Infectious bronchitis virus gel vaccination: evaluation of Mass-like (B-48) and 793/B-like (1/96) vaccine kinetics after combined administration at 1 day of age

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ABSTRACT Infectious bronchitis (IB) control has a strong impact on poultry farming, because of the necessary epidemiological knowledge for planning the best strategy, the optimal strain association, the priming and boosting interventions. Broiler farming is even more problematic given the short and intense productive cycle, which requires an early onset of protection against most of the infectious threats, possibly with limited respiratory post-vaccination reactions that would have a direct impact on the bird health and productivity. For this purpose, gel vaccination has been proposed as a new approach for infectious bronchitis virus (IBV) control and vaccine intake, kinetics and compatibility of combined strains administered by gel have been analyzed in this study. After gel vaccination with single and combined 1/96 and B-48 strains on 4 groups of commercial broilers, a 21-d-long experimental trial has been conducted to monitor the vaccine safety by clinical assessment and vaccine kinetics by strain-specific real-time RT-PCR on choanal cleft swabs. The vaccine strains administered by gel were safe and negligible respiratory signs were detected, even when combined. Vaccine titers were compared among groups and within the same group among a 10-bird pooled sample and 10 swabs from individually sampled birds. 1/96 strain early reached high titers in all animals, while B-48 presence was less constant even though it was detected in almost all birds before the trial end. The individual and pooled sample comparison revealed a partial overestimation of vaccine titers in the pooled samples and the loss of the prevalence data, although the trend portrayed by the pooled swabs closely followed the individual ones.

Key words: infectious bronchitis, vaccination, gel, quantification

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INTRODUCTION

Infectious bronchitis (**IB**) is probably one of the most economically relevant infectious diseases of poultry (World Bank, 2011; Assayag et al., 2012; Colvero et al., 2015). The major challenges in the disease control lie in infectious bronchitis virus (**IBV**) remarkable variability (Valastro et al., 2016), strain heterogeneity on local basis (Jackwood, 2012), and low cross-protection (De Wit et al., 2011), which hamper the management and control of the infection. Over the last years, many studies reported that Mass-like and 793/B-like-based vaccine association seems to ensure the widest heterologous protection (Cook et al., 1999; Terregino et al., 2008; Awad et al., 2015, 2016; Habibi et al., 2017) against the most widespread strains, such as QX (Franzo et al., 2017a) and Variant 2 (Franzo et al., 2017b; Lisowska et al., 2017) in Europe, where this protocol is commonly adopted (Jordan, 2017).

Despite these evidences, IBV control remains challenging and the virus is still widespread in all poultry productive types (Cook et al., 2012), suggesting that the major issue resides in suboptimal vaccination techniques or poor animal coverage, often occurring after vaccination at the farm (De Wit et al., 2010). Even though IBV is a respiratory virus, IBV vaccines have been also administered by drinking water, because IBV was proven to infect and stimulate immunity through the gut as well (Luginbuhl et al., 1955). Lately, an uptrend has been observed in the administration of combined vaccines at the hatchery (Franzo et al., 2016), where spray cabinets are commonly adopted and guarantee a more accurate coverage of the birds, which

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are easily managed individually or in boxes during the workflow. Another vaccine-delivery method is gel vaccination, which is widely adopted in coccidiosis control strategies (Danforth et al., 1997; Chapman et al., 2002) and consists in gel droplets, added with vaccines and sprinkled onto the birds. Gel droplets are quickly ingested by preening because of the dye appealing color. This approach might be advantageous because the gel could limit heat dispersion and act as a water supply (Chapman, 2000) to the chicks during the first day of life. In fact, the excessive feather moistening, which could occur after spray vaccination, could cause a significant decrease in the chick temperature, negatively affecting their initial growth period (Yahav and McMurtry, 2001; Lourens et al., 2005). The gel can also provide a stable environment for the combined administration of different vaccines, preventing possible alterations, thus gathering and limiting the number of interventions. Recently, gel vaccination has been tested also for immunization against IBV (Godoy et al., 2017; Jordan, 2017).

This study aims to evaluate the kinetics of B-48 (Mass-like) and 1/96 (793/B-like) vaccine strains co-administered by gel to day-old commercial broilers in experimental conditions, with particular attention to the interactions between the 2 different strains and vaccine titers recovered in the upper respiratory tract, assessed also by comparing pooled swabs and individual samples.

