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Efficacy of intranasal and spray delivery of adjuvanted live vaccine against infectious bronchitis virus in experimentally infected poultry

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Abstract

Live vaccines are widely used in the avian industry. Such vaccines can be either injected or delivered on animal mucosa and are usually not adjuvanted. In this study we show that live vaccines efficacy can be improved by formulation with adjuvants in a model of mucosal delivery of live infectious bronchitis vaccine in chicken. Three adjuvant technologies have been tested using intranasal and spray delivery methods to poultry. Those technologies are water in oil in water emulsion, nanoparticles and polymer adjuvants. Intranasal delivery of polymer and nanoparticles adjuvanted live vaccines improved significantly the antibody titer and protection to challenge observed compared to a commercial non-adjuvanted reference. Moreover, spray delivery of the polymer adjuvanted vaccine showed a significantly higher protection compared to the non-adjuvanted reference. Our data demonstrates that the use of Montanide\textsuperscript{TM} adjuvants in the formulation of live poultry vaccines for mucosal delivery can confer to vaccinated animals a significantly improved protection against pathogens.

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1. Introduction

Live vaccines are widely employed in veterinary practice. Generally used to vaccinate young animals [1], these types of vaccines are dedicated to a large spectrum of species with applications from farm animals such as pigs [2] or poultry [3] to companion animals such as horses [4] or pets [5]. Based on the infectious properties of the attenuated or genetically modified live micro-organism, these types of vaccines exist for bacterial [6], viral [7] and parasitic [8] pathogen models. The live pathogens delivered by vaccine inoculums are thought to initiate both humoral and cellular protective response after one delivery. Under experimental conditions, 100% efficacy has been demonstrated for live vaccine application. However, in field practice up to 10% of vaccinated animals present a lack of protection after live vaccine delivery [1]. This population represents a reservoir for pathogens and creates potential for new pathogenic strains to appear and challenge the vaccine strain in terms of herd protection. Live vaccines can also have an impact on the economical performance of herds as they will induce a transient infection necessary to trigger the immune response, and as reversions to virulence have already been observed in the field. Finally, whereas the cost of bacterial or viral vaccine production can be very low, the vaccine strains of parasitic pathogens can be very expensive to maintain and produce. Improvement of the safety and efficacy of live vaccines is therefore an important issue.

Several improvements are expected from the addition of adjuvants in live vaccine formulations. The most important would be a reduction of the antigenic dose delivered, which would lead to safety improvements, cost limitations and a better control of risks linked to the vaccination procedure. Adjuvant benefits in relation to this type of vaccine technology have already been demonstrated with model adjuvant molecules [9]. However, laboratory studies adjuvants are hardly compatible with industrial-scale production. On the contrary, the Montanide™ range of adjuvants is a well established brand of vaccine adjuvants [10] which is already used in all farm animal models at industrial scale in combination with diverse types of antigens. In this study we assessed the efficacy and safety of Montanide™ adjuvanted mucosal live infectious bronchitis (IB) vaccines in poultry.

IB virus is a coronavirus that affects airways, digestive system, kidneys and reproductive system in poultry [11]. Commercial live vaccines against IB are widely used in chicken breeding. We chose this model to study the effect of 3 families of adjuvants on the improvement of a IB commercial live vaccine in chicken delivered by individual intranasal or collective spray methods. Montanide™ ISA 201 VG (ISA 201) is a double water in oil in water emulsion that is an effective adjuvant for injectable vaccines in chicken [12], Montanide™ Gel 01 ST (Gel 01) is a polymer based adjuvant and Montanide™ IMS 1313 N VG (IMS 1313 N) consists of nanoparticles in an aqueous phase containing an immunostimulating compound. The adjuvants were used as extemporaneous diluents for the lyophilized IB antigen. We could show that Gel 01 and IMS 1313 N adjuvanted formulations improved significantly the antibody titers and protection provided by intranasal delivery of live commercial IB vaccine, whereas only the polymer adjuvanted vaccines showed a significantly better efficacy compared to the commercial reference in the spray assay.

2. Material and Methods

2.1. Animals

Trial 1: One day old SPF (specific pathogen free) chickens were purchased at Lohmann Tierzucht (Germany). Animals were seronegative to infectious bronchitis virus at day 0. 10 animals were included in each vaccine and control group.

Trial 2: 22 days old chickens of the egg cross Highsex Brown. 10 animals were included in each vaccine and control group.

