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Focus on :

Hen egg yolk antibodies (IgY), production and use for passive immunization against bacterial enteric infections in chicken: a review

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Enteric infections caused by *Salmonella* remain a major public health burden worldwide. Poultry, particularly chickens, are known to be the main reservoir for this zoonotic pathogen. Therefore, the prevention and monitoring of *Salmonella* infection during the live phase may greatly reduce the contamination of poultry meat during slaughter and processing. With the ban on sub-therapeutic antibiotic usage in Europe and the increasingly strictness of the European legislation on food hygiene, passive immunization by oral administration of pathogen-specific hen egg yolk antibody (IgY) may be a useful and attractive alternative. This review offers summarized information about IgY production and the use of these antibodies for passive immunization, particularly in poultry.
Keywords. Hen egg yolk antibodies, IgY production, passive immunization, enteric infections, feed additive.

Les anticorps du jaune d'œuf de poule (IgY), production et utilisation en immunisation passive contre les infections entériques bactériennes : une revue. Les infections entériques causées par *Salmonella* constituent un problème majeur de santé publique à travers le monde. Il est bien connu que la volaille, en particulier le poulet de chair, constitue le principal réservoir pour ce pathogène zoonotique. Par conséquent, la prévention et la surveillance de *Salmonella* au cours de la phase d'élevage pourrait réduire efficacement la contamination de la viande à l'abattoir et lors de la transformation. Avec l'interdiction de l'utilisation sub-thérapeutique des antibiotiques en Europe et la rigueur croissante de la législation européenne en matière d'hygiène alimentaire, l'immunisation passive par l'administration orale d'anticorps de jaune d'œuf (IgY) spécifiques du pathogène serait une alternative intéressante. Cette synthèse bibliographique donne des informations concises sur la production des IgY et leur utilisation en immunisation passive, en particulier chez la volaille.
Mots clés. Anticorps du jaune d'œuf, production de l'IgY, immunisation passive, infections entériques, additif alimentaire.

1. INTRODUCTION

Poultry meat is an important food product and the broiler chicken-related industry is an economically important component of the agro-industry in the European Union (EU). The industry however is under pressure and faces many challenges. Most importantly, there is a demand for meat of high nutritional value and free of microbiological and chemical hazards. Unfortunately poultry meat is one of the major sources of food borne bacterial infections in humans such as Salmonellosis (Mayrhofer et al., 2003).

In recent decades, Salmonellosis becomes a considerable burden to public health. *Salmonella* can cause a spectrum of pathological conditions such as acute gastroenteritis and bacteraemia in humans by the mechanisms of colonization, invasion and penetration of the intestinal epithelium (Roberts et al., 1996). In Europe, although Salmonellosis records a fall in the number of cases since 2005, it remains second in the list of human zoonotic diseases across the EU with 160,649 people infected in 2006 (35 cases per 100,000). Two serovars, *Salmonella* Enteritidis and *Salmonella* Typhimurium account for most Salmonellosis associated with foods of animal origin (EFSA, 2007). In Belgium, these two serovars accounted for almost 80% of identified serovars in humans in 2006 (Collard et al., 2008).

The contamination of poultry meat products originates primarily from chickens infected with *Salmonella* during processing. In this respect, *S*. Enteritidis and *S*. Typhimurium are of particular importance, since these pathogens can colonize the chicken host without causing discernible illness in the infected chickens (Barrow et al., 1987; Ziprin et al., 1989).

Since poultry meat will, due to EU legislation, not be brought as fresh poultry meat on the market from 12/12/2010 if *Salmonella* is detected, the broiler industry needs to take measures to decrease the colonization of the animals and their environment. These include pre-harvest, harvest and post-harvest measures. Harvest measures are essentially hygienic measures during catching and transport, while post-harvest measures include both hygienic measures and the application of decontaminating treatments on the meat. However, all carcass disinfectants are prohibited in the EU at present, thus decontamination is not an option. Therefore, prevention and monitoring during the live phase (pre-harvest phase) are of great importance in Europe.

There are three basic strategies that can be employed to control *Salmonella* in poultry production throughout the live phase. These are:
– preventive hygienic measures,
– vaccination,
– nutritional strategies or feed additives.

Preventive hygienic measures typically involve establishing effective farm-site biosecurity and poultry house sanitation protocols. Vaccination strategy cannot be applied in broilers due to the short life span of the animal. Since the ban on sub-therapeutic antibiotic usage in Europe, the use of feed additives is more and more accepted as a valuable way to combat *Salmonella* infection in broilers production. Among the candidate replacements for antibiotics are organic acids, prebiotics, probiotics, symbiotics, competitive exclusion products, and bacteriophages. These products are intended to reduce the intestinal colonization and subsequent faecal excretion of *Salmonella*. The faecal excretion reduction will lead to a decrease in the rates of the environment contamination and consequently the risk of horizontal contamination should decline.

Passive immunization by oral administration of hen egg yolk antibody (IgY) is an emerging and promising nutritional strategy that may serve to control *Salmonella* in broiler chicken industry. Some studies reported that *Salmonella*-specific IgY can prevent fatal Salmonellosis in mice or calves by oral administration (Peralta et al., 1994; Yokoyama et al., 1998a; 1998b).

The present review focuses on the use of IgY for passive immunization, particularly in poultry. The characteristics of these antibodies and their production are also discussed.

2. HEN EGG YOLK ANTIBODIES

The almost extreme properties of antibodies to recognize small specific structures on other molecules have made them an indispensable tool in laboratory in various applications such as research, diagnostic and therapy. Antibodies presently available for these purposes are mostly mammalian monoclonal or polyclonal antibodies. The production of these antibodies requires normally the use of laboratory animals. Nowadays, most classical chosen mammals for polyclonal and monoclonal antibodies are rabbits and mice, respectively. The procedure involves two steps, each of which causes distress to the animals: the immunization itself and repeated bleeding or sacrifying for spleen removal, which is a prerequisite for antibodies preparation.