MATERIALS AND METHODS

Animals

The experimental procedures were approved by the Institutional Review Board for Animal Research (Organismo Preposto al Benessere Animale; O. P. B. A.) at the University of Padua. A total of 500 Ross 308 day-old female chicks were selected from the same batch and breeder origin and brought to the Department of Animal Medicine, Production and Health (MAPS) facilities. The birds were divided into 5 boxes to mimic hatchery conditions and vaccination procedures. Each group was assigned to different vaccination protocols according to the experimental plan:

- group 1 (A) received CEVAC IBird (Ceva Sante Animale, Libourne, France) (1/96 strain, batch 1607E4S2KLB) vaccine by gel (Cevagel, Ceva Sante Animale) administration (standard gel amount);
- group 2 (B) received CEVAC MASS L (Ceva Sante Animale) (B-48 strain, batch 0307E2S1KLA) and CEVAC IBird (1/96 strain, batch 1607E4S2KLB) vaccines by 2 separate but consecutive gel administrations (double gel amount);
- group 3 (C) received CEVAC MASS L (B-48 strain) plus CEVAC IBird (1/96 strain) vaccines mixed into a single gel administration (standard gel amount);
- group 4 (D) received CEVAC MASS L (B-48 strain) vaccine by gel administration (standard gel amount);
- group 5 (E) received a mock vaccination by spray and was maintained as a control group for the vaccine intake evaluation.

Both gel and spray vaccine solutions were added with V9030 Sterile Blue Dye (NEL Northeast Laboratory, Waterville, ME) to mark the vaccine distribution. The vaccination was performed using DESVAC ELEC KIT (Ceva Desvac Campus, Saint-Barthélemy-d'Anjou, France) for spray vaccination and DESVAC GEL DISPENSER (Ceva Desvac Campus) for gel administration. The spray kit pressure was set at 1.5 atm and 25 cc of mock vaccine solution were distributed to 100 chicks. The supplied amount of gel was 25 cc for 100 chicks. After the vaccination, the birds were left in the boxes for 30 min allowing the vaccine ingestion and were kept apart from each other to avoid vaccine contaminations.

Gel/spray Intake

The birds were observed for the first 10 min after vaccination without interfering with droplet eating and preening actions. Thirty minutes after the vaccination procedures, the chicks were individually inspected for vaccine ingestion by tongue examination and dye presence evaluation.

Bird Housing

Once evaluated, 22 birds per box from the 4 gel-vaccinated groups (A–D) were housed in 4 separate rooms under high biosecurity measures for 21 d. The entire spray-vaccinated group and other animals were dismissed from the trial. Out of each housed group, 12 birds were marked individually (from 1 to 12). All birds were clinically examined for the first 11 d, when post-vaccination adverse reactions were expected. Symptoms were recorded and scored using the following scoring system:

0-absence of clinical signs
1-clear nasal exudate
2-turbid nasal exudate
3-swollen infraorbital sinuses and frothy eyes.

The aggregate score was intended as a clinical safety measure of the vaccination protocols. Rales, conjunctivitis, and the presence of enteric signs were also evaluated and recorded. The birds were fed ad libitum and weighed at days 5, 10, 14, and 20 to monitor growth and vaccine effect.

Samples

All animals were sampled at days 1, 2, 4, 7, 10, 14, 18, and 21 for vaccine quantification. At each sampling point, choanal cleft swabs were collected from marked animals and stored individually, whereas the remaining 10 were merged to be analyzed as a pooled sample for comparison. The pooled samples and 10 (from bird 1 to 10) out of the 12 single-swab samples were processed, whereas the remaining 2 single-swab samples were stored as spare samples, in case one of the chicks marked 1 to 10 would have died.

Swabs were left air-drying for 10 min to prevent bacterial and mold growth before –80°C storage until processing. Pooled swabs were eluted in 2 mL of PBS and single-swab samples in 200 μL. RNA extraction was performed using High Pure Viral RNA Kit (Roche, Basel, Switzerland), following the manufacturer's instructions.