All protocols were validated by internal ARRIAH’s ethics comity prior to launch according to OIE recommendations.
2.2. Antigen
The antigen used was IB virus strain H-120 from SPF egg embryos (ARRIAH). Vaccine antigen titer was log 4.0 egg infective dose 50 (EID50) per vaccine dose for trial 1 and log 7.0 EID50 per vaccine dose for trial 2. All adjuvanted test vaccines and the commercial non-adjuvanted positive control contained the same amount of antigen per dose delivered to chickens.

2.3. Adjuvants
Montanide™ IMS 1313 N VG (IMS 1313 N), Montanide™ ISA 201 VG (ISA 201) and Montanide™ Gel 01 (Gel 01) were used in this study.
Montanide™ IMS is a ready to dilute range of adjuvants consisting of liquid particles (10-500 nm) dispersed in an aqueous phase containing an immunostimulating compound. Montanide™ ISA is a ready to use range of oil adjuvants that can be used to manufacture different types of emulsions. ISA 201 allows the formulation of water-in-oil-in-water vaccines. Montanide™ Gel 01 is a ready to dilute polymeric adjuvant. It contains gel particles of sodium polyacrylate in water.

2.4. Vaccine formulations
All adjuvants were formulated extemporary to vaccination.

| Adjuvant     | Type of adjuvant | Adjuvant Ratio (weight) | Formulation process                      |
|--------------|------------------|-------------------------|------------------------------------------|
| IMS 1313 N   | Nanoparticles    | 50%                     | Manual shaking                           |
| ISA 201      | Water in oil in water emulsion | 50% | Specific procedure (available on request) |
| Gel 01       | Polymer          | 10%                     | Manual shaking                           |

A commercial live IB vaccine (strain H-120) produced by FGI ‘ARRIAH’, Russia (batch 211, control 211) was used as a positive control; this vaccine does not contain any adjuvant.

2.5. Vaccination and Experimental groups
In trial 1, 90 chickens were randomly separated in 9 groups of 10 chickens.
In groups 1 to 4, animals were vaccinated by intranasal delivery (IN) with the corresponding group test vaccine (i.e. IMS 1313 N adjuvanted vaccine, ISA 201 adjuvanted vaccine, Gel 01 adjuvanted vaccine or commercial non-adjuvanted control). Each animal received 1 antigen dose in 0.1 ml injected evenly in both nostrils.
In groups 5 to 8, animals were vaccinated by the spray method with the corresponding group vaccine (i.e. IMS 1313 N adjuvanted vaccine, ISA 201 adjuvanted vaccine, Gel 01 adjuvanted vaccine or commercial non-adjuvanted control). For each group, 2.5 cm³ of solution containing 10 doses of vaccine were sprayed over 10 animals in a box. Spray procedure was performed upon a high volume but the average of antigen dose received by the 10 vaccinated chickens was identical to the individual IN vaccination procedure dose. Group 9 (negative control) was left unvaccinated.
In trial 2, 50 chickens were randomly separated in 5 groups of 10 chickens.
In groups 1 to 4, animals were vaccinated by intranasal delivery (IN) with the corresponding group test vaccine (i.e. IMS 1313 N adjuvanted vaccine, ISA 201 adjuvanted vaccine, Gel 01 adjuvanted vaccine or commercial non-adjuvanted control). Each animal received 1 antigen dose in 0.1 ml injected evenly in both nostrils. Group 5 (negative control) was left unvaccinated.

2.6. Safety
For each trial, animal behavior was followed before and after vaccine delivery in order to identify any modification related to the vaccination procedure.

2.7. Serology
In both trials, blood samplings were performed at day 0, 7, 14, 21, 28, 35, 42, 49 and 56. Antigen specific antibodies were detected individually at each date by antigen specific ELISA (Enzyme Linked
Immunosorbent Assay). ELISA procedure was performed according to the manufacturer’s protocol. (IB antibody detection kit: ProFLOK® IBV ELISA, Synbiotics, Lyon, France).

2.8. Challenge

In both trials, an infectious challenge procedure was performed at day 56 by delivery of 0.1ml in each nostril of a highly virulent IB virus strain (M-41; titer: log 5.0 EID₅₀ per ml in trial 1, log 7.0 EID₅₀ per ml in trial 2). After challenge procedure, animals were observed over ten days post infection for IB specific clinical signs presence.

In trial 2 the strength and duration of clinical signs were followed after challenge. Briefly, the duration of illness was assessed by counting the animals presenting IB clinical signs. The strength of the infection was assessed by scoring for typical clinical signs as described by J.W. Macdonald [13]. Intensity of clinical symptoms was graduated as follows: short-breathed – 1 point; apparent short-breathed, tracheal rattle – 2 points; symptoms of respiratory failure, suppression, discharge from nostrils, tumescence of the head, rattles – 3 points. The sum of individual score was used to define a group scoring relative to the clinical signs intensity.

