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Comparative efficacy of intranasal and oral vaccines against *Bordetella bronchiseptica* in dogs

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ABSTRACT

In order to determine the comparative efficacy of vaccines administered intranasally or orally to protect puppies from disease subsequent to experimental infection with *Bordetella bronchiseptica* (*Bb*), a randomized controlled trial was performed using 48 approximately 8-week-old specific pathogen free, *Bb* naive Beagle puppies. Puppies were randomized into three groups and administered vaccines containing *Bb* intranasally or orally, or a placebo intranasally. Twenty-one days later, all dogs were challenge exposed via aerosol administration of *Bb*. Clinical signs, nasal bacterial shedding and immune responses were monitored for 28 days after challenge. Intranasally vaccinated puppies had significantly lower rates of coughing, nasal discharge, retching and sneezing (i.e. were less sick clinically) than control puppies. The distinction between the orally vaccinated puppies and the control puppies was less consistent. The orally vaccinated puppies had less coughing and less retching than the control puppies, but nasal discharge and sneezing did not differ from control animals. Orally vaccinated puppies had higher rates of coughing, nasal discharge, retching and sneezing than the intranasally vaccinated puppies. Although both intranasal and oral *Bb* vaccines stimulated immune responses associated with disease sparing following *Bb* infection, the intranasal route of delivery conferred superior clinical outcomes. The observed difference in clinical efficacy suggests the need to question the rationale for the use of currently available orally administered *Bb* vaccines.

Introduction

Bordetella bronchiseptica (*Bb*) is a Gram negative bacterium recognized as one of a constellation of agents etiologically associated with the canine respiratory disease complex (CIRD) (M'Gowan, 1911; Ford, 2006). Once its role as a canine respiratory pathogen was definitively established in the early 1970s (Wright et al., 1973), single component and combination vaccines for the agent, first parenteral, then intranasal, were developed (Ellis, 2015). Recently a single component oral *Bb* vaccine was licensed for commercial use (Hess et al., 2011; Ellis, 2015).

Given the relative ease of administration, the oral *Bb* vaccine has replaced vaccines for this pathogen administered by other routes in many veterinary practices. Since *Bb* vaccines first became available and commonly used in dogs, there have been differing opinions regarding the efficacy and mechanisms of protection of the various routes of administration (Ellis, 2015). Recently, there has been controversy regarding the relative efficacy of the oral and intranasal routes for mucosal administration; vaccines administered by these routes have been used as both primary immunogens and as 'last minute' prophylactics prior to commingling. The aim of this study was to compare the efficacy of representative intranasal and oral vaccines for *Bb*, and to examine immune responses, including those at the mucosa at the earliest documented onset of clinical immunity (72 h; Gore et al., 2005).

Materials and methods

Experimental subjects

Forty-eight (24 male, 24 female) weaned, specific pathogen free Beagle dogs, aged 56–62 days, were obtained from a commercial breeder (Ridglan Farms) and acclimated for 7 days at the study site. The puppies had received a single component vaccine against canine parvovirus (NeoPar, NeoTech) at 6 weeks of age. All dogs had low or no antibodies against *Bb* (<1:16 by microagglutination test, MAT; Ellis et al., 2001) and were determined to be free of *Bb* by deep nasal swab cultures on day 0 prior to vaccination. All dogs were maintained and handled using procedures consistent with the United States Department of Agriculture 9CFR, and approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and had access to ad libitum dry food and water.

Vaccines

A single component oral *Bb* vaccine (Bronchi-shield ORAL, Boehringer Ingelheim Vetmedica) was obtained commercially from a distributor. A triple component

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Table 1
Clinical scoring rubric.

