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Review

DNA vaccination of poultry: The current status in 2015

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ABSTRACT

DNA vaccination is a promising alternative strategy for developing new human and animal vaccines. The massive efforts made these past 25 years to increase the immunizing potential of this kind of vaccine are still ongoing. A relatively small number of studies concerning poultry have been published. Even though there is a need for new poultry vaccines, five parameters must nevertheless be taken into account for their development: the vaccine has to be very effective, safe, inexpensive, suitable for mass vaccination and able to induce immune responses in the presence of maternal antibodies (when appropriate). DNA vaccination should meet these requirements. This review describes studies in this field performed exclusively on birds (chickens, ducks and turkeys). No evaluations of avian DNA vaccine efficacy performed on mice as preliminary tests have been taken into consideration. The review first describes the state of the art for DNA vaccination in poultry: pathogens targeted, plasmids used and different routes of vaccine administration. Second, it presents strategies designed to improve DNA vaccine efficacy: influence of the route of administration, plasmid dose and age of birds on their first inoculation; increasing plasmid uptake by host cells; addition of immunomodulators; optimization of plasmid backbones and codon usage; association of vaccine antigens and finally, heterologous prime-boost regimens. The final part will indicate additional properties of DNA vaccines in poultry: fate of the plasmids upon inoculation, immunological considerations and the use of DNA vaccines for purposes other than preventing infectious diseases.

1. Introduction

There is a need to develop new, alternative vaccines for poultry. First, some conventional inactivated or attenuated vaccines are difficult to produce, store or distribute widely [1–3] or are costly [2,4]. Some of these conventional vaccines are not fully efficient due, for example, to the presence of maternal antibodies in young birds [5,6] or to the existence of multiple pathogenic strains and/or the emergence of new strains [7–10]. Furthermore, there is a risk with some conventional vaccines of reversion to virulence in certain cases [3,11] or of adverse reactions, especially in young birds [2,12,13]. Several vaccines offer no way of differentiating infected from vaccinated animals (DIVA) [13]. DNA vaccination is a suitable alternative to conventional treatments. It consists in inoculating plasmid DNA encoding the vaccine antigen that will be produced directly within the injected animal, the vaccine inducing humoral and cellular immune responses [14]. DNA vaccines can be suited to mass vaccination and are non-infectious, safe, inexpensive and DIVA. They can integrate multi-epitopes or modified epitopes and can be stably stored for several weeks or months, even at room temperature [15]. Furthermore, DNA vaccination may be used to replace drug treatment against coccidiosis to limit chemical residues in the meat [2,16,17].

2. Early and ongoing interest in poultry DNA vaccination

In 1990, Wolff et al. showed that injecting DNA plasmid in mouse muscle resulted in a significant expression of the protein encoded by the plasmid [18]. This was the first step in DNA vaccination. Studies were then carried out to develop DNA vaccines for humans and animals, including avian species. Progress in this field has resulted in the marketing of four mammalian or fish DNA vaccines or plasmid-related products since 2005 (West Nile Innovator, Canine Melanoma Vaccine, Apex-IHN and Life Tide-SW5). However, despite some of the first reports on DNA vaccination were

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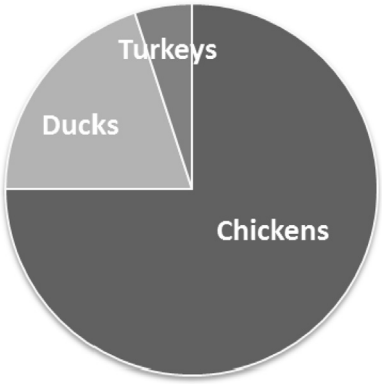

Fig. 1. Relative proportion of DNA vaccine studies performed in chickens, ducks and turkeys.

describing vaccines against avian influenza virus (AIV) in chickens [19] and huge, constant, effort made until now, no poultry DNA vaccine has reached the commercial development stage.

3. Different poultry DNA vaccination models

About 75% of poultry DNA vaccine studies in the literature were performed in chickens, 20% in ducks and the remainder are in turkeys (Fig. 1). About 75% of the studies concern viruses, 20% *Eimeria* and the remainder bacteria (Fig. 2). The main objective of these vaccines is to prevent infectious diseases.

The first DNA vaccine that was studied in poultry in 1993 was directed against AIV [19]. Thereafter DNA vaccination against AIV has been widely studied in chickens [6,19–28], and more modestly in ducks [10] (Table 1). Promising results have been obtained for this model. For example, 16 out of 17 chickens were protected from a very severe challenge after a single injection of plasmids expressing the HA protein [6]. A DNA vaccine carried by a salmonella vector has also proved effective as an oral vaccine to increase the levels of $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells on days 14 and 28 post-vaccination, to induce hemagglutinin inhibition and the production of Th1 cytokines [27]. DNA vaccination against infectious bursal disease virus (IBDV) has been also widely studied in chickens [5,9,29–36]. Very promising results have also been obtained here. Full protection against a highly virulent IBDV infection was induced by a DNA prime-recombinant protein boost regimen (with the VP2 antigen as a vaccine antigen) [35]. In ovo DNA vaccination (with the VP243 antigen) priming followed by a booster vaccination with a killed IBDV vaccine resulted in a complete protection against an experimental protection [34]. DNA vaccination against infectious bronchitis virus (IBV) has also been widely studied [7,37–41].

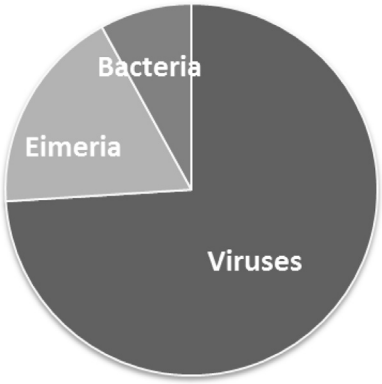

Fig. 2. Relative proportion of poultry DNA vaccine studies performed on viruses, eimeria and bacteria.

For example, between 60 and 90% of protection against experimental infections were achieved if plasmids encoding the S1, N and/or M proteins of the virus were inoculated [41]. In ducks, the most widely studied virus is duck hepatitis B virus (DHBV) as a model of chronic human hepatitis infection [42–47]. It has mainly been used to evaluate new treatments based on DNA vaccination (DNA vaccination alone or combined to lamivudine or entecavir). This resulted in the induction of anti-viral effects against chronical infection. DNA vaccines against other chicken viruses have only been evaluated more occasionally, and have met with mixed success. A DNA vaccine encoding avian reovirus σ protein resulted in 66.7–75% of protection [48]. Intranasal (IN) inoculation of a DNA vaccine encoding the F protein of Newcastle disease virus (NDV) and adsorbed to chitosan induced complete protection [49]. For the Marek disease virus, a DNA vaccine composed of a bacterial artificial chromosome encoding the herpesvirus genome permitted to 55% of the chickens to survive a highly virulent challenge (no survivors in the control group) [50]. Likewise, a DNA vaccine against infectious laryngotracheitis virus encoding the glycoprotein B resulted in the decrease of the mortality from 65–75% to 0% [12]. Finally, a DNA vaccine encoding the major component of the virion envelop gp90 of the reticuloendothelis virus induced the production of neutralizing antibodies and lymphoproliferation resulting in the control of the viremia after challenge [51]. Blood neutralizing antibodies, IL-2 and IFN-γ as well as proliferation of splenocytes were achieved upon DNA vaccination against chicken anemia virus (CAV) [52]. This resulted in a partial control of the viremia (13 chicken out of 15 controlled the viremia). Turkeys vaccinated with a DNA vaccine encoding the F protein of turkey rhinotracheitis produced neutralizing antibodies and the percentage of animals presenting clinical symptoms 7 days upon challenge decreased from 50–60% to 20–30% [53]. DNA vaccination with a DNA-prime protein-boost approach targeting TCoV coronavirus 4F/4R S fragment containing neutralizing epitopes reduced the number of turkeys showing clinical signs from 5/5 to 1/5 or 2/5, decreased the intensity, number and distribution of IFA-positive enterocytes and diminished the viral load in the ileum of TCoV-infected turkeys [54]. For duck viral enteritis DNA vaccination, serum IgG, IgA and neutralizing antibodies as well as bile IgA productions were induced [1]. This resulted in the survival of 80% of ducks experimentally infected with the virus. A DNA vaccine against plague virus encoding the glycoprotein C induced the production of IgG and neutralizing antibodies as well as lymphoproliferation [55].

