990). Around days 15–17 of the embryonic development, immature B cells progenitors leave the bone marrow and migrate to bursa of Fabricius. After properly homing in special sites of bursa of Fabricius, blocks of DNA are transferred from pseudo-V-genes to the recombined V regions of the Ig genes. Clones

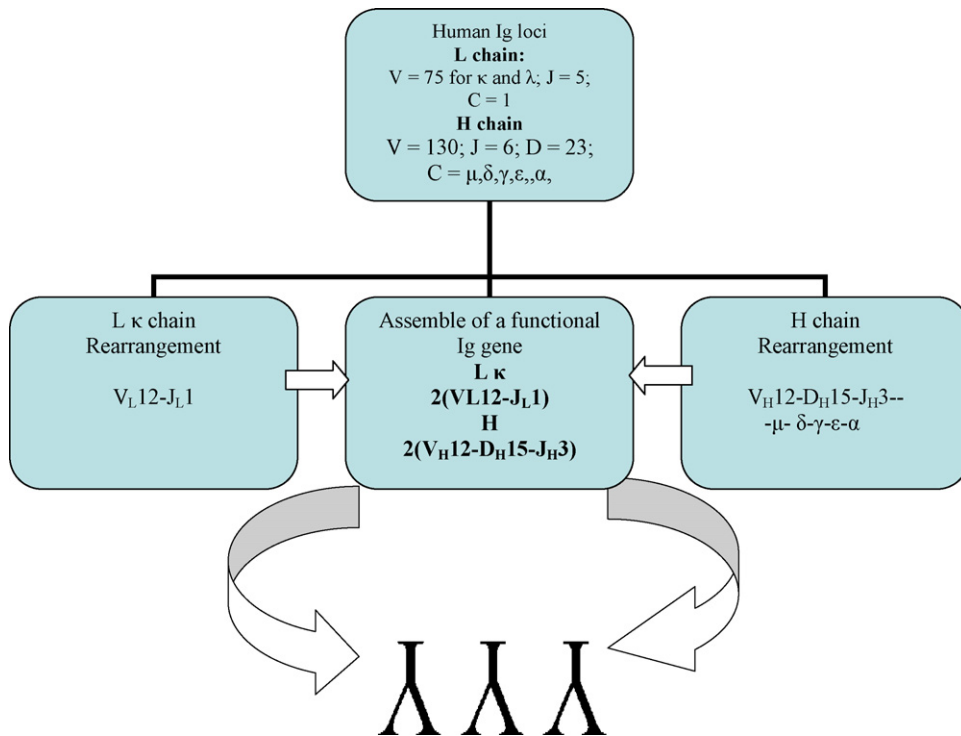


Fig. 2. The antibody diversification in human, as well as in other mammalian species, is brought about by gene somatic recombination. During this process DNA fragments encoding for V- and C-immunoglobulin protein that in germ line of all cells are separated (top), in lymphocytes of B lineage became rearranged in early ontogeny. One from the total V-DNA segments repertoire is randomly rearranged with individual DNA segments encoding for J (joining) for L chain (left), or with D (diversity) and J segments (right) for H chain. The resultant VJ_L and V_HDJ DNA rearranged segments are further rearranged with one of the C DNA fragments (μ - δ - γ - ϵ - α) (center). The resulting Ig functional rearranged gene encodes the synthesis of the functional immunoglobulin. This process occurs in B cells. Resulting Ig molecules are secreted into the blood circulation.

of mature competent B cells are produced and the immune humoral compartment is organized (Masteller et al., 1995).

Three exons separated by two introns encode the ν H-chain constant regions of IgY, IgA and IgM all located on chromosome E18C15W15. On this chromosome intervening DNA sequences of 18 and 15 kb separate the genes for IgA, IgY and IgM, respectively. The entire H Ig locus, V(D)J $\alpha\gamma\mu$ comprises a DNA segment with, approximately, 67 kb (Zhao et al., 2000). To construct the large number of V(D)J in chicken, equivalent to mammals, different mechanisms are used. First, by successive partial conversions of the rearranged V(D) containing the only V region and one of the 16 alternative D sequences available (Carlander, 2002). Next, by successive partial conversions of the rearranged V(D) segments by templates located in an upstream array of pseudo-V(D) genes and the V(D)J is rearranged (Carlander, 2002; McCormick et al., 1993) (Fig. 3). The mature competent immunoglobulin-producing B cells leave the bursa of Fabricius to populate the chicken's secondary immune system (Weill and Reynaud, 1987).