Clinical sign	Score	Description
Nasal discharge	0	Absent: Includes normal, moist nose
	1	Mild: Serous (clear, watery) discharge, must be extending approximately half way down the nasal philtrum
	2	Moderate: Serous discharge extending more than half way down the nasal philtrum, or evidence of mucopurulent discharge
	3	Severe: Mucopurulent discharge extending more than half way down the nasal philtrum, or bloody discharge, or a combination of mucopurulent and bloody discharge
Ocular discharge	0	Absent
	1	Mild: Evidence of excessive tear production (brimming and/or flowing out of the eye), such as some secretion in the corner of the eye or brimming with tears
	2	Moderate: Serous discharge extending more than half way down the nasal philtrum, or evidence of mucopurulent discharge
	3	Severe: Mucopurulent discharge extending more than half way down the nasal philtrum, or bloody, or bloody discharge, or a combination of mucopurulent and bloody discharge
Cough	0	Absent
	1	Mild: One cough episode
	2	Moderate: Spontaneous and frequent coughing; two or more coughing episodes
	3	Severe: Spontaneous coughing with frequent retching; animal had persistent and prolonged cough
Sneezing	0	Absent
	1	Mild: Animal sneezed once or twice
	2	Moderate: Animal sneezed repeatedly
	3	Severe: Animal presented paroxysmal sneeze
Depression	0	Absent
	1	Mild: Animal is slow to rise, lost interest in playing but still somewhat active
	2	Moderate: Animal is able to rise and move, but inactive other than to eat or drink
	3	Severe: Animal is recumbent, unable to rise, and refuses food and/or drink
Retching	0	Absent
	1	Mild: Animal retches or vomits once briefly or occasionally
	2	Moderate: Animal retches or vomits for a prolonged period
	3	Severe: Animal retches or vomits multiple times for a prolonged period
Respiration	0	Normal respiration
	2	Moderate: Small clicking, bubbling, or rattling sounds in the lung (rales)
	3	Severe: Difficult or labored breathing; shortness of breath (dyspnea)

('3-way') intranasal vaccine (Vanguard Rapid Resp, Zoetis) containing *Bb*, canine parainfluenza virus (CPIV) and canine adenovirus-2 (CAV-2) was obtained from the manufacturer. Both vaccines contain 'live avirulent cultures' of *Bb*; however, the specific isolates of the bacteria and the dose in the vaccines are considered proprietary.

Experimental design and housing

Dogs were randomized using a software program (SPSS, SAS Institute). Puppies were blocked in groups of six by date of birth and dam, and assigned to treatments within blocks (two per treatment). Treatments were randomly assigned to rooms for the vaccination phase of the study. Within vaccination rooms, blocks were randomly assigned to pens. During the vaccination phase, the 48 puppies were divided into three treatment groups: (1) the control group received 0.5 mL of sterile diluent (distilled water) intranasally; (2) the second group received 1.0 mL of the single component vaccine orally between the cheek and lateral gum; and (3) the third group received 0.5 mL of the three-way vaccine in one nare. The dogs were housed two per pen in three separate biosecure isolation rooms. To further reduce the chances for exposure to *Bb*, the room housing the control puppies was entered first; therefore, observers were not masked to treatment groups during the vaccination phase. On day 20 (the day before the challenge on day 21), the dogs were co-mingled in three rooms; three blocks were randomly assigned to two challenge rooms and two blocks to one challenge room. Within each room, there were six puppies per double pen (two dogs from each treatment group) (see Appendix: Supplementary Fig. S1). All personnel performing clinical evaluations (during the challenge phase), laboratory testing and analyses were 'masked' (unaware of treatment groups). This protocol was approved by IACUC committee at the study site (approval number KZ-1894e2013-10-ajb; year of approval 2013).

Bordetella bronchiseptica *inoculum and experimental infection*

The virulent Bihr (feline origin) *Bb* strain was used as the inoculum and was cultured on selective (Bordet Gengou, BG) agar from stock as previously described (Ellis et al., 2001). The number of bacteria was adjusted nephelometrically (optical density, OD, 600) to approximately 4×10^{10} colony forming units (CFUs)/mL. Dogs were challenged six at a time via aerosolization of 25 mL of inoculum containing approximately 6×10^{8} CFUs *Bb* (target of 1×10^{8} CFUs per dog) into a chamber. Dogs remained in the chamber for a total of 30–35 min.