In 1893, Klemperer first demonstrated that the immunization of a hen resulted in the transfer of specific antibodies from the serum to the egg yolk. For over a hundred years there was no scientific application for this knowledge. But when the animal welfare became a matter of serious ethical concern for the scientific community, the results of Klemperer have attracted a great attention, particularly since the 1980s.

In the sense of animal welfare, the use of laying hens for antibody production represents a refinement and a reduction in animal use. It is a refinement in that the painful and invasive blood sampling or sacrifying are replaced by collecting eggs. It entails a reduction in the number of animals used because the antibodies productivity in laying hens is nearly 18 times greater than that in rabbits (Schade et al., 1996).

Furthermore, because of the high yolk antibodies concentration, over 100 mg of antibodies can be obtained from one egg (Akita et al., 1992). Since a laying hen produces approximately 20 eggs per month, 2 g of antibody per month may be obtained from a single laying hen.

2.1. Avian immune system

Like in mammals, various mechanisms have been developed in birds to protect them from invading microorganisms and foreign substances.

The bird's immune system mainly consists of primary lymphoid organs and secondary lymphoid organs. Primary organs are the thymus, located in the neck along the jugular vein, and the bursa of Fabricius, located adjacent to the cloaca. Secondary organs are the spleen, bone marrow, Harderian gland, caecal tonsils, and organized lymphoid tissues associated

with mucosal surfaces (MALT), including bronchial-associated lymphoid tissues (BALT), gut associated lymphoid tissues (GALT), conjunctival associated lymphoid tissues (CALT) and lymphoid nodules. There is also a lymphatic circulatory system of vessels and capillaries that transport lymph fluid through the bird's body and communicate with the blood supply (Panada et al., 2007; Davison et al., 2008).

Functionally, the bird's immune system can be divided into two parts: one innate, but non-specific, while the other part is acquired and specific. The acquired immune system is characterized by specificity, heterogeneity, and memory. This system is divided into cellular branch and non-cellular (humoral) branch.

The non-cellular branch includes immunoglobulins (antibodies) and the cells which produce them. Antibodies are specific (specificity) for the foreign material (antigen) to which they attach. The cells which produce antibodies are the B-lymphocytes. These cells are produced in the embryonic liver, yolk sack and bone marrow. The cells move to the bursa of Fabricius after 15 days incubation through 10 weeks of age. The bursa of Fabricius programs these cells which then move to the blood, spleen, caecal tonsils, bone-marrow, Harderian gland, and thymus. When a foreign organism enters the body, it is engulfed by a phagocytic-type cell, the macrophage. The macrophage digests partially the foreign organism and becomes an antigen presenter showing particles of foreign antigen on its surface. These particles form complexes with the glycoproteins of the MHC class II, which are self markers already present on the surface of the macrophage. Then, the macrophage transports each complex and exposes it to the T-lymphocytes (helper T cells). The latter, that presents a receptor that can bind specifically to the antigen presented, binds to the self / non-self complex of the macrophage. Subsequently, the helper T cell enters into contact with a B-lymphocyte, which also presents on its surface the foreign particles at the same time that the glycoproteins of the MHC class II. This binding activates the differentiation of B-lymphocyte to a plasmocyte, i.e. an effecter cell secreting antibodies. The B-cells respond by producing antibodies after day 5 following exposure. The lag period occurs because the B-cells must be programmed and undergo clonal expansion to increase their numbers. If the chicken is exposed a second time to the same antigen, the response is quicker and a much higher level of antibody production occurs (memory).

Three immunoglobulin classes, which are distinguishable in concentration, structure, and immunochemical function, are found in birds: IgA, IgM, and IgY.

The IgA and IgM are similar to mammalian IgA and IgM in molecular weight, structure and electrophoretic mobility.

Historically, IgY, the major immunoglobulin class present in avian serum and egg yolk was called IgG, due to its function and serum concentration in comparison with the mammalian IgG. However, it has become clear that this is inappropriate, especially because of fundamental structural differences between IgG and IgY molecules, which will be discussed in more details later.

IgY makes up about 75% of the total immunoglobulin pool. The serum concentrations of IgY, IgA, and IgM have been reported to be 5.0, 1.25, and 0.61 $mg \cdot ml^{-1}$, respectively (Leslie et al., 1973).

The cellular branch of the bird's immune system includes all the cells that react with specificity to antigens, except those associated with antibody production. The cells associated with this system, the T-lymphocytes, begin as the same stem cells as the B-cells. However, the T-lymphocytes are programmed in the thymus rather than the bourse of Fabricius. The T-lymphocytes include a more heterogeneous population than the B-cells. Some T-cells act by producing lymphokines; others directly destroy foreign organisms; some T-cells act to enhance the response of B-cells, macrophages, or other T-cells (helpers); and others inhibit the activity of these cells (suppressors) (Carlander, 2002; Janeway et al., 2002).

2.2. Transfer of IgY into egg yolk

Antibodies are transferred from hen to chick via the latent stage of the egg, and play an important role in immunological function for the relatively immunoincompetent chick to resist various infectious diseases.

Serum IgY is selectively transferred from the hen's circulatory system across the oolemma into the maturing oocyte in the ovarian follicle (Rose et al., 1981). This transfer occurs via a specific receptor on the surface of the yolk sac membrane which allows the selective transport of all IgY subpopulations presented by the maternal blood (Tressler et al., 1987; Mohammed et al., 1998; Morrison et al., 2001). Morrison et al. (2001) have identified several regions within the antibody molecule important for its uptake into the egg yolk. Their data demonstrate that an intact Fc (Crystalizable (constant) region of antibody molecule) and hinge region but not the Fc-associated carbohydrate (Figure 1) are required for this transport. The same findings suggest that the CH2-CH3 domain (Figure 1) is recognized by the receptor responsible for IgY transport. The transovarial passage of IgY takes approximately 3-6 days (Patterson et al., 1962; Wooley et al., 1995). The exact "lag" curve probably depends upon the number of eggs undergoing their final processing, during which IgY is transferred to the yolk (Patterson et al., 1962).