Real-time Reverse Transcription Polymerase Chain Reaction

Samples were tested by 2 previously published strain-specific real-time reverse transcription polymerase chain reactions (**RT-PCRs**) (Tucciarone et al., 2018) in order to quantify the vaccine titers, expressed in EID_{50}/mL. The assays were performed in parallel using SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) on LightCycler Nano Instrument (Roche).

Serology

On the first day of the trial, 20-day-old-chicks from the same batch and breeder origin were bled at the hatchery, and sera were processed by Infectious Bronchitis Virus Antibody ELISA Test Kit (IDEXX, Westbrook, ME) according to the manufacturer's instructions to evaluate maternally derived antibody levels. Samples were considered negative if showing less than or equal to 0.2 S/P absorbance ratios (corresponding to an antibody titer of $10^{2.60}$).

Statistical Analysis

For each time point, differences among groups in cumulative clinical scores, viral, and antibody titers were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney test with Bonferroni correction. Analyses were performed independently for each vaccine strain. The mean body weight of the different groups was compared at each time point and the presence of statistically significant differences was tested by ANOVA followed by Student's t-test with Bonferroni correction. For all analyses, the significance level was set at $P < 0.05$.

RESULTS

Gel/spray Intake

Few minutes after gel administration, all of the gel droplets had already been ingested by the birds in groups A, B, C, and D. Spray-vaccinated birds of group E appeared uniformly wet and colored by the sprayed dye. Thirty minutes after the vaccination, 98% of the birds from group A, 99% from group B, 94% from group C, 100% from group D, and 99% from group E showed blue coloring of the tongue. The bird coverage was comparable between the 2 application methods, reaching almost all animals.

Vaccine Safety

Neither mortality nor enteric symptoms were recorded along the trial, and few respiratory signs were observed during the 11 d of monitoring. Conjunctivitis was detected occasionally, in association with nasal discharge and lasted no more than 2 d. Rales were hardly ever recorded. Cumulatively, at day 1 group A showed a significantly higher clinical score (P-value < 0.03), with 4 birds showing clear nasal exudate and 1 turbid exudate, compared to other groups; the same was true for group D (1 bird with clear nasal exudate and 1 with turbid exudate) compared to A and B groups at day 7. No clinical signs were detected from the 11th day on (data not shown).

The bird mean weights appeared in line with the Ross 308 AP Broiler Performance Objectives from 2017 and did not highlight any difference among the groups, regardless of the applied treatment (data not shown).

Vaccine Kinetics

Single swabs and pooled samples were collected from groups A, B, C, and D to study the vaccine kinetics and relations among pooled viral titers and individual values at each sampling point. No contamination from the other vaccine strain was detected in the single strain-vaccinated groups (A and D) (Figure 1a and b). In general, 1/96 titers resulted higher than B-48 titers, with the highest values reached around day 10. All pooled samples tested positive at all sampling points with the only exception of group C at day 1 (Figure 1b). 1/96 titers showed a quadratic (parabolic) curve in all groups, peaking at day 10 (group A and B) or 14 (group C) and then decreasing. B-48 titers showed a less characteristic pattern because, after the initial rise, titers remained almost constant with minor fluctuations over time. Group D was vaccinated only with B-48 strain and reached the highest titer of all groups on the last sampling day. After remaining quite low for the first 10 d, group C titers peaked twice, at day 14 and again at day 21. Until the seventh day, group B maintained high titers, which dropped to the first day levels at the