Many publications have focused on DNA vaccination against *Eimeria* in chickens [2,4,8,15–17,56–66] (Table 2). Specific immune responses and/or relatively high levels of clinical protection were obtained against this protozoan (attenuation of body weight loss, lesion scores and oocysts ratios upon *Eimeria* experimental challenge).

Very few DNA vaccines against bacteria have been evaluated (Table 3). In turkeys, a DNA vaccine against *Chlamydophila psittaci* encoding the MOMP induced the production of low levels of antibodies and 5 turkeys out of 15 were protected against an experimental challenge [67]. In chickens, a DNA vaccine against *Campylobacter jejuni* reduced bacterial cecum shedding by 2 logs [68]. 75% of the chickens were protected against an avian *Pasteurella multocida* experimental infection if a DNA vaccine encoding the Omp H and Omp A was inoculated [69].

In conclusion, and like for mammalian DNA vaccination, many poultry DNA vaccines induced only limited immune responses and/or protection. But others like for example one against IBDV [34] or one other against NDV [49] induced almost complete protections against experimental infections. Despite these vaccines and others seem to be highly efficient, to our best knowledge none of them achieved to be commercially developed. The same observations can be done for mammalian DNA vaccination where only 4 vaccines are

Table 1
Poultry DNA vaccination against viruses.

Pathogen	Host	Antigen	Route	Major immunological outcomes	References
Avian Influenza Virus	Chicken	HA (mainly) or H5 or H7 or M	IM Or IV + SC + IP Or ID/SC Or IM Or oral	Stimulation of $CD4^+$ and $CD8^+$ T-cells, Th1 cytokines. Hemagglutinin inhibition. High levels of protection (up to 16 out of 17 chickens protected from a severe challenge).	[6,19–28]
	Duck	HA	ID/IM	Induction of virus neutralizating antibodies and haemagglutination inhibition.	[10]
Infectious Bursal Disease Virus	Chicken	VP2 or VP243	IM Or IM + ID Or IM + IP Or oral Or In ovo/IM Or oral + eye	High level of protection against an experimental challenge, up to 100%.	[5,9,29–36]
Infectious Bronchitis Virus	Chicken	S1 and/or N and/or M or unique ORF with 7 epitopes	IM Or in ovo/IM	60–90% of protection against an experimental challenge.	[7,37–41]
Duck Hepatitis B Virus	Duck	preS/S and/or C	IM	Evaluation of new treatments based on DNA vaccination for humans.	[42–47]
Avian Reovirus	Chicken	σB and/or σC	oral	66.7–75% protection against experimental challenges.	[48,92]
Chicken Anemia Virus	Chicken	VP1 and/or VP2	IM	Induction of $CD8^+$ T-cells, virus neutralization antibodies, IL-2 and IFN-γ.	[3,52,93]
Newcastle Disease Virus	Chicken	F and/or H	IN + IM Or IN Or IM	High level of protection, up to 100% protection against an experimental challenge.	[13,49,72,74,77,90]
Marek Disease Virus	Chicken	Herpesvirus genome	IM/ID	55% of survivors after a severe experimental challenge.	[50]
Infectious Laryngotracheitis Virus	Chicken	gB	IM	Decrease of mortality from 65–75% to 0%.	[12]
Reticuloendotheliosis Virus	Chicken	Gp90	IM	Induction of neutralizing antibodies. Proliferation and production of IL4 and IFN-γ by lymphocytes. Partial control of viremia after challenge (13/15 vs 2/15 in the control group).	[51]
Turkey Rhinotracheitis Virus	Turkey	F and N	IM	Reduction in the level of virus shedding and induction of antibodies Decrease of animals presenting clinical symptoms from 50–60% to 20–30%.	[53]
Turkey Coronavirus	Turkey	4F/4R fragment of TCoVS	IM	Humoral immune response and decrease of turkeys showing clinical signs from 5/5 to 1/5 or 2/5.	[54]
Duck Enteritis Virus	Duck	gB, gC, gD or UL24	IG Or IM Or IN	IgG, IgA and neutralizing antibodies. Up to 80% of survivors after challenge.	[1,86,94]
Duck Plague Virus	Duck	gC	IM Or ID	Induction of virus specific serum IgG and neutralizing antibodies as well as lymphoproliferation.	[55]

Table 2
Chicken DNA vaccination against *Eimeria*.

Antigen	Route	Major immunological outcomes	References
3-E1	IM Or SC Or in Ovo	Stimulation of T-cells. Attenuation of body weight loss and formation of oocysts after challenge.	[17,56–59]
GAM56	IM	Induction of lymphoproliferation and GAM56 specific IgG. Increase of body weight gain (from 56% to 89.7% of normal weight) and decrease of 53.7% of the oocysts upon challenge.	[2]
SO7	IM	Induction of SO7 specific IgG, but no protection.	[60]
Rho	IM	Induction of Rho-specific IgG, IL-2 and IFN-γ. Stimulation of $CD4^+$ and $CD8^+$ T-cells. Reduction of the number of oocysts (75.8%), cecal lesions and body weight loss after challenge.	[64]
TA4	IM	Stimulation of T-cells. Attenuation of body weight loss and formation of oocysts. Multivalent vaccine with TA4 from 3 strains efficient in protecting against the 3 strain infections.	[8,15,16]
MZ5–7	IM	Induction of IL-2 and IFN-γ mRNA in spleen. Restoration of body weight gain and decreases of cecal lesions (60.0–62.5%) and formation of oocysts (about 60–66%) after challenge.	[63]
CSZ2	IM	Increase of body weight gain (up to 76.2–90.8% of normal weight) and attenuation of formation of oocysts (57–80%) after challenge.	[4,61]
LDH	IM	Induction of anti-LDH IgG in the blood and several cytokines in spleen and cecal tonsil (IFN-γ, IL-2, TNFSF15, IL-17D and TGF-β4). Increase of % of $CD3^+$ T cells. Attenuation of body weight loss, duodenal lesions and formation of oocysts (53.3–57.6%) after challenge.	[99]
S401Ag	Oral	*Salmonella typhimurium* as vector. Induction of anti-S401 IgG and stimulation of the proliferation of peripheral lymphocytes. 55.0–57.5% protection after challenge.	[66]
EtMIC2	In ovo	Induction of anti-EtMIC2 IgG. Restoration of the body weight gain and decreased fecal oocyst shedding) after challenge. Vaccine antigen froma *E. tenella*: cross-protection against *E. acervulina*, but not *E. maxima*.	[65]

Table 3
Poultry DNA vaccination against bacteria.

Pathogen	Host	Antigen	Route	Major immunological outcomes	References
Chlamydophila psittaci	Turkeys	MOMP	IM + IN Or ID Or IM	Induction of IgA, IgM and IgG. Induction of lymhoproliferation. Prevention, but not always abolition, of severe clinical symptoms upon challenge. Able to circumvent maternal antibodies.	[67,71,87,95,100]
Campylobacter jejuni	Chickens	Flagellin	IN	Induction of antibodies in the serum and intestinal mucosa. Reduction in cecum shedding of about 2 logs on days 18–21 after challenge.	[68]
Avian pasteurella multocida	Chickens	OmpH and OmpA	IM	Production of serum IgG antibodies. Induction of lymphoproliferation and IFN-γ. 70–75% of survivors after challenge.	[69]

marketed despite several thousand papers were published on DNA vaccination. The reasons for this lack of marketed vaccines may be many: competition with more classical vaccines for which pharmaceutic companies are used to develop them, often need of two or three injections of vaccine (sometimes at different sites on the body, see below) which is too much and expensive for poultry, the complexity and the cost to develop heterologous prime/boost vaccines, the need of vaccines that can used for mass vaccination, the difficulty to prove the safety of DNA vaccines, . . . One other potential explanation is that specific pathogen free (SPF) birds with no passive maternal antibodies against the pathogens targeted were often used to show the efficacies of the vaccines. It has been shown that these maternal antbodies can alter the induction of immune responses upon DNA vaccination [70,71]. So it may be possible that some of the efficient vaccines described above are not efficient for conventional birds with maternal antibodies.