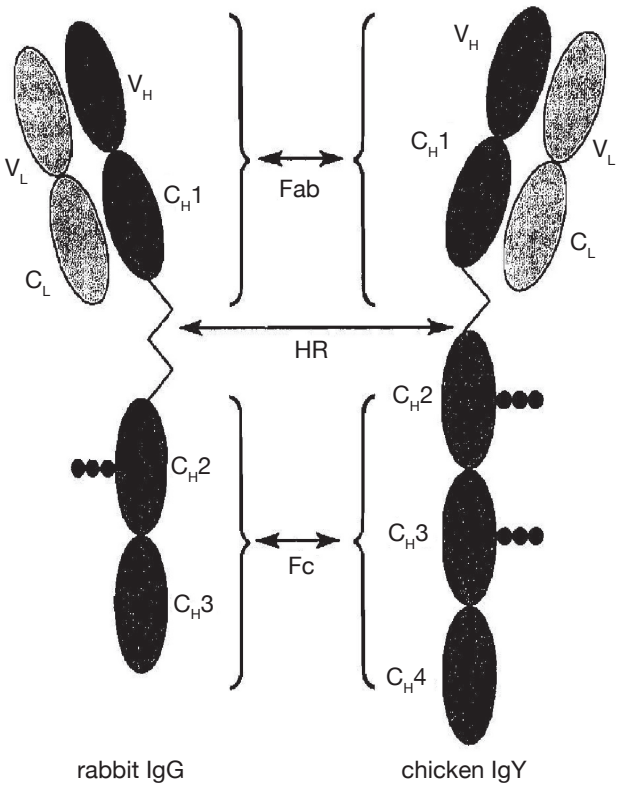

Figure 1. Comparison of the molecular structure of chicken IgY and rabbit IgG. (adapted from Schade et al., 2005) — *Comparaison entre la structure moléculaire de l'IgY de poule et de l'IgG de lapin (adapté d'après Schade et al., 2005).*

V: variable domain of the light chain (V_L) and the heavy chain (V_H) — *région variable de la chaine légère (V_L) et de la chaine lourde (V_H)*; C: constant domain of the light chain (C_L) and the heavy chain (C_H) — *région constante de la chaine légère (C_L) et de la chaine lourde (C_H)*; HR: hinge region, the black points symbolize carbohydrate chains — *région charnière, les points noirs symbolisent les chaines de carbohydrates*; Fab: Antigen Binding Fragment; Fc: Crystalizable (constant) region of antibody molecule.

Maternal IgA and IgM are incorporated into the egg white in the oviduct along with the egg albumen secretion. IgA and IgM in egg white are subsequently transferred to the embryonic gut via swallowed amniotic fluid (Rose et al., 1974) and IgY in egg yolk circulates in the blood of the chick via the endoderm of the yolk sac (Patterson et al., 1962). The concentrations of IgA (~0.7 mg·ml^{-1}) and IgM (~0.15 mg·ml^{-1}) in egg white are relatively low while that of IgY (8-25 mg·ml^{-1}) in egg yolk is considerably high (Rose et al., 1974).

2.3. Molecular properties of IgY

Structure of IgY. IgY has a molecular mass of ~180 kDa which is heavier than that of mammalian IgG (~150 kDa). The general structure of the IgY molecule consists of two identical heavy (H) chains and two identical light (L) chains, which are linked by disulfide bridge. The light chain of IgY consists of one variable (V_L), and one constant domain (C_L), like mammalian IgG. But, intra-chain disulfide linkage between the V_L region and C_L region of L-chain, which stabilizes the structure of the mammalian IgG L-chain is absent in the IgY L-chain and thus intra-molecular forces of IgY are weaker than those of mammalian IgG (Shimizu et al., 1993).

The heavy chain of IgY contains one variable domain (V_H), four constant domains (C_H1; C_H2; C_H3 and C_H4), unlike mammalian IgG which has three constant domains (C_H1; C_H2 and C_H3).

In the heavy chain of IgG, the C_H1 and the C_H2 domains are separated by a hinge region, which gives considerable flexibility to the Fab fragment (the portion which contains the antigen-binding activity). In contrast, the heavy chain of IgY does not have a hinge region, but there are regions near the boundaries of the C_H1-C_H2 and C_H2- C_H3 domains that contain proline and glycine residues. These regions have the potential to confer limited flexibility on the molecule.

Comparisons of C-domain sequences in IgG and IgY have shown that the C_H2 and C_H3 domains of IgG are the equivalents of the C_H3 and C_H4 domains of IgY, respectively. The equivalent of C_H2 domain of IgY is absent in the heavy chain of IgG.

The content of β-sheet structure in C domains of IgY is lower than that of mammalian IgG; therefore, the conformation of IgY domains is more disordered in comparison to mammalian IgG.

As for IgG, the Fc part of IgY is the site of most biological effector functions. It contains two carbohydrate side-chains, in contrast to only one in IgG (**Figure 1**).

Physico-chemical properties of IgY. The isoelectric point of IgY is lower than that of IgG (Polson et al., 1980). It is in the range of 5.7 to 7.6, whereas that of IgG lies between 6.1 and 8.5.

Since the Fc fragment (the most hydrophobic moiety of the antibody molecule) of the IgY is bigger than that of the IgG, the IgY molecule is more hydrophobic than the IgG molecule (Davalos et al., 2000).