2.9. Statistics

Statistical analysis was performed using Student’s t tests and proportion tests. Results were considered as significantly different when P≤0.05.

3. Results

In order to assess the safety and efficacy of Montanide™ adjuvanted mucosal IB live vaccines, 8 groups of 10 1-day old SPF chickens were vaccinated with Montanide ISA 201 VG, Montanide IMS 1313 N VG, Montanide Gel 01 ST or commercial non-adjuvanted IB formulations by mucosal delivery, either via individual intranasal delivery or by multi-animals spray delivery (Trial 1). One negative control group was left unvaccinated. A kinetic of blood sampling was performed to assess the immune response and antigen titers were measured via antigen specific IgG ELISA. All animals from all groups were submitted to an IB challenge at day 56.

To confirm the potential of adjuvants for live vaccines in field conditions, the same protocol was applied to 5 groups of 10 22-day old farm chickens via intranasal delivery (Trial 2).

![Figure 1: Trial 1. Efficacy of intranasal delivery of adjuvanted live IB vaccines. (a) Antibody titers (log scale) from day 7 to day 56 post vaccination. (b) Protection to challenge: animals without symptoms observed for 10 days after challenge. (* indicate significant differences compared to the commercial reference). The control group is the non-vaccinated group.](image-url)
3.1. Trials 1 and 2: General tolerance to the adjuvanted formulations

No modification of animal behavior (social, movements, feeding) could be observed after vaccine delivery in any of the protocols. Furthermore, no IB specific clinical signs could be observed after vaccine delivery. At last, no local reactions on the mucosa (eye, nostril, mouth) to vaccine delivery could be observed after IN or spray vaccination.

3.2. Trial 1: Efficacy of spray and IN delivery of IB adjuvanted vaccine in SPF chickens.

Using IN delivery, Gel 01 and IMS 1313 N adjuvanted vaccines were able to trigger a significantly stronger humoral immune response than the non-adjuvanted commercial reference (Figure 1a). IMS 1313 N group showed a faster response than the non-adjuvanted reference. Antibody titers of the ISA 201 vaccinated group were higher but not significantly different from the commercial control. After challenge, unvaccinated animals all showed clinical signs specific of IB infection as soon as 6 days post infection (Figure 1b). Gel 01 and IMS 1313 N adjuvanted vaccines conferred an improved protection to the vaccinated animals compared to the commercial reference (Figure 1b).

Spray delivery was not as efficient as IN delivery. Antibodies titers obtained after spray vaccination were lower than the IN induced titers for all groups. No adjuvanted group showed significant improvements compared to the commercial formulation (Figure 2a). A faster onset of the response could still be observed (as soon as day 14 for adjuvanted vaccines, at day 21 for the commercial formulation). After challenge, the Montanide™ Gel 01 based formula was the only formula able to induce a high rate of protection when used in spray (88% of animals without symptoms at day 10 post challenge, figure 2b) and to have a significantly higher efficacy than the commercial formulation (<15% of protection at day 10 post challenge).

3.3. Trial 2: Efficacy of IN delivery of IB adjuvanted vaccine in farm chickens.

In 22 days old farm chicken, individual IN delivery of all Montanide™ adjuvanted vaccines was able to trigger a significantly stronger humoral immune response than the non-adjuvanted commercial reference (Figure 3).
Figure 3: Trial 2. Efficacy of intranasal delivery of adjuvanted live IB vaccines in 22d old farm chickens: Antibody titers (log scale) from day 0 (before vaccination) to day 56 after vaccination (* indicate significant differences compared to the commercial reference). Control stands for the non-vaccinated group.

Table 1: Protection to challenge: animals were observed and scored for 10 days post challenge.

| Vaccine         | Average point per chicken | Average duration of illness (days) | Percentage of sick animals 10 days post challenge |
|-----------------|---------------------------|----------------------------------|-----------------------------------------------|
| ISA 201         | 0,50±0,70                 | 1,00±0,71                        | 40                                            |
| Gel 01          | 0,30±0,48                 | 0,30±0,48                        | 50                                            |
| IMS 1313 N      | 0,20±0,42                 | 0,20±0,42                        | 30                                            |
| Commercial reference | 2,44±1,94              | 2,33±1,73                        | 89                                            |
| Unvaccinated control | 9,08±1,83               | 6,92±1,44                        | 100                                            |

In trial 2, clinical symptoms after challenge were scored following J.W. Macdonald [13]. All the unvaccinated animals presented clinical signs at a high score. The commercial vaccine formulations reduce the scoring but 89% of the animals were still positive for clinical signs at day 10 post challenge. The scoring per group was strongly reduced for all adjuvanted vaccine groups. Illness duration was also reduced to less than one day when using Montanide™ adjuvants while unvaccinated controls were sick for almost a week on average and chickens receiving the commercial formulation for 2-3 days (Table 1).