4. Plasmids used for poultry DNA vaccination

DNA vaccines are basically composed of plasmids encoding the vaccine antigens and saline solutions. The plasmids efficiently used for poultry DNA vaccination (chickens, ducks and turkeys) are mainly the same as for mammalian DNA vaccination (pcDNA, pVAX or pCI plasmids) and usually induce immune responses. The plasmid transcription unit is composed of a strong, ubiquitous viral promoter and a termination-polyadenylation sequence. Very few plasmid backbones were specifically developed for poultry DNA vaccination. Several authors used a pCAGGS plasmid with which antigen transcription was under the control of the chicken β-actin promoter used to enhance vaccine efficacy in chickens [6,9,20,22,35,68,72,73]. Finally, in certain cases the bicistronic plasmid pIRES simultaneously encoding two vaccine antigens [74] or one vaccine antigen and one adjuvant cytokine [12] was used.

5. The different administration routes and doses of DNA vaccines for poultry

There are four main routes of administration for poultry DNA vaccines. Three are also used for mammalian DNA vaccination [75]: intramuscular (IM) injection, intradermal/subcutaneous (ID/SC) injection or application to mucosal surfaces (oral or intranasal (IN)). The age of birds on their first inoculation varies from the day of hatching to 5–6 weeks. The fourth, in ovo, route is specific to poultry.

In the vast majority of cases (about 75%), the plasmid DNA was administered in its naked form into the chest or thigh muscles. Naked DNA in saline solution was injected directly into skeletal muscle tissue using needles. This is a practical route of injection for experimental studies, but it is not adapted to mass vaccination apart it is automatically performed on one day old chicks during their transfers from the hatchering to the growing stations. Humoral and cellular protective immune responses were induced to a greater or lesser extent. In several studies, the DNA vaccine was injected at multiple IM sites [21,42,76,77]. Some injections were alternated between right and left thigh muscles [78]. Certain authors combined the IM route with IN [13,71], intraperitoneal (IP) [30], intradermal [29] or intravenous (IV) [19] routes, for example. Oral vaccination was the second most popular route. In this case, the plasmids were either carried by attenuated bacteria such as *Salmonella typhimurium* [25,79] or *Escherichia coli* [60], or by silver nanoparticles [26]. More rarely, plasmid DNA was inoculated via IN instillations alone. In this case, plasmids had to be formulated with microparticles such as PLGA [13] or chitosan [68]. Intragastric (IG) inoculation was also described in one report [1]. Mucosal vaccination is more adapted to mass vaccination since the bacteria or the particles can be nebulized in the air or loaded in the drinking water or the food.

It is also possible to inject the DNA vaccine in ovo on embryonation day 18, which is the best opportunity for mass vaccination since it can be automatically performed. This has been applied exclusively to chickens. Nevertheless, a single injection of DNA in ovo was not very efficient at inducing strong protective immunity, as found for the IBV model [37]. However, the booster injection of live attenuated vaccines or recombinant fowlpox virus at the age of 7 days [34,80] or 14 days [37] induced a highly efficient protective immune response. A booster with DNA vaccine was also able to induce some protective immunity [65]. It is a pity to add a boost injection after the in ovo priming because it reduces the possibility to use this vaccine regimen for mass vaccination, even if it is efficient.

Between 50 and 200 μg of plasmid DNA was inoculated per injection in about 70% of the cases, whatever the bird species. In about 10% of the other studies, smaller amounts of plasmids were used if plasmid entry into cells at the injection point was forced [47], which is an interesting way to reduce the cost of the vaccination. On the other hand, few studies using over 500 μg were published, though one used up to 10 mg per injection [5]. In these last studies, the cost of the vaccine is a break to industrial development. But, and as it will be explained below, high doses of vaccines are not always injected to develop a protective vaccine for poultry, but are often performed for other purposes.

Authors usually carried out at least two injections of the DNA vaccine via the same route to induce protective immunity. Many boosters in poultry were performed mainly to transfer maternal antibodies to progeny [43], to prepare antisera for diagnostic purposes [21] or to treat chronic infections (DHBV model) [45]. There was no standard interval between each vaccination, but a range from one week [3] to four weeks [21]. The general interval was one or two weeks, which is sufficient to permit to the immune system to be sufficiently and/or efficiently stimulated.

6. Strategies to increase poultry DNA vaccine efficacy

The protective immune responses induced by DNA vaccines in poultry are often not sufficient, and therefore need to be improved. Many strategies to increase the protective immune responses of

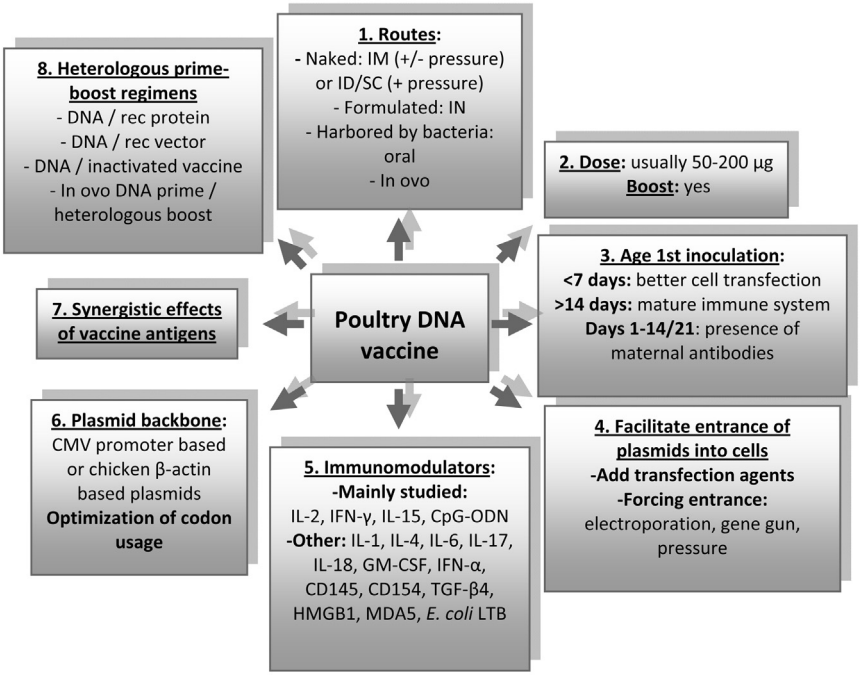

Fig. 3. The different strategies evaluated for enhancing poultry DNA vaccination.

poultry DNA vaccines have been evaluated (Fig. 3). They concern the route of inoculation, plasmid DNA dose, booster injections, age of the birds, uptake of plasmids by the host cells, addition of immune modulators, optimization of the plasmid backbone and host codon usage, association of vaccine antigens and use of a heterologous prime-boost regimen.

6.1. Optimization of the route of inoculation

In most cases, the DNA vaccine was injected via the IM route, with no reference to the underlying immunological rational. Nevertheless, some authors performed comparative studies. For naked DNA, the IM route was often described as the most efficient [15,29,81].

IN inoculation of plasmids associated with particles such as chitosan or PLGA were in some cases more efficient than an IM injection of naked plasmids [13,49]. The oral inoculation of plasmid harbored by *E. coli* was also described as more efficient than an IM injection of naked plasmids [33]. Likewise, the SC route was more efficient when the plasmids were adsorbed onto gold particles and injected with a gene gun [55,67].