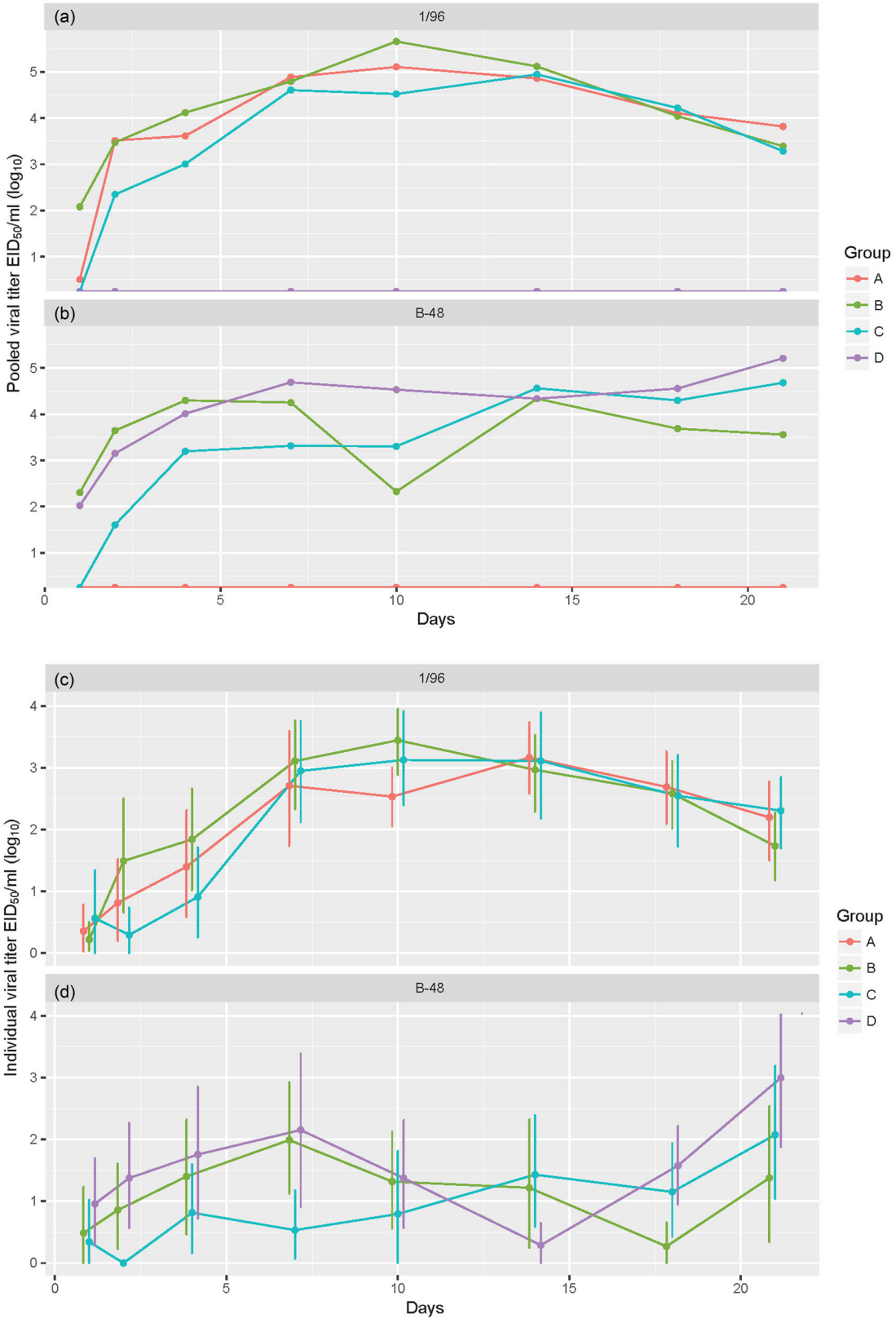

Figure 1. 1/96 and B-48 vaccine strain kinetics from pooled and individual samples for all four groups.

10th and then fluctuated until the end of the trial, displaying another peculiar behavior (Figure 1a and b).

The evaluation of single-animal samples demonstrated 1/96 vaccine presence in all the birds of group A from the fourth day of the trial and from the seventh in groups B and C until the trial end, with only 2 transitory negative results in group C at 2 different sampling points (days 14 and 18) (Table 1). For B-48 strain, the

Table 1. Real-time RT-PCR results for each group, bird, and sampling point.