4. Discussion

New tools are increasingly needed to manage the biosafety of avian production regarding viral and bacterial diseases. Indeed, viral diseases can cause zoonotic risks, and bacterial diseases may become difficult to control due to the recent evolution of regulation toward stronger restrictions on antibiotic use. Vaccination is one of the most powerful tools to save the farming efficiency and improve the protection of herds [14]. However, it is not possible to add multiple injection vaccination steps in avian farming procedures due to the cost and time consumed by such steps in farms containing several thousands of animals. Mucosal spray vaccination could then be a very useful tool for time and cost efficient vaccination of large groups of animals [15].
We have shown previously that nanoparticles vaccine adjuvants can enhance the efficacy of avian mucosal vaccination against parasitic disease [16, 17]. In this study we have demonstrated that following individual intranasal delivery of adjuvanted formulations containing live viral vaccines, nanoparticles or polymer based adjuvant technologies were able to improve the immune response and protection to IB challenge. Both Montanide™ Gel 01 ST and Montanide™ IMS 1313 N VG adjuvants conferred a significantly enhanced protection to challenge compared to the non-adjuvanted commercial reference, in SPF and farm chickens. Montanide™ ISA 201 VG is a mineral oil adjuvant used for the formulation of water in oil in water emulsion vaccines that can be used for injectable delivery in chicken [12]. Mineral oil based adjuvants are a reference for commercial injectable poultry vaccines, and we thought that the use of an oil emulsion vaccine with an aqueous continuous phase such as ISA 201 VG could be a good vector for mucosal delivery and enhance the efficacy of mucosal vaccines. However, we show in this study that nanoparticles and polymer adjuvants give much better results for mucosal delivery in poultry.

Using spray delivery, the polymer based adjuvant was the only adjuvant formulation able to confer protection at a very high rate and to be significantly more efficient that the commercial formulation. This study shows that Montanide™ Gel 01 ST can enhance the efficacy of mass spray delivery of live viral vaccines. Spray parameters such as particle size can have an impact on spray vaccination and may explain the poor performance of IMS 1313 N in spray delivery. These parameters were not controlled here and should be optimized in further studies. Particle sizing was previously shown to be important in the antigen/immune system contact and to have and impact on the immune response [18, 19]. Further studies should also assess if the protective stimulation using spray vaccination that we observed using polymer adjuvanted vaccine was restricted to the nasal mucosa or was also linked to the oral and on eye surfaces which would lead to different immune system / antigen contact [20]. Other criteria still remain to be studied, such as the ability to manage the antigen load or to combine several antigens in the same spray vaccine.

Benefits anticipated from the use of adjuvants in live vaccines concern both safety and efficacy improvements. The use of adjuvants in live vaccine could improve the efficacy and lead to a better management of the antigen load per vaccine dose. Such efficacy improvement could also improve the safety of the vaccine as the possible adverse reactions observed after delivery of live infectious vaccines could be lowered. Moreover, the risk of reversion to virulence that has already been observed in avian species would also be reduced [21, 22]. Moreover, the use of adjuvants should reduce the number of low or not responding animals and therefore reduce the possible reservoir for the disease [1]. Adjuvanted vaccine performance could also compensate the decrease of antigenic load in case of impaired vaccine delivery or formulation, a possible viricidal effect of storage or inappropriate resuspending conditions. Finally, it would be interesting to study whether adjuvants such as polymer formulae can also improve the survival and transmission of live vaccines.

Our work underlines the ability to use polymer adjuvants in mass vaccination for avian species, opening doors to improvements of live avian vaccines safety and efficacy.