Stability of IgY

pH stability. The stability of IgY to acid and alkali has been studied under various conditions. It was found that the activity of IgY was decreased at pH 3.5 or lower and almost completely lost with irreversible change at pH 3 (Shimizu et al., 1988; 1992; 1993). Similar results were reported by Lösch et al. (1986), Hatta et al. (1993) and Lee et al. (2002b). Rapid decrease of the IgY activity at low pH(s) indicated conformational

changes and damage in the Fab portion including the antigen-binding site.

Under alkaline conditions, the activity of IgY did not change until the pH increased to 11. However, it was markedly diminished at pH 12 or higher (Shimizu et al., 1988; 1992; 1993). Similar results about pH effect were presented by Lösch et al. (1986), Hatta et al. (1993) and Lee et al. (2002b).

Proteolysis stability. IgY is relatively resistant to trypsin or chymotrypsin digestion, but is fairly sensitive to pepsin digestion. Hatta et al. (1993) demonstrated that almost all of the IgY activity was lost following digestion with pepsin, but 39% and 41% of the activity remained even after 8 h of incubation with trypsin or chymotrypsin, respectively.

The stability of IgY against pepsin appears to be highly dependent on pH and the enzyme/substrate ratio. At pH 5 or higher, IgY was fairly resistant to pepsin and retained its antigen-binding and cell-agglutinating activities. However, at pH 4.5 or below, both activities were lost (Shimizu et al., 1988). The results of Hatta et al. (1993) who also observed the IgY behavior with pepsin under different incubation times and pH confirmed the susceptibility of IgY to pepsin at low pH. Digestion of IgY with pepsin at pH 2 resulted in complete hydrolysis of the antibody molecule, leaving only small peptides. However, IgY digested with pepsin at pH 4 retained 91% and 63% of its activity after 1 h and 4 h incubation time, respectively.

After tryptic digestion, IgY retained its antigen-binding and cell-agglutinating activities in spite of a definite breakdown of the polypeptides. Unlike the trypsin digestion, no definite cleavage of the IgY chains was observed for chymotryptic digestion and the activities of IgY remained high for these digests (Shimizu et al., 1988; Otani et al., 1991).

Temperature and pressure stability. IgY has been thermally treated at various temperatures for different periods of time. The binding activity of IgY with antigen decreased with increasing temperature and heating time. According to Shimizu et al. (1992) and Hatta et al. (1993), IgY is stable at temperature ranging between 60°C and 70°C. The activity of IgY decreased by heating for 15 min at 70°C or higher (Shimizu et al., 1988; 1992) and IgY denatured seriously when thermally treated at temperatures higher than 75°C (Chang et al., 1999).

IgY is relatively stable to pressure as reported with no detectable inactivation of IgY by pressure up to 4,000 kg per cm^2 (Shimizu et al., 1994).

Freeze and spray-drying stability. Freezing and freeze-drying are low temperature processes that are usually considered to be less destructive. However, proteins may suffer from a loss of activity as a result of conformational changes, aggregation or adsorption (Skrabanja et al., 1994). There have been some reports on the stability of IgY in regard to these methods. Freezing and freeze-drying did not affect the activity of IgY unless repeated several times (Shimizu et al., 1988). However, Chansarkar (1998) showed that frozen or freeze-dried IgY resulted in some loss of antigen-binding activity and a significant drop in the solubility under the conditions of high salt and protein concentrations. The same findings were observed by Sunwoo et al. (2002). Recently, Fu et al. (2006) examined the thermal stability of IgY at various temperatures ranging between 25 and 90°C for 15 min treatment, before and after freeze-drying process. Their results indicated that freeze-dried IgY had shown a good thermal stability with no significant reduction in reactive activity except at 90°C. But this was also observed for the non freeze-dried IgY.

Yokoyama et al. (1992) analyzed some properties of IgY powders obtained by spray-drying or freeze-drying the water-soluble fraction of egg yolks from *Escherichia coli* immunized hens. As compared to the freeze-dried powder, the spray-dried powder did not show a significant alteration in antibody titres and yields, even when several spray-drying temperatures were tried (140 to 170°C). However, a higher moisture content of powder was observed in the powder prepared by spray-drying than by freeze-drying.

2.4. IgY production

Immunization of the hens. Specific IgY development and production can be achieved by immunizing laying-hens with the target antigen. However, the resulting immune response of the immunized hens can not be very predictable. Mainly five factors influence this response: the antigen (dose and molecular weight), the type of adjuvant used, the route of application, the immunization frequency, and the interval between immunizations (Schade et al., 1996).

Antigen. The immune response is triggered by contact of the organism with antigen, which is a structure that is recognized by the immune system as foreign ("non-self"). The dose of antigen influences significantly the immune response and the antibody titre that is evoked. Too much or too little antigen may induce suppression, sensitization, tolerance or other unwanted immunomodulation (Hanly et al., 1995). Behn et al. (1996) achieved better results when injecting hens with 0.1 mg of mouse IgG, instead of 1.0 mg. Schwarzkopf et al. (2000) found that the injection of antigen concentrations ranging between 10 µg and 1 mg elicited good antibodies responses, and this was also reported by other researchers (Mahn, 1998).

Basically for each antigen, various concentrations have to be tested since the type of antigen should be also considered. Antigen can be presented to the immune system as complex multiantigens (e.g., bacteria, viruses and parasites) or as single antigens (e.g., proteins or polysaccharides) (Leenars et al., 1996). Proteins are recognized to be the most efficient immunogens because of the polymorphism of their structure and the differences existing between species and individuals (Goldsby et al., 2003). As a rule of thumb, only 10-100 µg of protein antigen should be used (Camenisch et al., 1999; Matsuda et al., 1999; Tini et al., 2002).

Peptides (molecular weight below 10 kDa) can also be used as antigen, but they should be coupled to carriers (e.g., bovine serum albumin or keyhole limpet haemocyanin) (Schade et al., 2005). Polysaccharides antigens are efficient too. However, lipid and nucleic acids are not potent immunogens unless they are coupled to proteins or polysaccharides (Goldsby et al., 2003).