Clinical assessment and sampling

General health observations were completed on all puppies from the day of arrival (day –7) to study completion (day 49). Puppies were observed prior to and approximately 3 h post-vaccination for any adverse reactions. Puppies were observed twice on day 20 (prior to and approximately 3–4 h after co-mingling), twice on day 21 (prior to and approximately 4–5 h post-challenge), then twice daily (morning and afternoon for 30 min per group) on days 22–48, and once on day 49. During the challenge phase, puppies were clinically scored according to a predetermined rubric (Table 1) focusing on the primary outcome variable, spontaneous coughing. Rectal temperatures were recorded during the morning observation period on days –1 and 0, and on days 20–49.

Nasal swabs for bacterial culture were collected on day 0, twice weekly (Tuesday, Thursday) until day 20, on day 21 and then thrice weekly (Monday, Wednesday, Friday) on days 21–49, and placed in tryptose phosphate broth transport medium. Nasal and oropharyngeal swabs were collected on days 0 and 3, and placed in 1 mL Dulbecco's modified Eagles transport medium for measurement of mucosal immunoglobulin (Ig) A (IgA) and interferon α (IFNα). Serum was obtained on days 0, 3, 20 and 49.

Bacteriological culture

Nasal swabs were streaked onto BG agar plates as previously described (Ellis et al., 2001). The identity of suspect colonies was confirmed by matrix-assisted laser desorption/ionization (MALDI; Patel, 2015) using a commercial apparatus (Microflex LT, Bruker Daltonics).

Quantitation of Bordetella bronchiseptica*-reactive antibodies*

MATs and ELISAs to measure *Bb* reactive IgG and IgA were performed as previously described (Harris and Switzer, 1972; Ellis et al., 2001).

Quantitation of canine interferon α

A capture ELISA for canine IFNα (Cloud Clone) was performed according to the manufacturer's instructions. The protein contents of nasal swab samples were determined using a colorimetric assay (Bradford, 1976) for comparison with OD values obtained in the capture (and IgA) ELISAs.

Statistical analyses

Descriptive statistics were conducted for the pre-challenge (vaccination) phase of the trial and descriptive and inferential statistics were conducted on the post-challenge data. In all inferential analyses, each treatment was compared to every other treatment (control versus oral vaccinates, control versus intranasal vaccinates, oral vaccinates versus intranasal vaccinates).

Post-challenge, the puppies were observed and scored for seven clinical signs using a Likert scale (score range 0–3; Table 1). Scoring was performed twice daily

Table 2
Summary of the number of observations (outcomes) post-challenge that puppies were scored as at least a 1, at least a 2, or at least a 3, using the scale in Table 1, and for clinical signs and treatment groups listed.

Puppy scored with at least a minimum value[a]	Outcome	Treatment group					
		Control		Orally vaccinated		Intranasally vaccinated	
		Median	Range	Median	Range	Median	Range
1 (at least mild clinical signs)	Cough	22	11–36	6	0–26	2	0–9
	Ocular discharge	13	0–51	12	3–45	16	0–49
	Sneezing	2	0–5	2	0–6	0	0–2
	Depression	0	0	0	0	0	0
	Retching	8	0–18	1	0–12	1	0–5
	Abnormal lung sounds	2	0–4	0	0–6	0	0
	Nasal discharge	1	0–5	0	0–2	0	0–1
2 (at least moderate or severe clinical signs)	Cough	20	0–31	4	0–19	1	0–6
	Ocular discharge	0	0–15	1	0–15	1	0–15
	Sneezing	0	0–1	0	0–2	0	0
	Depression	0	0	0	0	0	0
	Retching	1	0–5	0	0–4	0	0
	Abnormal lung sounds	2	0–4	0	0–6	0	0
	Nasal discharge	0	0–1	0	0	0	0
3 (severe clinical signs)	Cough	1	0–3	0	0–3	0	0
	Ocular discharge	0	0–1	0	0–1	0	0–2
	Sneezing	0	0	0	0	0	0
	Depression	0	0	0	0	0	0
	Retching	0	0	0	0	0	0
	Abnormal lung sounds	0	0	0	0	0	0

[a] Using the clinical sign scoring rubric over the 55 day post-vaccination observation period.