6.2. Dose of plasmid DNA and influence of booster injections

In most cases, the authors indicated a quantity of plasmids inoculated, but without a clear indication for the basis of the dose selection. This dose was generally between 50 and 200 µg of plasmids. Indeed, the few authors who compared the efficacy of the vaccines induced by different doses in chickens found that the best doses for IM injections lay within the 25–200 µg range [2,15,20]. Interestingly, the best results were not always obtained with the highest doses tested, which is interesting to limit the cost of the vaccine. For example Song et al. [15] found that the protection against *Eimeria tenella* afforded by 25–50 µg of DNA vaccine was stronger than that afforded by 100–200 µg. The authors indicated that the reason why the vaccine was less effective when higher doses were injected was unclear. Likewise, Suarez et al. [20] showed that 50–100 µg of DNA vaccine against AIV was more efficient than 250 µg. The authors did not comment on these results. Nevertheless, this highlights that dose-response studies could thus be very useful for optimizing DNA vaccine efficacy. For in ovo DNA vaccination, 40–60 µg of plasmids represented an efficient dose [82].

For oral DNA vaccination with plasmids carried by *Salmonella typhimurium*, the dose of bacteria used was also of major importance. For example, Yu et al. [1] showed that 10^{11} CFU of *Salmonella typhimurium* carrying a plasmid encoding duck viral enteritis UL24 was much more efficient in inducing protection than lower doses. Likewise, 10^9 CFU of this bacteria carrying IBDV [79] or *Eimeria tenella* [66] antigen encoding plasmids was much more efficient in inducing protection against their respective pathogens than 10^7 or 10^8 CFU.

Furthermore, a single injection of DNA vaccine was often insufficient to induce protective immunity. Therefore a booster immunization was often applied. This increased, for example, the level of antibodies induced by a DNA vaccine against AIV in chickens [22]. One or two booster injections were necessary to induce protection against avian *Pasteurella* [69] or IBDV [83] infections in chickens, but they were not always needed if other efficient strategies to enhance immune responses were used, like electroporation [6].

6.3. Age of birds on their first inoculation

It may be speculated that due to the age of the birds at slaughter (about 6 weeks for broilers for example), the younger the birds are the more the vaccination is gainful. Actually, there was no standard age for the first DNA vaccination in the different published experiments. It varied from embryonation day 18 or the day of hatching to 5–6 weeks. It is notewise to indicate that until week 2 or latest week 3, it is still worth to vaccinate the birds. On the other hand, the age of 5–6 weeks is not consistent with conventional breading of broilers. Nevertheless, the age of the first injection seemed to be of key importance from an immunological point of view. For example Lim et al. [84] found that the level of antibodies against AIV was

higher in chickens vaccinated for the first time at the age of 14 days than in those vaccinated on the day of hatching. In fact, Brownlie et al. showed that innate immunity is less developed in younger chickens [85]. On the other hand, cells of the immature muscles of 7-day-old chicks were more easily transformed by the plasmids than those of the more mature muscles of 14-day-old chicks [15].

6.4. Increasing plasmid DNA uptake by host cells

In mammals, it is known that injected naked plasmids are rapidly degraded post-administration, which decreases in the same time the efficacy of the vaccine [75]. Therefore, vaccine efficacy should be improved by increasing the uptake of plasmids by the cells of the injected birds before this degradation, just as for mammals. Cell transfection agents such as cationic liposomes [29], lipofectamine, lipotaxi [20], lipofectin [72] or lipoplex [86] were sometimes added to the DNA vaccines but no beneficial effects demonstrated.

Uptake has also been enhanced mechanically. Electroporation forces plasmids to enter cells at the injection site thanks to an electric field. In poultry, it induced acute local inflammation of the injected muscle, recruited $CD3^+$ T cells [47] and increased the intensity of both humoral and cellular immune responses [6,47]. Moreover, the quantity of plasmids needed was dramatically reduced (to 10 μg in certain cases [47]). Otherwise, ID injections of naked plasmid DNA were improved by administration with a gene gun, which propels DNA adsorbed onto gold beads onto the skin's surface [55,87,88]. This increases the protective immune response and/or reduces the quantity of plasmids needed to induce the immune response (up to 1 or 2 μg [87]) if the gold particles were smaller than 1.0 μm in diameter. A high-pressure liquid injection of the plasmid DNA using, for example, the Agrojet™ [24] or Medijector™ [23] devices also proved successful. All these strategies have also the advantage to reduce the costs of the vaccines, except for the gene gun which need to produce gold particles that are expensive.

6.5. Co-injection of immunomodulators

Immunomodulators are known to improve the efficacy of vaccines. Therefore co-injection of immunomodulators is also one of the most intensively studied strategies to improve DNA vaccination efficacy in poultry. Again, most of the efforts have focused on chickens, but several published studies have investigated duck DNA vaccination.

For chickens, interleukin (IL) 2 was the most frequently used in conjunction with DNA vaccines. This cytokine increased antibody production, lymphoproliferation, the level of $CD4^+$ T cells [38,89] and more generally, the protection afforded by vaccines against IBDV [31]. Antibody production and/or clinical protection were also improved in *Eimeria* DNA vaccination models [16]. Cytokines interferon-γ (IFN-γ) or IL-15 were also frequently and successfully used. IFN-γ improved protection against *Eimeria* [61] and the humoral and cellular responses against NDV [90]. IL-15 increased, for example, the levels of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, the humoral immune response against AIV [84] and the protective response against *Eimeria* [58]. CpG DNA sequences, recognized by chicken TLR21, effectively improved the immune protective response against IBDV [91] and IBV [7]. Immunomodulators IL-1 [57], IL-4 [77], IL-6 [32], IL-17 [63], IL-18 [56], GM-CSF [39], IFN-α [56], CD145 [78], TGF-β4 [56], the high mobility group box 1 (HMGB1) [3], the melanoma differentiation associated gene 5 (MDA5) [28] and the B-subunit of *E. coli* heat labile enterotoxin (LTB) [1] were also successfully used in different models of chicken DNA vaccination.

Much fewer studies investigated this aspect of duck DNA vaccination. IFN-γ [76], IL-2 [76] and CD154 [44] were positively associated with a DNA vaccine against DHBV.

6.6. Optimization of the plasmid backbone and codon usage

In the vast majority of cases, authors used the same kind of plasmids used for mammalian DNA vaccination, and this was efficient. But in order to optimize or increase immune responses in chickens, several authors used a pCAGGS plasmid for which antigen transcription is under the control of the chicken β-actin promoter [6,9,20,22,35,68,72,73]. In most cases, the efficacy of this plasmid was not compared to a CMV promoter based plasmid. It was able to induce protective immunity to a greater or lesser degree, depending on the model studied. When compared to classic plasmids, two controversial results were obtained for vaccination against AIV: in one case it induced higher antibodies and protection [22] and in the other, lower antibodies [20]. Therefore, in light of the current knowledge, it is difficult to draw conclusions on the beneficial effect of the chicken-adapted plasmid.

Vaccine efficacy was also enhanced by optimizing host codon usage, a technique employed for DNA vaccination against AIV in ducks [10]. Furthermore, the combination of codon usage optimization and increased mRNA synthesis enhanced vaccine efficacy against the reticuloendotheliosis virus [51] and IBDV [9] in chickens.

6.7. Combination of several vaccine antigens

In the majority of cases (about 60%), authors included a single antigen in their DNA vaccine, which was often sufficient to produce good protective immunity, especially if it was associated with an optimization strategy such as electroporation [6]. This was possible since the ability of these vaccine antigens to induce strong protective immune responses were strong. Nevertheless, it is not always possible to identify vaccine antigens that are powerful enough to induce alone efficient immune responses. Therefore many authors combined two or more vaccine antigens to increase the vaccine's efficacy. In chickens, for example, the combination of IBDV VP2, VP4 and VP3 induced significantly higher protective immune responses than VP2 alone [9,29,30,32]. Likewise, the synergistic effect of two or more vaccine antigens enhanced vaccine efficacy against IBV [41], avian reovirus [92], avian *Pasteurella multocida* [69] or CAV [93] in chickens. This was also the case for the DNA vaccine against duck enteritis virus [94].

The association of antigens also induced cross-protection. This was successful for AIV (HA from three different strains [24]) and IBDV (VP2 from two strains [36]).