Adjuvant. The induction of high and sustainable egg yolk antibody titre reclaims the use of adjuvant. There are more than 100 known adjuvants, which differ in their chemical characteristics, their efficacy in stimulating the immune system, and their secondary side-effects. Freund's complete adjuvant (FCA) remains the most effective adjuvant for antibodies production in laboratory animals. In mammals, the use of this adjuvant leads systematically to severe inflammation at the injection site. In birds, the use of FCA does not seem to result in the same severe lesions as in mammals. The results of Gassmann et al. (1990) suggest that chickens show higher resistance to tissue damaging potency of FCA than rabbits. Svendsen et al. (1996) also support this finding. However, the results of Wanke et al. (1996) and Olbrich et al. (2002) contradict these data. To avoid an eventual local tissue reaction, the Freund's incomplete adjuvant (FIA), which is the most effective substitute found to date, becomes now the most commonly used adjuvant to produce egg yolk antibody. Since FIA is less efficient than FCA, some investigators preferred the use of a combination of the two adjuvants: FCA for the first immunization and FIA for the booster immunizations (Kapoor et al., 2000; Li et al., 2006; Chalghoumi et al., 2008). In these studies good results were achieved and no adverse side-effects were reported.

Route of application. The most common route for antigen injection in hens for IgY production is the intramuscular route. Injection is usually performed in the breast muscle (for review, see Schade et al., 2005). Some authors inject antigen in the leg, but the general recommendation states that intramuscular injection in the leg should be avoided, since it can lead to lame.

Chicken can also be injected subcutaneously in the neck. With very young animals, it may be preferable to inject intramuscularly into the breast muscle, because subcutaneous injection is more difficult to perform and can therefore cause more distress (Schade et al., 1996).

Immunization frequency and interval between immunizations. The total number of immunizations required depend on the type and dose of the antigen as well as the adjuvant employed. At least two immunizations have to be given. Yolk antibody titres should be checked 14 days after the last immunization. If antibodies titres begin to decrease, booster immunizations can be given during the laying period to maintain production of high levels of specific antibodies up to year (Schade et al., 1996).

The success of an immunization protocol depends also on the interval between the first and second and subsequent immunizations. Often reported interval is two to four weeks (Camenisch et al., 1999; Matsuda et al., 1999; Tini et al., 2002).

IgY extraction. Lipids and proteins are the major constituents of egg yolk. The lipid fraction, including triglycerides, phospholipids, and cholesterol, constitutes approximately one third of the yolk. Proteins consist 15 to 17% of the yolk, which can be separated by centrifugation into two main fractions, the granule (precipitate on centrifugation) and the plasma (clear fluid supernatant on centrifugation) (Stadeklman et al., 1977).

Granules are about 22% of the total yolk proteins and composed of 70% high-density lipoproteins (HDL: α- and β-lipovitellins), 16% phosvitin (glycophospoprotein), and 12% low-density lipoproteins (LDL) (Burley et al., 1961).

Plasma is about 78% of the total yolk proteins and composed of 86% of LDL and 14% of livetins (McCully et al., 1962). Livetins are water soluble, lipid-free globular glycoproteins, which are divided into three classes: α-, β-, and γ-livetin. According to Bernardi et al. (1960) the relative proportion of the three livetins in the yolk is 2:5:3, respectively.

IgY is the predominant fraction of γ-livetin (Kovacs-Nolan et al., 2005). Separation of IgY requires therefore the removal of the lipoprotein and the recovery of the water-soluble fraction (WSF) followed by purification of the IgY from other livetins (Polson et al., 1980).

The removal of lipoproteins and the recovery of the WSF can be achieved by several methods, as presented in **table 1**. These various IgY crude extraction methods give different results towards recovery and purity. Akita et al. (1993) compared the water dilution methods to other methods such as polyethylene glycol, dextran sulphate, and aliginates methods in terms of yield,

Table 1. Summary of the IgY crude extraction methods — *Sommaire des méthodes d'extraction brute de l'IgY*.

Method	References
Water dilution method	
Centrifugation	Akita et al., 1992
Filtration	Kim et al., 1996
Lipoprotein precipitation	
Polyethylene glycol precipitation	Polson et al., 1980; 1985
Anionic polysaccharide precipitation	Jensenius et al., 1981; Hatta et al., 1990
Lipoprotein removal by organic solvents	Bade et al., 1984; Polson et al., 1990; Horikoshi et al., 1993
Lipoprotein removal by specific chemicals	Vieira et al., 1984; Yokoyama et al., 1993; McLaren et al., 1994

purity and activity of IgY. The water dilution method yielded IgY in the highest level (91%), purity (31%) and with similar activity to that obtained by using other methods. Deignan et al. (2000) evaluated the dextran sulphate, the phosphotungstic acid, the polyethylene glycol and the isopropanol-aceton methods. The dextran sulphate, the phosphotungstic acid methods were both the better methods with regard to IgY recovery, followed by Polson method (Polson et al., 1985). The use of isopropanol-aceton method gave the lowest recovery.

The choice of suitable IgY extraction method is mainly influenced by the quality of extraction (preservation of antibodies activity, purity and recovery of antibodies), scale of extraction (laboratory or industrial), cost effectiveness and technology. For example for a wide use of IgY in food application, a large-scale production of IgY with high recovery and purity is necessary. Such a separation process should be simple, economical and requiring few chemicals. In view of these requirements, the water dilution method appears the most appropriate technique.

The recovery of the WSF can be followed by an additional step to separate IgY (γ-livetins) from the other water-soluble proteins (α- and β-livetins), and the remaining LDL. Three kinds of separation can be used to eliminate these contaminants: precipitation, chromatography and filtration. Several of these techniques could be included in one total clean-up procedure.