for 27 days and once on day 28 for a total of 55 observation periods. For the post-vaccination time frame, for each clinical outcome in Table 1, data were summarized by counting the number of times each puppy was observed to have scored ≥1 (at least mild signs), ≥2 (moderate or severe signs) or 3 (severe signs) (Table 2) (Dohoo et al., 2009). After summarizing each clinical sign in this manner, for each of the above three categories, a separate generalized linear mixed model (GLMM), with a negative binomial distribution and the offset set as the log of the number of observations, was performed (Table 3). Potential clustering within room and pen was accounted for using random intercepts and the only independent variable in each model was treatment group. A GLMM model with a Poisson distribution was performed for the outcome count of days that a puppy shed *Bb* post vaccination, with an offset of the log of the total number of observations, and room and pen effects accounted for as random intercepts.

All GLMM model results were reported as incidence rate ratios (IRRs) with 95% confidence intervals (95% CIs) (Dohoo et al., 2009). An incidence rate ratio measures the number or counts of new events in a defined population in a specified period of time. For example, in comparing mild coughing between control and oral vaccinated puppies, an IRR of 1.5 would indicate that the frequency of coughing was 50% higher in the control group than in the oral vaccinates; alternatively, the interpretation would be that the rate of coughing in control puppies was 1.5 times higher than in orally vaccinated puppies.

To obtain an approximately normal distribution, serum IgG and IgA were log transformed and a linear multilevel mixed effects model with the log transformation for either IgG or IgA, separately, was constructed with treatment group as the independent variable, and room and pen as random effects. Transformation of the MAT serum dilutions to approximate a normal distribution was unsuccessful; therefore, a nonparametric Kruskal–Wallis test was performed. All analyses were performed using commercial statistical software (STATA V13.1, StataCorp). As a result of the multiple outcomes of interest, a Bonferroni adjustment was made to provide conservative estimates of statistical significance ($P \leq 0.002$) and offset the chance of a type I error (Dohoo et al., 2009).

Results

No adverse effects were noted following vaccine administration. For each clinical sign, results are presented as a count of the number of times that puppies within treatment groups were scored as ≥1 (at least mild clinical signs), ≥2 (moderate to severe clinical signs) or 3 (severe clinical signs) over the 55 observations conducted in the post-vaccination period. The median and range (minimum and maximum) of counts are summarized in Table 2. These data demonstrate that coughing scores were particularly variable between each group. Intranasally vaccinated puppies had lower counts of coughing, nasal discharge, retching and sneezing than the control group or the orally vaccinated puppies.

Statistically significant differences between the treatment groups and the clinical signs are summarized in Table 3. As an example of the interpretation of the data in Table 3 for a comparison of control puppies with oral vaccinated puppies, the rate of mild coughing in control puppies was 2.2 (95% CI 1.5–3.5) times higher than in orally vaccinated puppies ($P < 0.0001$). Intranasally vaccinated puppies had significantly lower rates of nasal discharge, coughing, sneezing and retching than control puppies. Orally vaccinated puppies had less coughing and retching than control puppies, but nasal discharge and sneezing did not differ from controls. Orally vaccinated puppies had higher rates of coughing, nasal discharge, retching and sneezing than intranasally vaccinated puppies (Fig. 1).

No differences were detected in the rates of ocular discharge between the groups for any score of ocular discharge (at least mild, at least moderate or severe) ($P > 0.30$). All puppies in the intranasally vaccinated group had no abnormal lung sounds over the duration of the study. In puppies with abnormal lung sounds, there were no significant differences in the frequency of having moderate to severe lung sounds between the control group and the orally vaccinated puppies ($P = 0.02$). No puppies in any group were scored as having severe labored breathing and dyspnea. One dog in the three-way intranasally vaccinated group had a single day of pyrexia (>39.5 °C; day 47); no signs of systemic illness were observed in any of the dogs after challenge.

Bacteriological findings

The median duration of shedding *Bb* was 12 (range 10–12) days for control dogs, 12 (range 1–13) days for orally vaccinated dogs and 9 (range 2–12) days for intranasally vaccinated dogs. There was no significant difference in the rate of shedding between the orally vaccinated dogs and the control group ($P = 0.37$). The rate of shedding of *Bb* in control puppies was 1.5 (95% CI 1.2–1.8) times higher than in intranasally vaccinated puppies ($P = 0.001$), whereas there was no significant difference in the rate of shedding between orally vaccinated and intranasally vaccinated puppies ($P = 0.02$).