3. Active transfer of IgY from serum to egg yolk

IgY secretion in the young chick starts 6 days after hatching. The IgY secreted by mature B cells is delivered directly into the circulation, attaining a constant concentration of 1.0–1.5 mg/ml of serum (Davies et al., 1995). Along the hen's productive life, the serum IgY concentration is maintained stable due to an equilibrium

resulting from its continued synthesis and transfer processes.

The IgY stored in egg yolk, at the time of incubation, is the antibody source produced by the hen to protect the young chick during the first days after hatching. The IgY antibody concentrations range from 50 to 100 mg per egg yolk. In contrast, IgM and IgA are present in egg-white but almost undetectable in egg yolk (Carlander, 2002). These antibodies are first adhered in the yolk sac from days 7 to 18 of incubation, where they bind and fix in a domain-specific Fc receptors-pH-dependent manner (McCormick et al., 1993). This association of IgY-Fc and yolk sac receptors is first mediated by low affinity sac receptors with K_D of 3.4×10^{-7} (on the 8th day of incubation), and then by receptors of high affinity, with K_D 3.4×10^{-8} (on the 18th day of incubation) (McCormick et al., 1993). The IgY-Fc receptors continually attract and bind all IgY reaching the hen serum (Davies et al., 1995).

4. Methods for purification and quantification of IgY antibodies

Although IgY can be purified from serum or plasma, egg yolk is by far the indicated source for purification (Brambell, 1970; Rose et al., 1974). Eggs can be collected daily from the same hen and processed either individually or, as desired, from hens belonging to a similar immunized group. Over 100 mg of purified IgY can be obtained from a single egg (Tressler and Roth, 1987). As hens continue producing eggs along at least 10 months, it is possible to obtain enormous amounts of specific IgY directed to the

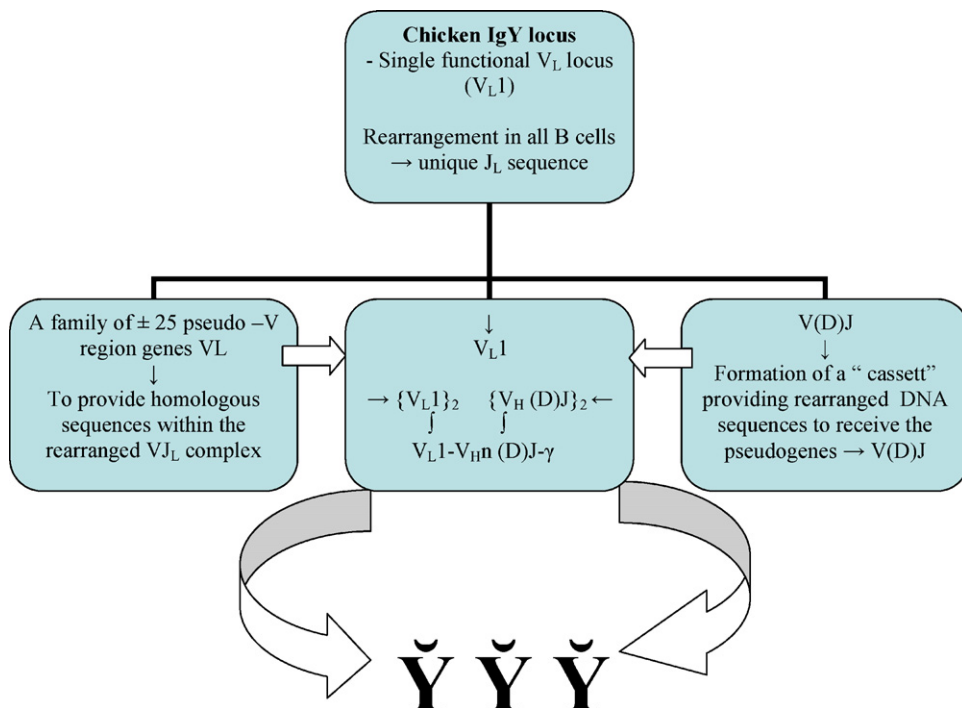


Fig. 3. The antibody diversification in chicken by gene conversion. During this process pseudo-derived genes sequences (left) replace homologous sequences in rearranged immunoglobulin genes (right), giving rise to rearranged complete IgG (IgY) functional gene (center). This process occurs in bursa of Fabricius resident B cells. Resulting IgY molecules are secreted into the blood circulation and subsequently stored on egg yolk.

same or related antigens (Loeken and Roth, 1983; Polson et al., 1980).