2.5. IgY use for passive immunization

Passive immunity is the transfer of active humoral immunity in the form of ready-made antibodies, from one individual to another. Passive immunity can occur naturally, when maternal antibodies are transferred to the offspring. It can also be induced artificially, when high levels of antibodies specific for a pathogen or a toxin are recovered from immunized individual or from patient recovering from the infection and administered to non-immune individual. The antibodies transfer may be carried out via systemic, intravenous or oral route. This latter, is the route of choice for localized treatment of the digestive tract infections. Immunity derived from passive immunization lasts for only a short period of time as long as the antibodies remain in the organism, but it provides immediate protection.

In both humans and animals, the administration of preformed specific antibodies is an attractive approach to establish protective immunity against viral and bacterial pathogens. It is becoming a more and more interesting alternative to control the increasing number of antibiotic-resistant organisms. Passive immunization can also be used against organisms that are non-responsive to antibiotic therapy.

Antigen-specific IgY can be produced on a large-scale from eggs laid by chickens immunized with selected antigens (Hatta et al., 1997). Therefore, the use of IgY for passive immunization has been studied extensively, demonstrating its effectiveness in preventing or treating infectious diseases caused by various pathogens in animal models, especially those of the intestinal tract (**Table 2**). In this case, antibodies are usually administered in the feed in several forms: whole eggs powder, whole yolks powder, water-soluble fraction powder or purified IgY material. The utilization of preformed antigen-specific IgY for passive immunization in poultry can be schematized as presented in **figure 2**.

As we can see in the **table 2**, although positive results have been obtained against some pathogens in mice, pigs and calves, very little research has been done to assess the ability of hen-egg antibodies in reducing enteric pathogen shedding and gastrointestinal infections in poultry.

Tsubokura et al. (1997) used chicken immunoglobulin preparation from the eggs yolks of immunized hens for prophylactic and therapeutic treatment of chickens infected with *Campylobacter jejuni*. In prophylactic trial, 0.5 g of IgY preparation with a purity rate of 95% were preincubated with 10^6 CFU bacteria and administrated as a single dose to 14-days-old chickens. In the therapeutic experiment, *C. jejuni*-infected chickens were given 0.2 g of the same IgY preparation, as a single dose, 4 days after

Table 2. Effect of passive immunization by enteric pathogen-specific IgY — *Effet de l'immunisation passive par le biais de l'IgY spécifique du pathogène*.

Pathogen	Animal specie	Effects	Reference
Salmonella	Mice	Preventing mice challenged with *S*. Enteriditis from experimental salmonellosis	Peralta et al., 1994
		Protecting mice challenged with *S*. Enteritidis or *S*. Typhimurium from experimental salmonellosis	Yokoyama et al., 1998a
	Calves	Preventing fatal salmonellosis in neonatal calves exposed with *S*. Typhimurium or *S*. Dublin	Yokoyama et al., 1998b
	Laying hens	Reducing *S*. Enteritidis contaminated eggs rate in experimentally infected hens	Gürtler et al., 2004
		Elimination of *S*. Enteritidis shedding in faeces	Kassaify et al., 2004a
	Broiler chickens	Failure in the elimination of the intestinal colonization by *S*. Enteritidis	Wilkie, 2006
		Reducing caecal colonization and faecal shedding in *S*. Enteritidis challenged chickens	Rahimi et al., 2007
Campylobacter jejuni	Broiler chickens	Inhibiting the faecal shedding of *C. jejuni*	Tsubokura et al., 1997
		Reducing the faecal shedding of *C. jejuni*	Tsubokura et al., 1997
		Failure in the elimination of the intestinal colonization by *C. jejuni*	Wilkie, 2006
Escherichia coli	Pigs	Curing diarrhoea affected piglets in a field study	Wiedemann et al., 1991
		Preventing K88+, K99+, 987P+ ETEC infection in neonatal piglets	Yokoyama et al., 1992
		Protecting pigs challenged with K88+ ETEC from *E. coli*-induced enterotoxernia	Yokoyama et al., 1993
		Inhibiting shedding of F18+ *E. coli* in infected piglets	Imberechts et al., 1997
		Reducing intestinal colonization by F18 + ETEC in infected weaned pigs	Zúniga et al., 1997
		Prevention of ETEC K88+ infection in neonatal and early weaned piglets	Marquardt et al., 1999
	Calves	Protecting neonatal calves from fatal enteric colibacillosis by K99-piliated ETEC	Ikemori et al., 1992
	Rabbits	Preventing diarrhoea in rabbits challenged with ETEC	O'Farrelly et al., 1992
Rotavirus	Mice	Preventing murine rotavirus (MRV)	Yolken et al., 1988
		Preventing human rotavirus (HRV)-induced gastroenteritis in mice	Ebina et al., 1990
		Preventing mice from HRV-induced diarrhoea	Hatta et al., 1993
		Protecting mice from bovine rotavirus (BRV)-induced diarrhoea	Kuroki et al., 1993
		Preventing HRV-induced gastroenteritis in mice	Ebina et al., 1996
	Calves	Protecting calves from BRV disease	Kuroki et al., 1994
		Preventing BRV-induced diarrhoea in neonatal calves	Özpinar et al., 1996
Coronavirus	Calves	Protecting neonatal calves from bovine Coronavirus (BCV)-induced diarrhoea	Ikemori et al., 1997

contamination. A marked prophylactic effect was noted using passive oral administration of anti-*C. jejuni* IgY in that it resulted in a 99% decrease in the number of bacteria in faeces as compared to controls. 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 approach in the elimination of this infection in an industrial or farm setting.