6.8. Heterologous prime-boost strategies

DNA vaccination alone was not always sufficiently efficient to induce protective immune responses. Therefore it was associated with a boost inoculation performed with the same antigen as a protein or carried by a recombinant live vector: this corresponds to the heterologous prime-boost strategy. Like for mammals, this strategy has been shown to be particularly effective in improving vaccine efficacy. The authors first inoculated poultry with the DNA vaccine. The subsequent booster inoculation either used a recombinant protein corresponding to the protein encoded by the DNA vaccine, or a recombinant fowlpox virus expressing this antigen [8,23,35,77,80]. The DNA prime-recombinant protein regimen was particularly efficient for an IBDV model where 100% of the chickens were protected (VP2 antigen) [35]. It also induced high protective immunity against *Eimeria* infection [8] and avian influenza [23] in chickens. In turkeys, this regimen gave some protection against *Chlamydophila psittaci*

infections [95]. Alternatively, booster immunizations with inactivated vaccines also gave good protection against AIV [25], IBDV [40] or IBV [37] infections. Furthermore, heterologous booster immunizations were also performed after in ovo DNA prime inoculations in IBDV [34,80], IBV [37] and *Eimeria* [65] models. This resulted in highly effective vaccines. Finally, a heterologous DNA prime followed by a recombinant fowlpox virus booster was successfully used for a therapeutic DNA vaccine designed to fight chronic DHBV infections in ducks [45,77].

7. Additional properties of poultry DNA vaccination

Few studies investigated how the DNA vaccine was able to induce protective immune responses in poultry, unlike the situation for mammalian vaccination [75]. Nevertheless, the fate of the plasmids upon inoculation was described in several studies. Furthermore, some immune considerations were taken into account, especially the stimulation of certain immune cells upon vaccination, maturity of the host immune system and influence of maternal antibodies on the induction of immunity. Finally, DNA vaccination can be used for other applications than to induce protective immunity.

7.1. Fate of the plasmids upon DNA vaccine inoculation

Contrary to what has been done for mammals, there are few studies describing the fate of the plasmids after DNA vaccination of birds. In chickens, the plasmids were found in the blood, liver, spleen, lungs, kidney, muscle and heart on days 5 and 15 post-immunization [40,59]. This is quite different to what was observed in mammals, where tissues other than the injected muscles were found transiently positives only during the first one to seven days after injection, whereas plasmid DNA was detected only in the injected muscle for up to three weeks or more [75]. No association of plasmid DNA with genomic DNA (as a marker of the integration of the plasmid DNA into the host genome DNA) or transfer of the plasmid DNA to bacteria in chicken faeces (as a marker of the transfer of DNA plasmids in environment) were observed [59]. If *Salmonella* bacteria were used as vectors for DNA vaccines, the plasmids were found from 3 to 10 days [92] or even 6 weeks [66] after oral inoculation in the spleen, liver and cecum. For in ovo chicken vaccination, the amniotic fluid was completely free of plasmids within 2 h [80]. Nevertheless, a sufficient quantity of plasmids reached the embryo and was distributed throughout the body [65].

In ducks, the plasmids were found in the injected muscle, liver, kidney, lungs, heart, cecum and spleen from 1 h to 10 days after IM injection [86]. The concentration of plasmids was higher in the injected muscle.

7.2. Some immune considerations

DNA vaccination stimulated both humoral and cellular immune responses in birds. This was observed for example for NDV [49], IBDV [9], AIV [27] or *Eimeria* [2] DNA vaccination. The plasmids were either inoculated intramuscularly, intranasally or orally (harbored by *Salmonella*).

The stimulated immune cells were rarely characterized. It has been shown that $CD8^+$ and $CD4^+$ T cells proliferated in chickens [27,38,41] and ducks [94].

It has also been shown that the innate immune system is immature in young chickens [85] and is fully mature in 4-week-old birds. Therefore, the young birds need passive maternal antibodies against some pathogens for protection during the first two to three weeks of age. In most cases, authors used SPF birds or birds without maternal antibodies against the pathogen targeted by the DNA vaccine. In real life, these vaccines may be used in young birds with maternal antibodies. However, few studies were performed to study the influence of maternal antibodies on the induction of immunity by DNA vaccines. It has been shown that, contrary to mammals, maternal antibodies hindered the induction of an effective protective immune response. For example, Hsieh et al. had to inject a massive dose of 7.5 mg of a DNA vaccine at least three times to induce protection against IBDV [5]. In SPF chickens, only two injections of 100–400 μg of the same kind of DNA vaccine were sufficient for good protection [9,30,83]. Likewise, maternal antibodies hindered the induction of protective immunity against NDV [74]. Finally, in turkeys, the presence of maternal antibodies affected the production of antibodies induced by a DNA vaccine against *Chlamydophila psittaci*, but not the cellular and/or protective immune responses [70,71].

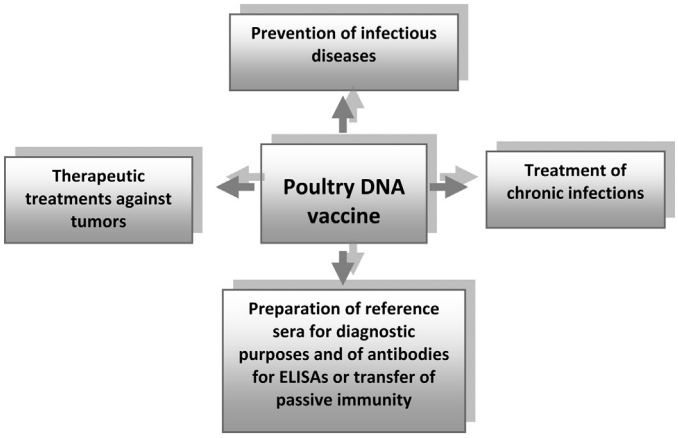

Fig. 4. The different applications of poultry DNA vaccination.

7.3. Other applications of DNA vaccination in poultry

In most cases, DNA vaccination was considered to induce preventive protective immune responses against infectious diseases. Nevertheless, DNA vaccination was also carried out for other purposes (Fig. 4). It was used to test new treatments combining a DNA vaccine and drugs against chronic infections induced by DHBV [45]. DNA vaccines were also used to produce reference sera for diagnostic purposes, such as determining AIV strains [21], and to produce antibodies for ELISA (anti-HSP70 [96]). They were furthermore used for transferring passive immunity against the white spot syndrome virus to shrimps [97]. Finally, tumor growth induced by the v-src gene was retarded by DNA vaccination in chickens [98].

8. Concluding remarks

DNA vaccination is an appropriate and simple concept that could be developed to better advantage in poultry vaccination, especially for chickens, ducks and turkeys. It has most often been evaluated in the literature in relation to fighting viral infections, but promising results have also been obtained for anti-bacterial and anti-protozoan properties. Several strategies designed to enhance the protective immune response have also been shown to be successful. Some would be very useful for mass vaccination, such as DNA vaccines transported by *Salmonella typhimurium*, in ovo or IN vaccination. However, despite the substantial efforts made since 1993, no vaccine has reached the industrial development stage. This may be due to the fact that the vast majority of vaccines were injected intramuscularly and needed at least two injections, which is not feasible in the field. Furthermore, the assays were mainly

performed with SPF birds. In real life, young birds may harbor passive maternal antibodies against the pathogen of interest that have been shown to hinder, at least partially, the induction of immunity. Protection was nonetheless achieved in a number of cases, proving that DNA vaccination in the presence of maternal antibodies is still possible, at least against certain pathogens. Therefore, further efforts should be made to develop DNA vaccines suited to the mass vaccination of birds with or without maternal antibodies.