B-48	Birds	Day								1/96	Birds	Day							
		1	2	4	7	10	14	18	21			1	2	4	7	10	14	18	21
B	1	-	-	-	+	+	+	-	+	B	1	+	+	+	+	+	+	+	+
	2	-	+	+	+	+	+	+	+		2	+	+	+	+	+	+	+	+
	3	-	-	+	+	-	-	-	-		3	-	-	+	+	+	+	+	+
	4	-	+	-	+	-	-	+	+		4	+	+	+	+	+	+	+	+
	5	-	+	+	+	+	+	-	-		5	-	+	+	+	+	+	+	+
	6	-	-	-	+	+	-	-	-		6	+	-	+	+	+	+	+	+
	7	-	-	-	+	-	-	-	+		7	-	+	+	+	+	+	+	+
	8	-	-	-	-	-	-	-	-		8	-	-	+	+	+	+	+	+
	9	+	+	+	+	+	-	-	-		9	-	-	-	+	+	+	+	+
	10	+	+	+	+	+	-	-	-		10	+	+	+	+	+	+	+	+
C	1	-	-	-	-	+	+	+	+	C	1	-	-	+	+	+	+	+	+
	2	-	-	-	-	-	+	+	+		2	-	-	-	+	+	+	+	+
	3	+	-	+	+	+	+	+	+		3	+	+	+	+	+	+	+	+
	4	-	-	+	-	-	-	-	-		4	-	-	+	+	+	+	+	+
	5	-	-	+	-	-	+	+	+		5	-	-	+	+	+	+	+	+
	6	-	-	+	-	-	-	-	-		6	-	-	+	+	+	+	+	+
	7	-	-	-	+	-	+	-	-		7	-	+	-	+	+	+	+	+
	8	-	-	+	+	-	+	+	+		8	-	-	-	+	+	+	-	+
	9	-	-	+	+	-	+	+	+		9	-	-	+	+	+	+	+	+
	10	-	-	-	-	+	-	-	-		10	+	+	+	+	+	-	+	+
D	1	+	+	+	+	+	+	-	+	A	1	-	-	+	+	+	+	+	+
	2	+	+	-	+	+	+	+	+		2	-	-	+	+	+	+	+	+
	3	-	-	-	-	+	-	+	+		3	+	+	+	+	+	+	+	+
	4	-	-	-	-	-	-	+	+		4	-	-	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+		5	-	-	+	+	+	+	+	+
	6	-	-	-	-	-	-	+	+		6	-	+	+	+	+	+	+	+
	7	+	+	+	+	-	-	+	+		7	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	-	+	-		8	+	+	+	+	+	+	+	+
	9	-	+	+	+	+	-	+	+		9	+	+	+	+	+	+	+	+
	10	-	-	+	-	-	-	-	+		10	-	-	+	+	+	+	+	+

vaccine detection was less consistent over groups and sampling points, with some birds showing an intermittent vaccine presence but only one bird never became positive (Table 1). 1/96 showed a complete animal coverage and the likelihood of recovering a non-infected bird was null at 7 d, although for B-48 the likelihood persisted until the last day of the trial (Figure 2). Differences between the 2 strains emerged also in terms of titer trend, because 1/96 titers displayed a lower variance within a sampling point and among the groups A, B, and C, whereas B-48 titers were more scattered (Figure 3).

Comparing the pooled sample with the individual sample value means, the tendency for 1/96 strain seemed to follow partially the pooled sample curves, although showing lower titers overall (Figures 1a–c and 3). For B-48 strain, high and low peaks appeared at different time points in all groups instead. The highest and lowest peaks of group B individual titer means were at day 7 and 18, respectively, instead of 14 and 10 for the pooled samples (Figure 1b–d). In group C, some individual samples were positive at day 1 but they were all negative at day 2, oppositely to the pooled samples, which were negative at the beginning but positive at day 2 (Figure 1a–c and 3). With few exceptions, pooled sample titers were comparable or slightly higher than the highest individual value at each sampling point (Figure 3).