Table 3
Summary of the statistical comparisons of the three treatment groups for each outcome of interest by categorization of clinical scores as ≥1, ≥2 or 3.

Outcome	Treatment group comparisons	Incident rate ratio	95% Confidence interval	*P* value
Score ≥1				
Coughing	Control versus oral vaccination	2.2	1.5–3.5	<0.0001
	Control versus intranasal vaccination	6.9	4.2–11.3	<0.0001
	Oral vaccination versus intranasal vaccination	3	1.8–5.3	<0.0001
Nasal discharge	Control versus oral vaccination	No difference	–	0.87
	Control versus intranasal vaccination	1.8	1.3–2.3	<0.0001
	Oral vaccination versus intranasal vaccination	1.7	1.3–2.3	<0.0001
Sneezing	Control versus oral vaccination	No difference	–	0.9
	Control versus intranasal vaccination	5.8	2.2–14.9	<0.0001
	Oral vaccination versus intranasal vaccination	5.6	2.2–14.5	<0.0001
Retching	Control versus oral vaccination	3.2	1.7–6.3	<0.0001
	Control versus intranasal vaccination	10.6	4.7–23.8	<0.0001
	Oral vaccination versus intranasal vaccination	No difference	2.2–14.5	0.005
Score ≥2				
Coughing	Control versus oral vaccination	2.8	1.7–4.5	<0.0001
	Control versus intranasal vaccination	11.6	6.5–21.0	<0.0001
	Oral vaccination versus intranasal vaccination	4.2	2.3–7.7	<0.0001
Nasal discharge	Control versus oral vaccination	No difference	–	>0.02
	Control versus intranasal vaccination	No difference	–	>0.02
	Oral vaccination versus intranasal vaccination	No difference	–	>0.02
Coughing	Control versus oral vaccination	2.8	1.7–4.5	<0.0001
	Control versus intranasal vaccination	11.6	6.5–21.0	<0.0001
	Oral vaccination versus intranasal vaccination	4.2	2.3–7.7	<0.0001
Sneezing	Control versus oral vaccination	No difference	–	0.9
	Control versus intranasal vaccination	No dogs scored as moderate	–	NA
	Oral vaccination versus intranasal vaccination	No difference	–	NA
Retching	Control versus oral vaccination	No difference	–	0.15
	Control versus intranasal vaccination	No difference	–	0.99
	Oral vaccination versus intranasal vaccination	Model won't converge	–	NA
Score 3				
Coughing	Control versus oral vaccination	No difference	–	>0.02
	Control versus intranasal vaccination	No difference	–	>0.02
	Oral vaccination versus intranasal vaccination	No difference	–	>0.02
Nasal discharge	Control versus oral vaccination	No dogs scored as severe	–	NA
	Control versus intranasal vaccination	No dogs scored as severe	–	NA
	Oral vaccination versus intranasal vaccination	No dogs scored as severe	–	NA
Sneezing	Control versus oral vaccination	Model won't converge	–	NA
	Control versus intranasal vaccination	No dogs scored as severe	–	NA
	Oral vaccination versus intranasal vaccination	No dogs scored as severe	–	NA
Retching	Control versus oral vaccination	No dogs scored as severe	–	NA
	Control versus intranasal vaccination	No dogs scored as severe	–	NA
	Oral vaccination versus intranasal vaccination	No dogs scored as severe	–	NA

NA, not applicable.