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References

[1] Yu X, Jia R, Huang J, Shu B, Zhu D, Liu Q, et al. Attenuated *Salmonella typhimurium* delivering DNA vaccine encoding duck enteritis virus UL24 induced systemic and mucosal immune responses and conferred good protection against challenge. Vet Res 2012;43:56.
[2] Xu J, Zhang Y, Tao J. Efficacy of a DNA vaccine carrying *Eimeria* maxima Gam56 antigen gene against coccidiosis in chickens. Kor J Parasitol 2013;51:147–54.
[3] Sawant PM, Dhama K, Rawool DB, Wani MY, Tiwari R, Singh SD, et al. Development of a DNA vaccine for chicken infectious anemia and its immunogenicity studies using high mobility group box 1 protein as a novel immunoadjuvant indicated induction of promising protective immune responses. Vaccine 2015;33:333–40.
[4] Shah MA, Song X, Xu L, Yan R, Li X. Construction of DNA vaccines encoding *Eimeria acervulina* cSZ-2 with chicken IL-2 and IFN-gamma and their efficacy against poultry coccidiosis. Res Vet Sci 2011;90:72–7.
[5] Hsieh MK, Wu CC, Lin TL. DNA-mediated vaccination conferring protection against infectious bursal disease in broiler chickens in the presence of maternal antibody. Vaccine 2010;28:3936–43.
[6] Ogunremi O, Pasick J, Kobinger GP, Hannaman D, Berhane Y, Clavijo A, et al. A single electroporation delivery of a DNA vaccine containing the hemagglutinin gene of Asian H5N1 avian influenza virus generated a protective antibody response in chickens against a North American virus strain. Clin Vaccine Immunol 2013;20:491–500.
[7] Tian L, Wang HN, Lu D, Zhang YF, Wang T, Kang RM. The immunoreactivity of a chimeric multi-epitope DNA vaccine against IBV in chickens. Biochem Biophys Res Commun 2008;377:221–5.
[8] Ding J, Qian W, Liu Q, Liu Q. Multi-epitope recombinant vaccine induces immunoprotection against mixed infection of *Eimeria* spp. Parasitol Res 2012;110:2297–306.
[9] Li K, Gao L, Gao H, Qi X, Gao Y, Qin L, et al. Codon optimization and woodchuck hepatitis virus posttranscriptional regulatory element enhance the immune responses of DNA vaccines against infectious bursal disease virus in chickens. Virus Res 2013;175:120–7.
[10] Guionie O, Niqueux E, Amelot M, Bougeard S, Jestin V. H5-based DNA constructs derived from selected highly pathogenic H5N1 avian influenza virus induce high levels of humoral antibodies in Muscovy ducks against low pathogenic viruses. Virol J 2014;11:74.
[11] Fu Y, Chen Z, Li C, Liu G. Protective immune responses in ducklings induced by a suicidal DNA vaccine of the VP1 gene of duck hepatitis virus type 1. Vet Microbiol 2012;160:314–8.
[12] Chen HY, Zhao L, Wei ZY, Cui BA, Wang ZY, Li XS, et al. Enhancement of the immunogenicity of an infectious laryngotracheitis virus DNA vaccine by a bicistronic plasmid encoding glycoprotein B and interleukin-18. Antiviral Res 2010;87:235–41.
[13] Zhao K, Li W, Huang T, Luo X, Chen G, Zhang Y, et al. Preparation and efficacy of Newcastle disease virus DNA vaccine encapsulated in PLGA nanoparticles. PLOS ONE 2013;8:e82648.
[14] Dufour V. DNA vaccines: new applications for veterinary medicine. Vet Sci Tomorrow 2001:1–20.
[15] Song X, Xu L, Yan R, Huang X, Shah MA, Li X. The optimal immunization procedure of DNA vaccine pcDNA-TA4-IL-2 of *Eimeria tenella* and its cross-immunity to *Eimeria necatrix* and *Eimeria acervulina*. Vet Parasitol 2009;159:30–6.
[16] Xu Q, Song X, Xu L, Yan R, Shah MA, Li X. Vaccination of chickens with a chimeric DNA vaccine encoding *Eimeria tenella* TA4 and chicken IL-2 induces protective immunity against coccidiosis. Vet Parasitol 2008;156:319–23.
[17] Ma D, Gao M, Li J, Ma C, Li G. Construction of novel cytokine by fusion of chicken IL-2 signal peptide to mature chicken IL-15 and comparison of the adjuvant effects by DNA immunization against *Eimeria* challenge. Vet Immunol Immunopathol 2013;156:114–20.
[18] Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle in vivo. Science 1990;247:1465–8.
[19] Robinson HL, Hunt LA, Webster RG. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine 1993;11:957–60.
[20] Suarez DL, Schultz-Cherry S. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Dis 2000;44:861–8.
[21] Lee CW, Senne DA, Suarez DL. Development and application of reference antisera against 15 hemagglutinin subtypes of influenza virus by DNA vaccination of chickens. Clin Vaccine Immunol 2006;13:395–402.
[22] Jiang Y, Yu K, Zhang H, Zhang P, Li C, Tian G, et al. Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector. Antiviral Res 2007;75:234–41.
[23] Le Gall-Recule G, Cherbonnel M, Pelotte N, Blanchard P, Morin Y, Jestin V. Importance of a prime-boost DNA/protein vaccination to protect chickens against low-pathogenic H7 avian influenza infection. Avian Dis 2007;51:490–4.
[24] Rao S, Kong WP, Wei CJ, Yang ZY, Nason M, Styles D, et al. Multivalent HA DNA vaccination protects against highly pathogenic H5N1 avian influenza infection in chickens and mice. PLoS ONE 2008;3:e2432.
[25] Pan Z, Zhang X, Geng S, Fang Q, You M, Zhang L, et al. Prime-boost immunization using a DNA vaccine delivered by attenuated *Salmonella enterica* serovar typhimurium and a killed vaccine completely protects chickens from H5N1 highly pathogenic avian influenza virus. Clin Vaccine Immunol 2010;17:518–23.
[26] Jazayeri SD, Ideris A, Zakaria Z, Shameli K, Moeini H, Omar AR. Cytotoxicity and immunological responses following oral vaccination of nanoencapsulated avian influenza virus H5 DNA vaccine with green synthesis silver nanoparticles. J Control Release 2012;161:116–23.
[27] Jazayeri SD, Ideris A, Zakaria Z, Yeap SK, Omar AR. Improved immune responses against avian influenza virus following oral vaccination of chickens with HA DNA vaccine using attenuated *Salmonella typhimurium* as carrier. Comp Immunol Microbiol Infect Dis 2012;35:417–27.
[28] Liniger M, Summerfield A, Ruggli N. MDA5 can be exploited as efficacious genetic adjuvant for DNA vaccination against lethal H5N1 influenza virus infection in chickens. PLOS ONE 2012;7:e49952.
[29] Li J, Huang Y, Liang X, Lu M, Li L, Yu L, et al. Plasmid DNA encoding antigens of infectious bursal disease viruses induce protective immune responses in chickens: factors influencing efficacy. Virus Res 2003;98:63–74.
[30] Kim SJ, Sung HW, Han JH, Jackwood D, Kwon HM. Protection against very virulent infectious bursal disease virus in chickens immunized with DNA vaccines. Vet Microbiol 2004;101:39–51.
[31] Li J, Liang X, Huang Y, Meng S, Xie R, Deng R, et al. Enhancement of the immunogenicity of DNA vaccine against infectious bursal disease virus by co-delivery with plasmid encoding chicken interleukin 2. Virology 2004;329:89–100.
[32] Sun JH, Yan YX, Jiang J, Lu P. DNA immunization against very virulent infectious bursal disease virus with VP2-4-3 gene and chicken IL-6 gene. J Vet Med B: Infect Dis Vet Public Health 2005;52:1–7.
[33] Mahmood MS, Hussain I, Siddique M, Akhtar M, Ali S. DNA vaccination with VP2 gene of very virulent infectious bursal disease virus (vvIBDV) delivered by transgenic *E. coli* DH5alpha given orally confers protective immune responses in chickens. Vaccine 2007;25:7629–35.
[34] Park JH, Sung HW, Yoon BI, Kwon HM. Protection of chicken against very virulent IBDV provided by in ovo priming with DNA vaccine and boosting with killed vaccine and the adjuvant effects of plasmid-encoded chicken interleukin-2 and interferon-gamma. J Vet Sci 2009;10:131–9.
[35] Gao H, Li K, Gao L, Qi X, Gao Y, Qin L, et al. DNA prime-protein boost vaccination enhances protective immunity against infectious bursal disease virus in chickens. Vet Microbiol 2013;164:9–17.
[36] Negash T, Liman M, Rautenschlein S. Mucosal application of cationic poly(D,L-lactide-co-glycolide) microparticles as carriers of DNA vaccine and adjuvants to protect chickens against infectious bursal disease. Vaccine 2013;31:3656–62.
[37] Kapczynski DR, Hilt DA, Shapiro D, Sellers HS, Jackwood MW. Protection of chickens from infectious bronchitis by in ovo and intramuscular vaccination with a DNA vaccine expressing the S1 glycoprotein. Avian Dis 2003;47:272–85.
[38] Tang M, Wang H, Zhou S, Tian G. Enhancement of the immunogenicity of an infectious bronchitis virus DNA vaccine by a bicistronic plasmid encoding nucleocapsid protein and interleukin-2. J Virol Methods 2008;149:42–8.
[39] Tan B, Wang H, Shang L, Yang T. Coadministration of chicken GM-CSF with a DNA vaccine expressing infectious bronchitis virus (IBV) S1 glycoprotein enhances the specific immune response and protects against IBV infection. Arch Virol 2009;154:1117–24.
[40] Guo Z, Wang H, Yang T, Wang X, Lu D, Li Y, et al. Priming with a DNA vaccine and boosting with an inactivated vaccine enhance the immune response against infectious bronchitis virus. J Virol Methods 2010;167:84–9.
[41] Yan F, Zhao Y, Hu Y, Qiu J, Lei W, Ji W, et al. Protection of chickens against infectious bronchitis virus with a multivalent DNA vaccine and boosting with an inactivated vaccine. J Vet Sci 2013;14:53–60.
[42] Rollier C, Charollois C, Jamard C, Trepo C, Cova L. Early life humoral response of ducks to DNA immunization against hepadnavirus large envelope protein. Vaccine 2000;18:3091–6.
[43] Thermet A, Rollier C, Zoulim F, Trepo C, Cova L. Progress in DNA vaccine for prophylaxis and therapy of hepatitis B. Vaccine 2003;21:659–62.