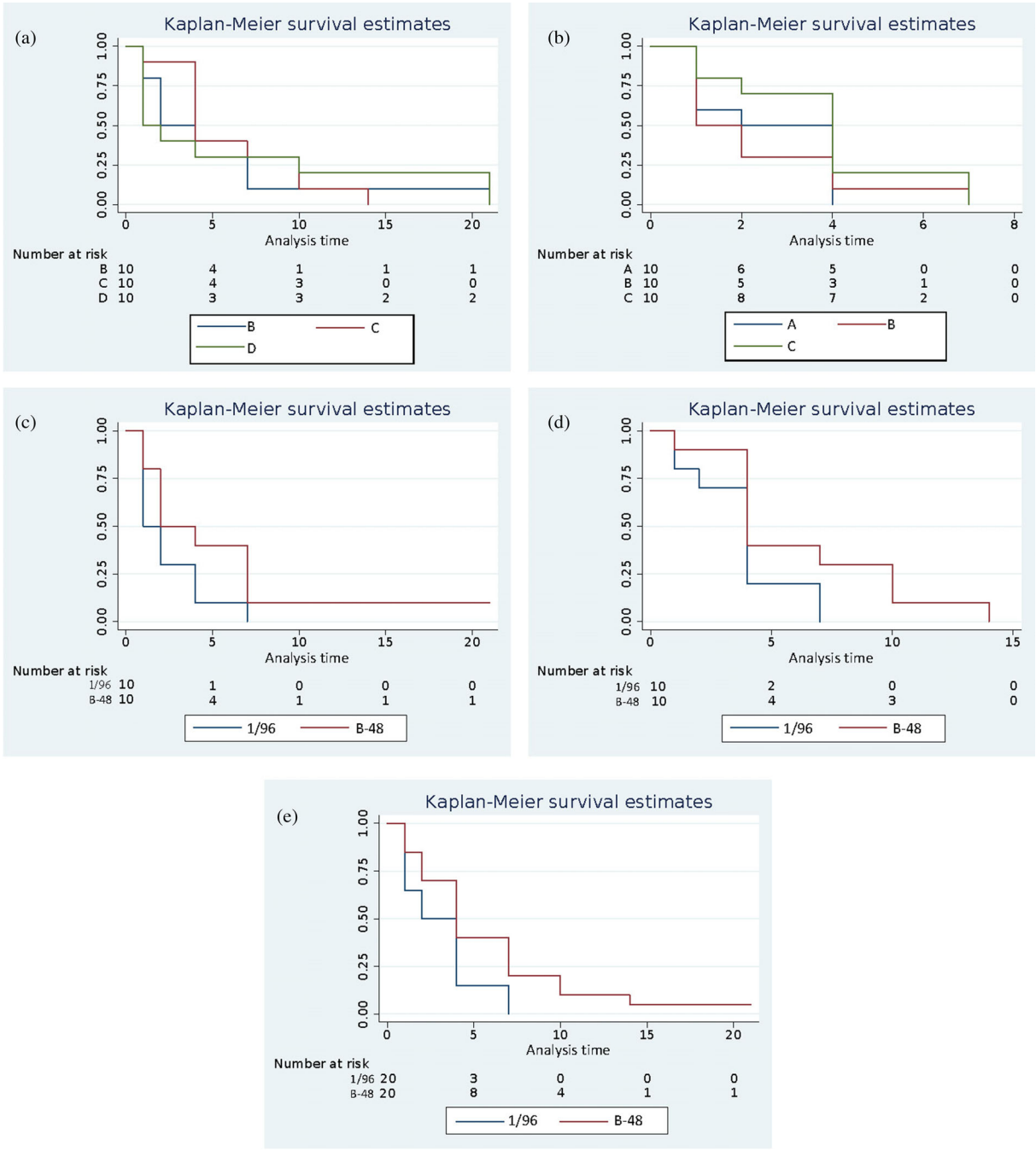

Figure 2. Kaplan-Meier survival estimates: a) comparison among groups B, C, D for B-48 strain; b) comparison among groups A, B, C for 1/96 strain; c) comparison between B-48 and 1/96 strains within group B; d) comparison between B-48 and 1/96 strains within group C; e) cumulative comparison of group B and C between B-48 and 1/96 strains.

Serology

On the first day of the trial, maternally derived antibody levels were homogeneous among birds, with a mean antibody titer of $10^{3.49}$.

DISCUSSION

A correlation between the protection conferred by IBV vaccines and challenge detection by quantitative real-time RT-PCR (Jackwood et al., 2015) has been suggested, and vaccine quantification with a similar tool has also been proposed as an indicator of correct spray vaccination procedures and protection in broilers, because lower vaccine titers were more frequently detected in field strain-positive samples (Tucciarone et al., 2018). Being actively replicating in the respiratory tract, IBV live vaccines could also compete with field strains, providing an additional protective effect other than just eliciting the classical immune response (Tucciarone et al., 2018). However, for the same reason, a certain competition among vaccine strains is possible. Therefore, vaccine kinetics evaluation could provide a measure of vaccination procedure accuracy, strain