Gürtler et al. (2004) investigated the influence of oral application of anti-*S*. Enteritidis egg yolk antibodies on the *Salmonella* contamination rate of eggs laid by experimentally infected hens at an infection dose of $2x10^8$ CFU per hen. Antibodies were administrated as a whole egg powder, mixed with feed, at dose of 3 g per day per hen (~2.5% of the feed) for 23 or 26 days. In the experimental group 13.3% of the eggs were *S*. Enteritidis positive, which was significantly different from 29.4% *S*. Enteritidis-positive eggs in the control group. These results indicated that oral administration of whole egg powder containing *S*. Enteritidis-specific antibodies could reduce the rate of *S*. Enteritidis contaminated eggs

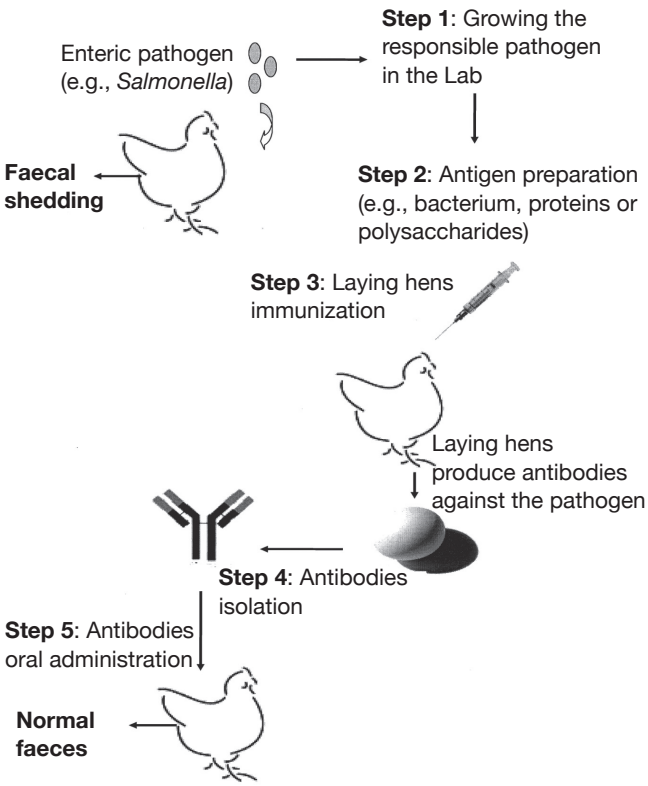

Figure 2. Summary of the egg yolk antibodies utilization for passive immunization in poultry — *Sommaire de l' utilisation des anticorps du jaune d' œuf en immunisation passive chez la volaille*.

and offer a potential tool for controlling *S*. Enteritidis infection.

In multi-trials study, Kassaify et al. (2004a) examined the effects of feed supplementation with non-immunized egg yolk powder and immunized egg yolk powder (containing anti-*S*. Enteritidis antibodies) on the elimination of *S*. Enteritidis infection in laying hens. Hens were orally infected with 10^9 CFU *S*. Enteritidis. Four weeks after the bacterial challenge, hens were given daily a supplemented feed of 15% (wt/wt) 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. In another work, Kassaify et al. (2004b) investigated the efficacy of non-immunized egg yolk powder supplement in controlling the colonization of laying hens with *S*. Typhimurium, *C. jejuni*, and *E. coli*. In the elimination study, four weeks after the bacterial challenge, hens were given the non-immunized egg yolk powder at concentrations ranging between 1 and 10% (wt/wt) for *S*. Typhimurium and *C. jejuni* challenge tests, and between 5 and 10% (wt/wt) for *E. coli* challenge test. Egg yolk powder at concentration higher than or equal to 5% (wt/wt) was capable to eliminate *S*. Typhimurium or to reduce significantly the bacteria shedding after 2 weeks of feeding. Egg yolk powder at concentration higher than or equal to 7.5% (wt/wt) is also capable to reduce significantly the colonization of *C. jejuni* and *E. coli* after 1 week of feeding. In the prevention trial, hens were fed supplemented feed (containing 10% of egg yolk powder) for 4 weeks and were then infected with the same pathogens. *S*. Typhimurium was prevented from colonization the intestine throughout the four-weeks following the challenge test, and *C. jejuni* and *E. coli* populations in the intestine were significantly suppressed. These two studies are the first and only studies to demonstrate that oral administration of non-immunized egg yolk powder can reduce the frequency of colonization of foodborne pathogens and prevent these organisms from colonizing the intestinal tract, and to suggest that the egg yolk contains other anti-infectious factors besides IgY. Subsequent *in vitro* work conducted by the authors (Kassaify et al., 2005) identified the protective yolk fraction against the foodborne pathogens as the granule component, HDL. However, previous studies suggested that other egg yolk components are the protective factors. Indeed, an antibacterial activity of fractionated hen egg yolk LDL from non-immunized egg yolk against two pathogenic *Streptococcus* strains *in vitro* had been reported by Brady et al. (2002). Furthermore Sugita-Konish et al. (2002) suggested that egg yolk plasma derived sialyoligosaccharides (YDS) and their derivatives are useful in preventing *Salmonella* infection when ingested continuously.

Wilkie (2006) studied the effectiveness of using hen egg antibody administration on *C. jejuni* and *S*. Enteritidis colonization in the gastrointestinal tract of broiler chickens. In separate trials, day-of-hatch broiler chicks were experimentally infected with either *S*. Enteritidis or *C. jejuni*, and following challenge, anti-*S*. Enteritidis or anti-*C. jejuni* IgY were administered in form of spray-dried egg yolks powder mixed in feed at 0.05% (wt/wt) or via oral gavage with either phosphate buffered saline extracted (1/10 diluted) or ammonium-sulphate precipitated (10x concentrated) antibody. On days 4, 7, and 11 jejunum, ileum, and cecum contents were collected and plate counts obtained for the appropriate pathogen. Despite investigator observed a measurable IgY activity *in vivo* and *in vitro*, they were unable to demonstrate any significant reduction in the intestinal colonization by either *S*. Enteritidis or *C. jejuni*.