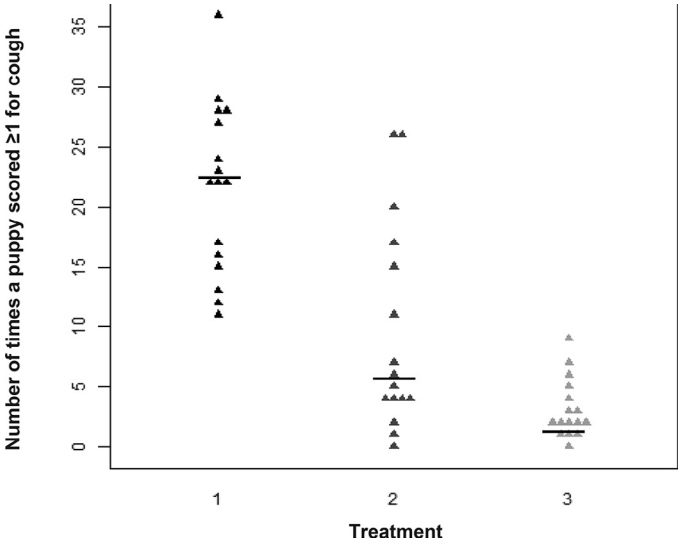

Fig. 1. Scatter (bee swarm) plot of coughing in puppies after infection with *Bordetella bronchiseptica*. Each symbol represents the count of observations post-challenge in which an individual puppy was scored as having at least a mild cough (i.e. ≥1; Table 1) in each treatment group (controls: black triangles, $n = 16$; orally vaccinated: dark gray triangles, $n = 16$; intranasally vaccinated: light gray triangles, $n = 16$). The black horizontal lines represent the median number of observations that puppies scored ≥1 for coughing for each treatment group.

Onset of local immunity

Mucosal *Bb* reactive IgA and IFN-α concentrations were low (<5 U in IgA ELISA; few picograms in IFN assay) and undetectable in most nasal swab samples at day 0 prior to vaccination. There was no increase in either of these soluble mediators at 3 days after vaccination; values were consistently low or undetectable. Hence, any responses were considered biologically irrelevant and there were no apparent differences among treatment groups (data not shown); no further analyses were performed.

Antibody responses

No significant differences between any of the groups were detected in the MAT results at day 0 ($P = 0.51$; Fig. 2). The control group had significantly lower MAT serum dilutions at days 20 ($P < 0.0001$) and 49 ($P = 0.002$) than the intranasally vaccinated group. The control group also had significantly lower MAT serum dilutions at day 20 ($P < 0.0001$), but not at day 49 ($P = 0.06$), than the orally vaccinated dogs. No differences were detected between orally and intranasally vaccinated groups for either day 20 ($P = 0.03$) or day 49 ($P = 0.32$). All dogs had moderate to high concentrations of *Bb* reactive IgA in serum from day 49 (control: median 96 U, range 39–440 U; orally vaccinated: median 89 U, range 40–200 U; intranasally vaccinated: median 97 U, range 38–181 U). There were no significant

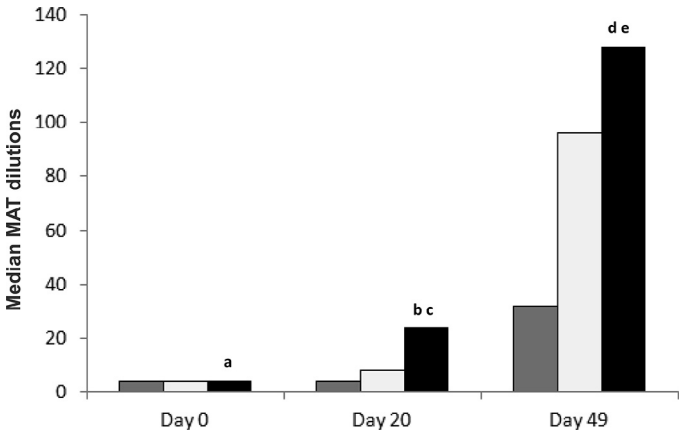

Fig. 2. Median microagglutination titer (MAT) serum dilutions for each treatment group on days 0, 20 and 49 of the study. The dark gray bars represent the control group, the light gray bars represent the orally vaccinated group and the black bars represent the intranasally vaccinated group. Significant differences: [a]No significant differences detected between any of the groups ($P \geq 0.05$); [b]Significant differences detected between the control and orally vaccinated groups and between the control and the intranasally vaccinated groups ($P < 0.05$); [c]No significant differences detected between the orally and intranasally vaccinated groups ($P \geq 0.05$); [d]Significant differences detected between the control and the intranasally vaccinated groups ($P < 0.05$); [e]No significant differences detected between the control and the orally vaccinated groups and between the orally and intranasally vaccinated groups ($P < 0.05$).

differences among groups in *Bb* reactive IgG on day 49 (control: median 65 U, range 46–91 U; orally vaccinated: median 60 U, range 29–80 U; intranasally vaccinated: median 57 U, range 42–80 U; $P > 0.07$).