5. References

[1] Cavanagh D. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol 2003;32:567-582.
[2] Dong XN, Chen YH. Marker vaccine strategies and candidate CSFV marker vaccines. Vaccine 2007;25:205-230.
[3] Rauw F, Gardin Y, Palya V, van Borm S, Gonze M, Lemaire S, Van den Berg, T, Lambrecht, B. Humoral, cell-mediated and mucosal immunity induced by oculo-nasal vaccination of one-day-old SPF and conventional layer chicks with two different live Newcastle disease vaccines. Vaccine 2009;27:3631-3642.
[4] Jacobs AA, Goovaerts D, Nuijten PJ, Theelen RP, Hartford OM, Foster TJ., Investigations towards an efficacious and safe stranggles vaccine: submucosal vaccination with a live attenuated Streptococcus equi. Vet Rec 2000;147:563-567.
[5] Schultz RD, Thiel B, Mukhtar E, Sharp P, Larson LJ. Age and long-term protective immunity in dogs and cats. *J Comp Pathol* 2010;142 Suppl 1:S102-108.

[6] Frey J. Biological safety concepts of genetically modified live bacterial vaccines. *Vaccine* 2007;25:5598-5605.

[7] Bhanuprakash V, Indrani BK, Hosamani M, Balamurugan V, Singh RK. Bluetongue vaccines: the past, present and future. *Expert Rev Vaccines* 2009;8:191-204.

[8] Dalloul RA, Lillehoj HS. Recent advances in immunomodulation and vaccination strategies against coccidiosis. *Avian Dis* 2005;49:1-8.

[9] Rauw F, Gardin Y, Palya V, Anbari S, Gonze M, Lemaire S, Van den Berg T, Lambrecht B. The positive adjuvant effect of chitosan on antigen-specific cell-mediated immunity after chickens vaccination with live Newcastle disease vaccine. *Vet Immunol Immunopathol* 2010;134:249-258.

[10] Aucouturier J, Ganne V, Laval A. Efficacy and safety of new adjuvants. *Ann N Y Acad Sci* 2000;916:600-604.

[11] Cavanagh D. Coronavirus avian infectious bronchitis virus. *Vet Res* 2007;38:281-297.

[12] Jang SI, Lillehoj HS, Lee SH, Lee KW, Park MS, Bauchan GR, Lillehoj EP, Bertrand F, Dupuis L, Deville S. Immunoenhancing effects of Montanide ISA oil-based adjuvants on recombinant coccidia antigen vaccination against Eimeria acervulina infection. *Vet Immunol Immunopathol* 2010;127:379-388.

[13] Macdonald JW, Randall CJ, McMartin DA, Dogless MD. Immunity following inoculation of the H120 and H52 vaccine strains of infectious bronchitis virus into the crop of the domestic fowl. *Avian Pathol* 1983;12:379-388.

[14] Glisson J, Kleven S. Vaccines for veterinary application. In *Poultry vaccines* 1st ed. Oxford: Butterworth-Heinemann Ltd, 1993, p165-179.

[15] Branton SL, Roush WB, Lott BD, Evans JD, Dozier WA 3rd, Collier SD, Bearson SM, Bearson BL, Pharr GT. A self-propelled, constant-speed spray vaccinator for commercial layer chickens. *Avian Dis* 2005;49:147-151.

[16] Jang SI, Lillehoj HS, Lee SH, Lee KW, Lillehoj EP, Bertrand F, Dupuis L, Deville S. Montanide™ IMS 1313 N VG PR nanoparticle adjuvant enhances antigen-specific immune responses to profilin following mucosal vaccination against Eimeria acervulina. *Vet Parasitol* 2011;182:163-170.

[17] Jang SI, Lillehoj HS, Lee SH, Lee KW, Lillehoj EP, Bertrand F, Dupuis L, Deville S. Mucosal immunity against Eimeria acervulina infection in broiler chickens following oral immunization with profilin in Montanide™ adjuvants. *Exp Parasitol*. 2011;129(1):36-41.

[18] Corbanie EA, Matthijs MG, van Eck JH, Remon JP, Landman WJ, Vervaet C. Deposition of differently sized airborne microspheres in the respiratory tract of chickens. *Avian Pathol* 2006;35:475-485.

[19] Corbanie EA, Remon JP, Van Reeth K, Landman WJ, van Eck JH, Vervaet C. Spray drying of an attenuated live Newcastle disease vaccine virus intended for respiratory mass vaccination of poultry. *Vaccine* 2007;25:8306-8317.

[20] Purswell JL, Mayer JJ, Evans JD, Branton SL, Davis JD. Eye surface area and dosage rates for spray vaccination. *Avian Dis* 2010;54:1310-1315.

[21] Guy JS, Barnes HJ, Smith L. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. *Avian Dis* 1991;35:348-355.

[22] Hopkins SR, Yoder HW Jr. Reversion to virulence of chicken-passaged infectious bronchitis vaccine virus. *Avian Dis* 1986;30:221-223.