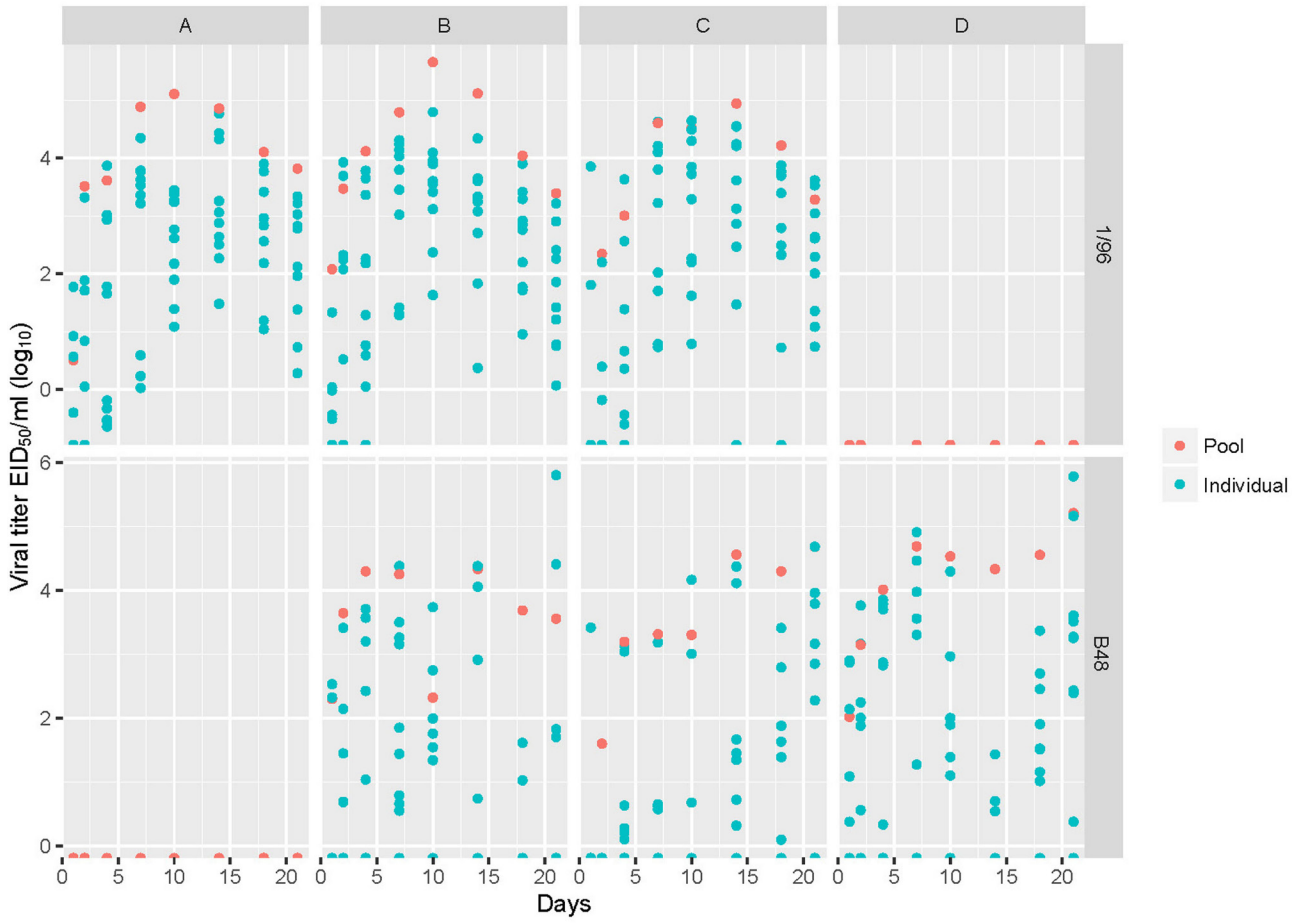

Figure 3. 1/96 and B-48 vaccine titer comparison among pooled and individual samples for each group and sampling point.

interactions, and protection. Consequently, the kinetics of the most frequently combined vaccine strains should be outlined for comparison.

The vaccination route could influence the kinetics and potentially the vaccine efficacy, especially if administration routes and viral tropism seem in conflict. Gel vaccination is an already applied procedure to the control of enteric disease like coccidiosis but great interest is growing around IBV vaccination by this method, which could theoretically also allow the simultaneous administration of different substances, otherwise chemically incompatible, like some vaccines. Nevertheless, the effectiveness of this administration route has never been investigated in case of multistrain respiratory pathogen vaccination. The present study aimed to portray B-48 and 1/96 strain kinetics, the vaccine safety, and trend of the vaccine titers after administration by gel.