Discussion

The results of this study confirm previous observations (Ellis, 2015) that mucosal delivery of modified live *Bb* by either the intranasal or oral route can effectively immunize dogs and confer sparing of CIRD, as assessed in a robust challenge model. In the present study, the intranasal route engendered superior protective immunity against clinical signs typical of *Bb* associated 'kennel cough'. These results contrast with the equivalent clinical outcomes following intranasal or oral immunization reported in the one other published comparative study (Larson et al., 2013). However, the previous study had issues with experimental design and no statistical analyses were undertaken, rendering the conclusions drawn therein questionable (Ellis, 2015).

Arguably, a more relevant comparison than the one reported herein would have been to evaluate the response to a three-way vaccine administered either intranasally or orally; however, currently there are no licensed three-way oral vaccines. Despite this, a three-way combination vaccine is likely to represent a rational approach to immunoprophylaxis, given the commonality of multiple infections currently documented in dogs with respiratory disease (Schulz et al., 2014; Joffe et al., 2016).

Strictly speaking, the results reported herein are only applicable to the vaccines tested. It is possible that different oral and intranasal *Bb* vaccines (i.e. different formulations or different doses of *Bb*) may engender different immune responses and affect different clinical outcomes after challenge. However, notwithstanding that caveat, we think these results truly reflect generic differences between the relative efficacy of oral versus intranasal delivery in stimulating disease-sparing responses to *Bb* and probably other respiratory pathogens.

In large part, the superior efficacy of intranasal versus oral administration may simply be a consequence of more extensive distribution of antigen, exposing both nasal associated lymphoid tissue (Kiyono and Fukuyama, 2004), as well as the retropharyngeal tonsil (adenoid), which is unique among tonsils with its overlying layer of respiratory epithelium (Billen et al., 2006). Both sites are important sites of immune induction and neither would be exposed to antigen by oral administration of vaccine (Kiyono and Fukuyama, 2004). Beyond their use as primary immunogens, mucosally delivered vaccines are frequently used immediately prior to boarding (housing dogs in communal kennels) or other potentially high challenge situations, the rationale being that mucosal delivery induces a local and more rapid response.

It is often assumed, from a mechanistic standpoint, that IgA is responsible for any disease reduction (Gore et al., 2005; Davis et al., 2007). Our examination of innate and adaptive immune responses at 0 and 72 h after vaccination readdressed this issue. The 72 h time point was specifically chosen because it was the earliest onset of immunity reported in the one published study (Gore et al., 2005); however, local immune responses were not reported in that study. Perhaps predictably, based on the current understanding of antibody production involving class switching and maturation of plasma cells from stimulated B cells (Strungell and Wijburg, 2010) we found little, or mostly no, detectable IgA 72 h after either intranasal or oral immunization in either nasal or oral secretions.

In one of few studies reporting local immune responses, *Bb* reactive IgA in nasal secretions was not detected until more than 21 days after primary intranasal vaccination (Davis et al., 2007). On the basis of studies in cattle (Todd et al., 1973) and *Bb* infected cats (Bradley et al., 2012), it is probable that innate immune responses are responsible for disease reduction in the first few days after mucosal vaccination. We attempted to measure type I interferon (IFN-α). Unfortunately little or mostly no INF-α was detected in nasal or oral secretions of mucosally vaccinated puppies at 72 h. This could be the result of inadequate sample volume, the failure of the commercial capture ELISA to detect INF-α in the types of samples tested or the possibility that other soluble mediators of the innate immune system are the effectors stimulated by mucosal vaccination (Kumar et al., 2011).