Recently, Rahimi et al. (2007) examined the effect of *S*. Enteritidis-specific IgY administration on the bacterial fecal shedding, for 42 days of experiment, in 3-days old experimentally infected birds. Animals received 15 ml of yolk contained antibody mixed per 3.84 ml of drinking water on day 1 and continuing for

duration of the experiment. Compared to the control animals, who did not receive antibodies, the treated animals had significantly lower faecal shedding (0% *versus* 14%) and lower cecal concentration of *S*. Enteritidis (0.27 $\log_{10}$ CFU *versus* 3.98 $\log_{10}$ CFU). They also had a lower isolation of *S*. Enteritidis from the liver, spleen and ileum. Under the condition of this study, the use of *S*. Enteritidis-specific IgY had a beneficial effect in reducing the colonization of *Salmonella* in market-aged broiler.

The mechanism of action for orally applied IgY for pathogen reduction is still unknown. According to Peralta et al. (1994) and Tsubokura et al. (1997) antibodies are not characterized by either a bactericidal or a bacteriostatic impact. In contrast, many *in vitro* studies demonstrated that specific IgY have an inhibitory effect on bacterial growth of *Salmonella* spp. (Lee et al., 2002a; Chalghoumi et al., 2009) and *E. coli* (Sunwoo et al., 2002; Amaral et al., 2008; Wang et al., 2008). Agglutination, which is the interaction between antibodies and particulate antigens resulting in visible clumping, may be one mediator of growth inhibition; bacteria competing with each other in large aggregates conceivably grow more slowly than their free-swimming, single counterparts. However, agglutination may not be an important mediator of growth inhibition since the steric hindrance of two Fab arms of IgY precludes the cross-linking of bacteria (Kubo et al., 1973; Gallagher et al., 1974).

The major mode of action is obviously the binding of antibodies to certain specific components on the bacterial surface such as outer membrane protein (OMP), lipopolysaccharide (LPS), flagella, and fimbriae (or pili). It is hypothesized that these cell surface components can easily be recognized and bound by antibodies. This binding may lead to the impairment of the biological functions of those components that play an essential role in the bacterial growth (Sim et al., 2000) and attachment to the intestinal cells (Yokoyama et al., 1998b). In this way, the antibodies protect against adhesion of bacteria at the intestinal cells (Sugita-Konishi et al., 2000; Girard et al., 2006; Chalghoumi et al., 2009) and prevent the invasion into epithelial cells (Sugita-Konishi et al., 2000).

3. CONCLUSION

Salmonella is an important concern to the poultry industry mainly because of the associated contamination of poultry products for human consumption. With the EU ban on sub-therapeutic antibiotic usage in livestock industry, there is increasing interest in finding alternatives to antibiotics for poultry production. Passive immunization is one of those strategies. Egg yolk antibody prophylaxis and therapy have a natural place in poultry industry. In poultry, maternal antibodies are transmitted to the offspring via the yolk of the egg.

Although the above success stories of prophylactic and therapeutic use of IgY in experimental models are undisputed, it would be an exaggeration, today, to consider oral IgY therapy as a common practice in livestock production, especially in poultry.

This is undoubtedly linked to the fact that the results of experimental application of these antibodies in poultry have not always been consistent. Another interesting point is that the number of studies concerning possible beneficial effect of the use of IgY in poultry is quite low compared to studies in other animal species. Indeed, despite the fact that the European legislation does not prohibit the use of egg products in feed, since the mad cow crisis, there is still a wide spread misconception that egg yolk antibodies can not be used in chicken feed, just like animal flour in cattle feed.

Furthermore, the production of standardized IgY, although theoretically and technically relatively simple, is labour demanding and, thus, cost intensive. Therefore, producing highly specific IgY in the most cost-effective manner possible is a key to justify commercial application of these antibodies. For example, this can be achieved by improving the immunostimulatory effect and the choice of the adjuvant used in IgY production in order to maximize the production of specific IgY (Lévesque et al., 2007). It may be possible as well to develop IgY directed against a mixture of poultry common pathogen microorganisms (Chalghoumi et al., 2008).

Another important concern is stability of these antibodies in the gastrointestinal tract when they are fed to poultry. Orally administered antibodies, like any other protein, are susceptible to denaturation by the acidic pH of the stomach and degradation by proteases. Although, it has been shown that some immunological activity of the administrated antibodies remained detectable in the caecum, it is imperative to protect these antibodies in order to increase the fraction of immunoreactive antibodies delivered locally in the gastrointestinal tract. It has been demonstrated that microencapsulation may be an effective method for protecting IgY from gastrointestinal inactivation (Chang et al., 2002; Cho et al., 2005; Kovacs-Nolan et al., 2005), but it will induce certainly additional cost. Given that the use of IgY in the form of whole egg yolk powder might provide protection to IgY against the gastrointestinal conditions (Jaradat et al., 2000), this way of incorporation in feed seems to be the most practical and economic one, especially since it has been shown that anti-bacterial properties were associated with egg yolk components other than the IgY. Among these components are the granule HDL (Kassaify et al., 2005), the plasma LDL (Brady et al., 2002), and

the YDS (Sugita-Konish et al., 2002). However, the anti-bacterial properties of these components are not yet confirmed.

Evidence suggest that with the increasing resistance of microorganisms to antibiotics, research on all aspects related to the development of specific IgY against pathogenic microorganisms will have to be intensified.

Abbreviations

BALT: Bronchial Associated Lymphoid Tissues
CALT: Conjunctival Associated Lymphoid Tissues
FCA: Freund's Complete Adjuvant
FIA: Freund's Incomplete Adjuvant
GALT: Gut Associated Lymphoid Tissues
HDL: High-Density Lipoproteins
LDL: Low-Density Lipoproteins
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IgY: Immunoglobulin Y
MALT: Mucosal Associated Lymphoid Tissues
MHC II: Major Histocompatibility Complex
YDS: Sialyoligosaccharides
WSF: Water-Soluble Fraction

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