Interestingly, although almost all birds immediately ingested the vaccine, as demonstrated by the tongue coloring, the infection timing and vaccine kinetics differed substantially among the individuals (Table 1, Figure 3). The simple contact with the vaccine cannot be assumed as a proxy of proper vaccination and other factors, such as the actually ingested vaccine dose, could explain the individual-level differences. Additionally, the variations in the replication patterns were likely attributable to the biological features of the considered strains. In fact, high 1/96 titers were soon detected in all birds (within 7 d), whereas in one bird B-48 was never evidenced in spite of the 100% tongue coverage (Table 1). The fluctuating presence of B-48 in the same bird could indicate a different replication ability or tropism of this strain, as previously proposed (Tucciarone et al., 2018). This evidence is particularly relevant for group C, where both strains were co-administered in the same gel dose, excluding differences in the vaccine intake.

Despite almost all animals were infected by the end of the study, only a limited chick subset tested positive during the first 48 h in all groups. B-48 curve resembles a cubic function, with an initial rise (vaccination) followed by a decline (physiological decline in viral titers after infection) and then a new mean titer increase (infection of new animals after contact) (Figure 1b). This dynamic would surely be amplified in a large farm population, granting higher vaccine circulation and pressure, with stronger possibility of replication and infection. Thus, a longer sampling period exploring the whole replication trend of B-48 should be performed in the field, accounting for the achievement of complete coverage by re-infections before the end of

the productive cycle. Moreover, a possible increase in B-48 titers could find space in the second half of the cycle, when a decrease in 1/96 and other Mass-like strain titers has been previously attested in field conditions over the same period (Tucciarone et al., 2018). Further studies would be required to evaluate the plausibility of this hypothesis and its consequences.

Besides, the study design had the purpose of comparing the viral titers estimated individually and as a pool, as no studies have yet examined the relationship between these 2 sampling strategies. This issue was investigated because diagnostic samples, for practical and economic constraints, are usually collected as pools, enclosing the whole farm or shed epidemiological information. Interestingly, the results reported herein demonstrated a tendency of pooled samples to overestimate the viral titers (Figure 3). Actually, the pool titer was more closely related to the highest sample titer rather than to the individual sample mean. Even if individual and pooled samples originated from different individuals (in order to reduce animal stress), the identical environmental conditions and random selection of the individually tested animals allow the assumption of a comparable situation between the 2 groups. Additionally, the consistency of the results among experimental groups and vaccine strains further supports the generalization of these data.

Unfortunately, because of the dependence from the highest sample titer, the pool-sampling scheme totally misses the infection prevalence. In fact, particularly for B-48, the pool titers remained constant from the fourth day onward, despite the extremely variable number of positive individuals. These evidences should be taken into account when planning the evaluation of vaccination coverage and efficacy in field conditions. Nevertheless, the pool samples, even if imprecise in absolute values, could be considered a fair trade-off between costs and viral titer dynamics estimation at population level.

Once again, no competition between the 2 strains occurred in the coupled administration, because both vaccines displayed overlapping behaviors in the double and single-strain vaccinated groups, supporting the practicability of B-48 and 1/96 strain combination. Even if experimental conditions created a controlled environment, limiting the presence of other predisposing factors and losing peculiar elements of the multifactorial picture of poultry farming, the negligibility of respiratory clinical signs was encouraging and endorsed the safety of this administration route. Nevertheless, further studies will be necessary to investigate the vaccine distribution after gel administration and the conferred protection, even if the kinetics does not suggest particular differences with more traditional vaccination routes. On the other hand, the evaluation of protection should be tested ultimately by challenge, because the actual correlation between vaccine titers and field strain presence should be demonstrated and no basic interpretation can be drawn by the immune response without a dedicated study.

CONCLUSIONS

The present study described the application of a gel-based administration technique for the vaccination against a respiratory virus. The obtained results support the efficacy and safety of the evaluated method as well as the feasibility of combining different, commonly used, strains in a single administration without establishing any competition. The vaccine kinetics curves, evaluated both at pool and individual level, could provide a useful model for the evaluation of vaccination coverage and efficacy in field conditions.

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