[44] Gares SL, Fischer KP, Congly SE, Lacoste S, Addison WR, Tyrrell DL, et al. Immunotargeting with CD154 (CD40 ligand) enhances DNA vaccine responses in ducks. Clin Vaccine Immunol 2006;13:958–65.
[45] Miller DS, Boyle D, Feng F, Reaiche GY, Kotlarski I, Colonno R, et al. Antiviral therapy with entecavir combined with post-exposure prime-boost vaccination eliminates duck hepatitis B virus-infected hepatocytes and prevents the development of persistent infection. Virology 2008;373:329–41.
[46] Thermet A, Buronfosse T, Werle-Lapostolle B, Chevallier M, Pradat P, Trepo C, et al. DNA vaccination in combination or not with lamivudine treatment breaks humoral immune tolerance and enhances cccDNA clearance in the duck model of chronic hepatitis B virus infection. J Gen Virol 2008;89:1192–201.
[47] Khawaja G, Buronfosse T, Jamard C, Guerret S, Zoulim F, Luxembourg A, et al. Enhanced magnitude and breadth of neutralizing humoral response to a DNA vaccine targeting the DHBV envelope protein delivered by in vivo electroporation. Virology 2012;425:61–9.
[48] Wan J, Wang C, Wen X, Huang X, Ling S, Huang Y, et al. Immunogenicity of a DNA vaccine of Avian Reovirus orally delivered by attenuated *Salmonella typhimurium*. Res Vet Sci 2011;91:382–3.
[49] Zhao K, Zhang Y, Zhang X, Li W, Shi C, Guo C, et al. Preparation and efficacy of Newcastle disease virus DNA vaccine encapsulated in chitosan nanoparticles. Int J Nanomed 2014;9:389–402.
[50] Tischer BK, Schumacher D, Beer M, Beyer J, Teifke JP, Osterrieder K, et al. A DNA vaccine containing an infectious Marek's disease virus genome can confer protection against tumorigenic Marek's disease in chickens. J Gen Virol 2002;83:2367–76.
[51] Li K, Gao L, Gao H, Qi X, Gao Y, Qin L, et al. Protection of chickens against reticuloendotheliosis virus infection by DNA vaccination. Vet Microbiol 2013;166:59–67.
[52] Moeini H, Omar AR, Rahim RA, Yusoff K. Improving the potency of DNA vaccine against chicken anemia virus (CAV) by fusing VP1 protein of CAV to Marek's Disease Virus (MDV) type-1 VP22 protein. Virol J 2011;8:119.
[53] Kapczynski DR, Sellers HS. Immunization of turkeys with a DNA vaccine expressing either the F or N gene of avian metapneumovirus. Avian Dis 2003;47:1376–83.
[54] Chen YN, Wu CC, Yeo Y, Xu P, Lin TL. A DNA prime-protein boost vaccination strategy targeting turkey coronavirus spike protein fragment containing neutralizing epitope against infectious challenge. Vet Immunol Immunopathol 2013;152:359–69.
[55] Lian B, Cheng A, Wang M, Zhu D, Luo Q, Jia R, et al. Induction of immune responses in ducks with a DNA vaccine encoding duck plague virus glycoprotein C. Virol J 2011;8:214.
[56] Min W, Lillehoj HS, Burnside J, Weining KC, Staeheli P, Zhu JJ. Adjuvant effects of IL-1beta, IL-2, IL-8, IL-15, IFN-alpha, IFN-gamma TGF-beta4 and lymphotactin on DNA vaccination against *Eimeria acervulina*. Vaccine 2001;20:267–74.
[57] Lillehoj HS, Ding X, Quiroz MA, Bevensee E, Lillehoj EP. Resistance to intestinal coccidiosis following DNA immunization with the cloned 3-1E Eimeria gene plus IL-2, IL-15, and IFN-gamma. Avian Dis 2005;49:112–7.
[58] Ma D, Ma C, Pan L, Li G, Yang J, Hong J, et al. Vaccination of chickens with DNA vaccine encoding *Eimeria acervulina* 3-1E and chicken IL-15 offers protection against homologous challenge. Exp Parasitol 2011;127:208–14.
[59] Zhao Y, Bao Y, Zhang L, Chang L, Jiang L, Liu Y, et al. Biosafety of the plasmid pcDNA3-1E of *Eimeria acervulina* in chicken. Exp Parasitol 2013;133:231–6.
[60] Klotz C, Gehre F, Lucius R, Pogonka T. Identification of *Eimeria tenella* genes encoding for secretory proteins and evaluation of candidates by DNA immunisation studies in chickens. Vaccine 2007;25:6625–34.
[61] Shah MA, Song X, Xu L, Yan R, Song H, Ruirui Z, et al. The DNA-induced protective immunity with chicken interferon gamma against poultry coccidiosis. Parasitol Res 2010;107:747–50.
[62] Song H, Song X, Xu L, Yan R, Shah MA, Li X. Changes of cytokines and IgG antibody in chickens vaccinated with DNA vaccines encoding *Eimeria acervulina* lactate dehydrogenase. Vet Parasitol 2010;173:219–27.
[63] Geriletu Xu L, Xurihua Li X. Vaccination of chickens with DNA vaccine expressing *Eimeria tenella* MZ5-7 against coccidiosis. Vet Parasitol 2011;177:6–12.
[64] Liu Y, Zheng J, Li J, Gong P, Zhang X. Protective immunity induced by a DNA vaccine encoding *Eimeria tenella* rhomboid against homologous challenge. Parasitol Res 2013;112:251–7.
[65] Ding X, Lillehoj HS, Dalloul RA, Min W, Sato T, Yasuda A, et al. In ovo vaccination with the *Eimeria tenella* EtMIC2 gene induces protective immunity against coccidiosis. Vaccine 2005;23:3733–40.
[66] Du A, Wang S. Efficacy of a DNA vaccine delivered in attenuated *Salmonella typhimurium* against *Eimeria tenella* infection in chickens. Int J Parasitol 2005;35:777–85.
[67] Vanrompay D, Cox E, Volckaert G, Goddeeris B. Turkeys are protected from infection with *Chlamydia psittaci* by plasmid DNA vaccination against the major outer membrane protein. Clin Exp Immunol 1999;118:49–55.
[68] Huang JL, Yin YX, Pan ZM, Zhang G, Zhu AP, Liu XF, et al. Intranasal immunization with chitosan/pCAGGS-flaA nanoparticles inhibits *Campylobacter jejuni* in a White Leghorn model. J Biomed Biotechnol 2010:2010.
[69] Gong Q, Qu N, Niu M, Qin C, Cheng M, Sun X, et al. Immune responses and protective efficacy of a novel DNA vaccine encoding outer membrane protein of avian *Pasteurella multocida*. Vet Immunol Immunopathol 2013;152:317–24.
[70] Van Loock M, Lambin S, Volckaert G, Goddeeris BM, Vanrompay D. *Chlamydophila psittaci* DNA vaccination in turkeys in the presence of maternal antibodies. Commun Agric Appl Biol Sci 2003;68:573–8.
[71] Van Loock M, Lambin S, Volckaert G, Goddeeris BM, Vanrompay D. Influence of maternal antibodies on *Chlamydophila psittaci*-specific immune responses in turkeys elicited by naked DNA. Vaccine 2004;22:1616–23.