Different methods are currently used to isolate IgY from egg yolk. A simple non-expensive method was published by Bhanushali et al. (1994); the egg yolk is diluted 10-fold with distilled water, pH 5.5 and the suspension incubated overnight at 4 °C. The supernatant containing the IgY is collected by centrifugation ($10,000 \times g$ at 4 °C for 30 min), and the temperature adjusted to 20 °C and subjected to 29% ammonium sulfate. Precipitate proteins are recovered by centrifuging the solution ($10,000 \times g$ at 4 °C for 30 min), re-dissolving in 0.15 M NaCl and dialyzing against this same solution. The material can be further purified by Q-Sepharose Fast Flow chromatography to remove non-immunoglobulin proteins. The resulting IgY preparations are re-dissolved in 0.15 M NaCl and dialyzed against this same solution. When specific purified IgY antibodies are desired, immune-affinity chromatography can be used. The antibodies are applied, at neutral pH, to a column prepared with a matrix, where the antigen of interest is bound. After washing with isotonic phosphate-saline, pH 7.2, to remove non-specific antibodies, specific bound IgY antibodies are eluted with 0.1 M glycine, pH 2.2–2.8 (Akita and Nakai, 1992; Kim et al., 1999; Almeida et al., 1998, 2003, 2008).

IgY, like mammalian IgG, is a reasonably stable protein. Diluted in saline-containing substances that preserve the protein structure, IgY antibody activity can be stored at 2–4 °C. When lyophilized, the IgY antibody activity is not diminished even after several months of storage at temperatures of –20 °C or less, or even for 1 month at 37 °C. IgY, however, is not very stable at temperatures of higher than 70 °C, and at pH below 4.0 (Shimitzu et al., 1994; Olovsson and Larsson, 1993; Larsson et al., 1999). IgY can be stored for over 10 years in 0.15 M NaCl containing 0.02% NaN_3 at 4 °C (Shimitzu et al., 1994). The IgY antibodies, such as mammalian IgG, can be labeled by routinely described methods with biotin or horseradish (Olovsson and Larsson, 1993; Larsson et al., 1999).

Similar to mammalian IgG, IgY is bivalent, but its hinge region is more rigid due to a very different amino acid sequence (Warr et al., 1995). This region is probably responsible for the particular behavior that occurs when the IgY antibody combines with the antigen.

The IgY antibodies are identified and quantified by the usual immunochemical methods. Reliable methods for characterizing or quantifying IgY antibodies include agglutination of particulate antigens, precipitation of soluble antigens, identification of cell-bound antigens by immunofluorescence techniques (Kim et al., 1999) and the blocking of some exposed active domains in well-characterized molecules, such as toxins, enzymes, hormones, or even other immunoglobulins (Kubbo et al., 1973; Shimitzu et al., 1998; Akita et al., 1998; Hata et al., 1993). Agglutination and precipitation of the antigens are particularly affected by the rigidity of IgY hinge region. The immune precipitin reaction, for instance, expresses better when performed in the presence of 1.5 M NaCl (Shimitzu et al., 1998). IgY antibodies are, however, very effective at neutralizing

toxins present in animal venoms (Almeida et al., 1998, 2003, 2008; Shimitzu et al., 1998; Akita et al., 1998; Hata et al., 1993), and block some virulence factors such as BfpA, expressed by enteropathogenic *Escherichia coli* (Almeida et al., 2003).

5. IgY and immunodiagnostics

The IgY concentration in the serum of adult hens is approximately 5–7 mg/ml. One hen of a high egg-laying strain can produce around 20 eggs *per* month. Such amounts correspond to 2 g of IgY *per* month equivalent, therefore, to the IgY content of 300 ml of serum or 600 ml of total blood (Shimitzu et al., 1994). Such amounts of blood only can be obtained from large mammals. Chicken antibodies, therefore, constitute a much less expensive vehicle for use in diagnostic proposals.