Nevertheless, these negative results and still unresolved mechanistic uncertainties should not preclude the use of mucosally delivered vaccines in this common application. Again, from an immunologically rational standpoint, the use of intranasal combination vaccines makes more sense than a single component oral vaccine based on the number of different biochemical motifs or pathogen associated molecular patterns (PAMPs; Kumar et al., 2011) in the three-way intranasal formulation, including negative stranded viral RNA, viral DNA, and viral glycoproteins, in addition to the CpG DNA motifs, endotoxin and flagellin in *Bb*. Intranasal delivery would result in more extensive exposure to PAMPs and stimulation of the innate immune response in both the nasal cavity and oropharynx.

The MAT is one of the classic tests used to assess the efficacy of vaccines for human whooping cough caused by *B. pertussis* (*Bp*), the closely related descendent of *Bb* (Miller et al., 1943; van der Ark et al., 2012). It is often stated or implied that serum antibody responses are largely irrelevant to clinical immunity in *Bb* infections in dogs (Ford, 2006; Davis et al., 2007; Hess et al., 2011; Larson et al., 2013); however, the correlation between serum antibody, notably MAT, and disease-sparing was documented more than 70 years ago in large numbers of vaccinated and naturally *Bp* infected children (Miller et al., 1943). The protective role of serum antibodies in *Bp* vaccines was further characterized with antigen specific ELISAs 20 years ago (Cherry et al., 1998).

Previously, we demonstrated a similar relationship between clinical immunity and serum antibodies in *Bb* infected dogs, following immunization with either or both intranasal and parenteral vaccines (Ellis et al., 2001). In the present study, the intranasally vaccinated dogs had numerically higher MATs than the control and

orally vaccinated animals; however, the stringency of the analyses for significance ($P < 0.002$) required when conducting multiple comparisons (Dohoo et al., 2009) most likely precluded the detection of significant differences between vaccine groups. Furthermore, the time of sampling may have been a factor in not detecting differences, which may have been revealed in anamnestic responses within days to a week after challenge (Ellis et al., 2001). Overall, the immunological results of this study replicated previous findings (Ellis et al., 2001), albeit, as in the previous study, mucosal immunization resulted in less pronounced systemic (MAT) responses (than parenteral immunization), as would be expected.

At least in part based on our previous experience with high variation in sampling of mucosal secretions in dogs (Ellis et al., 2002), and difficulties in obtaining consistent nasal samples, especially in young puppies with small nares, we used serum IgA as a surrogate for mucosal IgA to further characterize the anamnestic response following challenge. This approach is validated by classical studies with canine IgA in normal dogs and dogs with selective IgA deficiency, as well as studies in human beings infected with influenza virus (Vaerman and Heremans, 1970; Brown et al., 1985; Batt et al., 1991; Olsson et al., 2014). IgA in dog serum is predominately dimeric (Vaerman and Heremans, 1970) and is thought to be primarily produced at mucosal surfaces (Vaerman and Heremans, 1970; Batt et al., 1991; Olsson et al., 2014).

Our finding of no differences among treatment groups in the concentrations of *Bb* reactive IgA (or IgG) in serum 28 days after challenge could also be a function of the timing of sampling. Earlier sampling after challenge may have revealed differences in anamnestic responses, as we previously demonstrated in samples collected on day 3 after experimental exposure to *Bb* (Ellis et al., 2001). Nevertheless, the finding of moderate to high concentrations of *Bb* reactive IgA in serum of all dogs following only mucosal exposure to the non-invasive bacteria supports the seminal observations that IgA is primarily produced by plasma cells in the lamina propria of mucosae. It then 'spills over' into the systemic circulation (Vaerman and Heremans, 1970), where it can be more easily and consistently measured by sampling of plasma.

Conclusions

The results of this study indicate that, although mucosal delivery of the intranasal and oral *Bb* vaccines resulted in significant disease sparing as a result of primary immunization, the intranasal vaccine conferred superior clinical immunity. Furthermore, these results should question the medical rationale for routine use of oral *Bb* vaccine, notwithstanding its apparent easier administration.

Conflict of interest statement

Zoetis funded this study and five of the coauthors (SS, SW, AB, ZX, EB) are employees of Zoetis. None of the other authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2016.04.004.

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