[72] Sakaguchi M, Nakamura H, Sonoda K, Hamada F, Hirai K. Protection of chickens with linear plasmid by vaccination with a linear plasmid DNA expressing the F protein of Newcastle disease virus. Vaccine 1996;14:747–52.
[73] Shan S, Jiang Y, Bu Z, Ellis T, Zeng X, Edwards J, et al. Strategies for improving the efficacy of a H6 subtype avian influenza DNA vaccine in chickens. J Virol Methods 2011;173:220–6.
[74] Rajawat YS, Sundaresan NR, Ravindra PV, Kantaraja C, Ratta B, Sudhagar M, et al. Immune responses induced by DNA vaccines encoding Newcastle virus haemagglutinin and/or fusion proteins in maternal antibody-positive commercial broiler chicken. Br Poult Sci 2008;49:111–7.
[75] Faurez F, Dory D, Le Moigne V, Gravier R, Jestin A. Biosafety of DNA vaccines: new generation of DNA vectors and current knowledge on the fate of plasmids after injection. Vaccine 2010;28:3888–95.
[76] Saade F, Buronfosse T, Pradat P, Abdul F, Cova L. Enhancement of neutralizing humoral response of DNA vaccine against duck hepatitis B virus envelope protein by co-delivery of cytokine genes. Vaccine 2008;26:5159–64.
[77] Sawant PM, Verma PC, Subudhi PK, Chaturvedi U, Singh M, Kumar R, et al. Immunomodulation of bivalent Newcastle disease DNA vaccine induced immune response by co-delivery of chicken IFN-gamma and IL-4 genes. Vet Immunol Immunopathol 2011;144:36–44.
[78] Zhang R, Zhang S, Li M, Chen C, Yao Q. Incorporation of CD40 ligand into SHIV virus-like particles (VLP) enhances SHIV-VLP-induced dendritic cell activation and boosts immune responses against HIV. Vaccine 2010;28:5114–27.
[79] Li L, Fang W, Li J, Huang Y, Yu L. Oral DNA vaccination with the polyprotein gene of infectious bursal disease virus (IBDV) delivered by the attenuated Salmonella elicits protective immune responses in chickens. Vaccine 2006;24:5919–27.
[80] Haygreen EA, Kaiser P, Burgess SC, Davison TF. In ovo DNA immunisation followed by a recombinant fowlpox boost is fully protective to challenge with virulent IBDV. Vaccine 2006;24:4951–61.
[81] Song KD, Lillehoj HS, Choi KD, Yun CH, Parcells MS, Huynh JT, et al. A DNA vaccine encoding a conserved *Eimeria* protein induces protective immunity against live *Eimeria acervulina* challenge. Vaccine 2000;19:243–52.
[82] Oshop GL, Elankumaran S, Vakharia VN, Heckert RA. In ovo delivery of DNA to the avian embryo. Vaccine 2003;21:1275–81.
[83] Chang HC, Lin TL, Wu CC. DNA-mediated vaccination against infectious bursal disease in chickens. Vaccine 2001;20:328–35.
[84] Lim KL, Jazayeri SD, Yeap SK, Mohamed Alitheen NB, Bejo MH, Ideris A, et al. Antibody and T cell responses induced in chickens immunized with avian influenza virus N1 and NP DNA vaccine with chicken IL-15 and IL-18. Res Vet Sci 2013;95:1224–34.
[85] Brownlie R, Zhu J, Allan B, Mutwiri GK, Babiuk LA, Potter A, et al. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol Immunol 2009;46:3163–70.
[86] Sun K, Li X, Jiang J, Cheng A, Wang M, Zhu D, et al. Distribution characteristics of DNA vaccine encoded with glycoprotein C from Anatid herpesvirus 1 with chitosan and liposome as deliver carrier in ducks. Virol J 2013;10:89.
[87] Vanrompay D, Cox E, Vandenbussche F, Volckaert G, Goddeeris B. Protection of turkeys against *Chlamydia psittaci* challenge by gene gun-based DNA immunizations. Vaccine 1999;17:2628–35.
[88] Kodihalli S, Kobasa DL, Webster RG. Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines. Vaccine 2000;18:2592–9.
[89] Kumar S, Ahi YS, Salunkhe SS, Koul M, Tiwari AK, Gupta PK, et al. Effective protection by high efficiency bicistronic DNA vaccine against infectious bursal disease virus expressing VP2 protein and chicken IL-2. Vaccine 2009;27:864–9.
[90] Yin J, Jin H, Yang F, Ding Z, Huang C, Zhu Q, et al. Synergistic effects of adjuvants interferon-gamma and levamisole on DNA vaccination against infection with Newcastle disease virus. Viral Immunol 2007;20:288–99.
[91] Mahmood MS, Siddique M, Hussain I, Khan A, Mansoor MK. Protection capability of recombinant plasmid DNA vaccine containing VP2 gene of very virulent infectious bursal disease virus in chickens adjuvanted with CpG oligodeoxynucleotide. Vaccine 2006;24:4838–46.
[92] Wan J, Wen X, Huang X, Tang Y, Huang Y, Yan Q, et al. Immunogenic analysis of two DNA vaccines of avian reovirus mediated by attenuated *Salmonella typhimurium* in chickens. Vet Immunol Immunopathol 2012;147:154–60.
[93] Moeini H, Omar AR, Rahim RA, Yusoff K. Development of a DNA vaccine against chicken anemia virus by using a bicistronic vector expressing VP1 and VP2 proteins of CAV. Comp Immunol Microbiol Infect Dis 2011;34:227–36.
[94] Zhao Y, Cao Y, Cui L, Ma B, Mu X, Li Y, et al. Duck enteritis virus glycoprotein D and B DNA vaccines induce immune responses and immunoprotection in Pekin ducks. PLOS ONE 2014;9:e95093.
[95] Loots K, Loock MV, Vanrompay D, Goddeeris BM. CpG motifs as adjuvant in DNA vaccination against *Chlamydophila psittaci* in turkeys. Vaccine 2006;24:4598–601.
[96] Brujeni GN, Gharibi D. Development of DNA-designed avian IgY antibodies for detection of *Mycobacterium avium* subsp. paratuberculosis heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal cattle. Appl Biochem Biotechnol 2012;167:14–23.

[97] Lu Y, Liu J, Jin L, Li X, Zhen Y, Xue H, et al. Passive protection of shrimp against white spot syndrome virus (WSSV) using specific antibody from egg yolk of chickens immunized with inactivated virus or a WSSV-DNA vaccine. Fish Shellfish Immunol 2008;25:604–10.
[98] Plachy JV, Hejnar JV, Trtkova K, Trejbalova K, Svoboda J, Hala K. DNA vaccination against v-src oncogene-induced tumours in congenic chickens. Vaccine 2001;19:4526–35.
[99] Song H, Yan R, Xu L, Song X, Shah MA, Zhu H, et al. Efficacy of DNA vaccines carrying *Eimeria acervulina* lactate dehydrogenase antigen gene against coccidiosis. Exp Parasitol 2010;126:224–31.
[100] Verminnen K, Loock MV, Cox E, Goddeeris BM, Vanrompay D. Protection of turkeys against *Chlamydophila psittaci* challenge by DNA and rMOMP vaccination and evaluation of the immunomodulating effect of 1 alpha,25-dihydroxyvitamin D(3). Vaccine 2005;23:4509–16.