It is a well-known concept that the immune response is more potent when the distance between the antigen source and the immune system increases. Therefore, to obtain immunoreagents containing antibody titers against mammalian antigens, chickens are better and cheaper than mammals (Shimitzu et al., 1994; Olovsson and Larsson, 1993; Svendsen and Hau, 1996; Ericka, 1999; Larsson et al., 1999). Furthermore, chicken antibodies recognize more epitopes when mammalian proteins are used as antigens, than the corresponding mammalian antibodies (Svendsen and Hau, 1996; Hadge and Ambrosius, 1984; Horton et al., 1984; Song et al., 1985). The absence of immunological cross-reactivity between chicken IgY and mammalian IgG (Hadje and Ambrosius, 1984), determined by the evolutionary distance, reinforces the advantages of using IgY over IgG as the first antibody in some types of immunological reactions. For instance, in immunohistochemical analysis the usually common cross-reactions observed between tissue IgG and epitopes, shared by the primary antibody and recognized by the secondary mammalian antibody, are not observed when IgY is used as the secondary antibody (Larsson and Lindahl, 1993). Chicken antibodies exhibit high avidity (10^9 L/mol) even after the first immunization. In order to reach similar avidity values (10^{10} L/mol), sheep must receive four boosters (Wooley and Landon, 1995).

Despite the significant advantage of chickens over sheep as antibody producers, the antibody half-lives of chicken antibodies are approximately 36 h, while sheep antibodies half-lives are of about 15 days.

Chickens can be immunized through different routes, as desired by the immunization protocols (Wooley and Landon, 1995). The injection of the antigen by the intramuscular route results in higher antibody levels by day 28 after immunization, and the resulting antibodies also exhibit higher specificity, being over 10 times more specific when compared with chickens immunized with the same antigen but by the sub-cutaneous via (Wooley and Landon, 1995). Chickens, immunized by the intramuscular via, continue producing specific antibodies during more than 200 days (Horton et al., 1984). Chickens can also tolerate the use of common immunological adjuvants, such as Freund's adjuvant, Specol, Hunters

TiterMax and lipopeptide Pam₃-Cys-(lys)₄ (Losch et al., 1986). The percentage of antigen specific antibodies in one egg yolk is close to 10% (Losch et al., 1986; Hass and Aspöck, 1988; Thalley and Carrol, 1990; Akita et al., 1998).

6. IgY antibodies for passive immunization against enteric infections

Effective protection against *Salmonella enteritidis*, *Salmonella e. typhimurium*, *Campylobacter jejuni*, *Escherichia coli* ETEC, murine and bovine rotavirus, and bovine corona virus infections in mice, pig and calves has been obtained with the use of passively-administered egg yolk-derived antibodies (Chalghoumi et al., 2009).

Purified IgY preparations, obtained from hens immunized with *C. jejuni*, were able to induce both prophylactic and therapeutic effects in chickens. The prophylactic protection was analyzed by preincubating 0.5 g of IgY with 10⁶ CFU of the bacteria, while the therapeutic effect was analyzed by administering the bacteria 4 days before the administration of 0.2 g IgY antibodies. Antibodies and bacteria were administered orally and protection against infection was followed by counting the bacteria in faeces. A decrease in the number of bacteria in faeces, as compared with controls, was observed. In the therapeutic experiment, a marked reduction of 80–95% was also observed. These observations suggest that oral passive immunization with anti-*C. jejuni* IgY may be a prophylactic and therapeutic tool to protect chickens against this bacterial infection (Tsubokura et al., 1997).

Anti-*S. enteritidis* egg yolk antibodies, administered in powdered form in hens feed, were found to reduce the rate of *S. enteritidis* contaminated eggs (13.3% contaminated eggs), in comparison with the control group (26.0% contaminated eggs). Doses of 3 g of egg powder per day per hen (~2.5% of the feed) were ingested *ad libitum* for 23 or 26 days. The experimental group received egg powder from immunized hens. In contrast, the control group received egg powder from non-immunized hens (Gürtler et al., 2004).

The protective effect of feed supplementation with non-immunized egg yolk powdered and immunized egg yolk powder (containing anti-*S. enteritidis* antibodies) on the elimination of *S. enteritidis* infection in laying hens was further investigated in a multi-trial study. Hens were orally infected with 10⁹ CFU *S. enteritidis*. Four weeks after the bacterial challenge, hens were given daily a supplemented feed of 15% (w/w) of either non-immunized egg yolk powder or immunized egg yolk powder for 28 days. Oral administration of both powders resulted in a rapid decrease in the number of *S. enteritidis* in faeces and an elimination of the organism after 2 weeks of feeding (Brady et al., 2002; Sugita-Konishi et al., 2000).

In order to investigate the specificity of the egg yolk IgY antibodies for protecting hens against *S. e. typhimurium*, *C. jejuni*, and *E. coli* infections, hens were given the non-immunized egg yolk powder at concentrations ranging between 1 and 10% (w/w) for *S. e. typhimurium* and *C. jejuni* challenge tests, and between 5 and 10% (w/w) for the *E. coli* challenge test. Egg yolk powder, at a concentration of close

to 5% (w/w) was able to eliminate *S. e. typhimurium* and significantly reduce the bacterial shedding after 2 weeks of feeding. Higher concentrations (7.5%, w/w) of egg yolk powder from non-immune hens were also able to reduce the colonization of *C. jejuni* and *E. coli*. Therefore, oral administration of non-immunized egg yolk powder can reduce colonization of the intestinal tract, indicating that the egg yolk may contain additional anti-infectious factors, besides IgY.

Among the non-specific antibacterial activities present in egg yolk, the lipoprotein-derived anti-microbial factor from hen-egg yolk (LDAMF) and egg plasma-derived sialyoligosaccharides (EPDS) have been identified. LDAMF inhibits *Streptococcus* strain growth, *in vitro*, while (EPDS) prevents *Salmonella* infection (Gürtler et al., 2004; Sugita-Konishi et al., 2000, 2002).

Experiments to observe the effect of *S. enteritidis*-specific IgY administration on the bacterial fecal shedding have been performed recently (Sugita-Konishi et al., 2002; Schade et al., 2005; Rahimi et al., 2007). Fifteen milliliters of yolk-containing antibody were mixed with 3.84 ml of drinking water on day one continuing for duration of the experiment. In comparison to control animals, who did not receive antibodies, the treated animals had significantly lower faecal shedding (0% versus 14%) and lower cecal concentrations of *S. enteritidis* (0.27 log₁₀ CFU versus 3.98 log₁₀ CFU). They also presented lower isolation of *S. enteritidis* from the liver, spleen and ileum. Under the conditions of this study, the use of *S. enteritidis*-specific IgY had a beneficial effect in reducing the colonization of *Salmonella* in market-aged broilers.

In experiments conducted *in vitro*, chicken IgY-specific antibodies directed against the *E. coli* enteropathogenic BfpA virulence factor have also been shown to block, in a dose-effect manner, the virulence factor-induced apoptosis of Vera cells (Melo et al., 2005).

7. Concluding remarks

The use of chicken IgY, instead of IgG mammalian antibodies, to detect non-self or even self antigens, certainly may help lower costs of clinical or research immunological tests (Table 1). In addition, chicken antibodies do not activate the mammalian complement system nor interact with rheumatoid factors, or bacterial and human Fc receptors.

The advantages of chicken antibodies over mammalian antibodies include: (a) reduction in animal use, since chickens produce larger amounts of antibodies than laboratory animals; (b) the elimination of painful blood collections in animals; (c) the utility of IgY in many immunological assays without loss of specificity and sensitivity; (d) the considerably lower cost of feeding and handling of chickens than mammals; (e) crude egg may be used as an antibody source. Items (a) and (b) meet the recommendations of the European Centre for the Validation of Alternative Methods (ECVAM), which specify that yolk antibodies should be used instead of mammalian antibodies for animal welfare reasons (Schade et al., 2005).

Table 1
Advantages of IgY as immunoreagent antibody.

Comparison items	Mammalian IgG	Avian IgY
Main sources	Plasma	Egg yolk
Steps imposing physical suffering to the antibody-producing animals	Antigen injection, blood collections, sometimes blood transfusions	Antigen injection
Antibody titer to mammalian antigens	Frequently low	Frequently high
Binding to Protein A/Protein G	++++	–
Binding to mammalian cell Fc receptor	++++	–
Immunocomplexes activate mammalian complement system	++++	–
Sustained antibody production	From 60th day in rabbits	From 30th day in chickens
Antibody production capacity	50–70 ml sera/90 immunization day; 500–700 mg of IgG	1 egg per day and 60 eggs/90 immunization days: 3–6 g of IgY
Antibody source collection	Production of the antisera involves complexes and expensive several steps	Production of the antisera involves simple and inexpensive few steps
Applications	RIA, IRMA, ELISA, Western blot, FACS, etc.	RIA, IRMA, ELISA, Western blot, FACS, etc.

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