

CAPÍTULO REIVINDICATORIO MODIFICADO

1. Una vacuna combinada, caracterizada por que comprende:
parvovirus canino vivo modificado (CPV MLV); y
partículas de tipo virus de parvovirus canino (CPV VLPs) en una cantidad eficaz para superar el efecto neutralizante de los anticuerpos derivados por vía materna (MDAs) contra el CPV MLV en un animal,
en donde el CPV MLV es un parvovirus de la cepa Cornell 780916, y
en donde las CPV VLPs comprenden un polipéptido que tiene la secuencia como se indica en SEQ ID NO.: 1, 3, 4, 6, 8, 9 o 10, o fragmentos de los mismos.
2. La vacuna combinada de la Reivindicación 1, caracterizada por que provee inmunidad protectora con una sola dosis.
3. La vacuna combinada de las Reivindicaciones 1 o 2, caracterizada por que las CPV VLPs corresponden por lo menos a un 10% (p/p) del contenido total de proteínas de la vacuna combinada.
4. La vacuna combinada de cualquiera de las Reivindicaciones 1 a 3, caracterizada por que las CPV VLPs corresponden por lo menos a un 20% (p/p) del contenido total de proteínas de la vacuna combinada.
5. La vacuna combinada de cualquiera de las Reivindicaciones 1 a 4, caracterizada por que las CPV VLPs fueron producidas por un vector de expresión de baculovirus en células de insecto.
6. La vacuna combinada de cualquiera de las Reivindicaciones 1 a 5, caracterizada por que las CPV VLPs comprenden por lo menos una proteína de cápside.
7. La vacuna combinada de cualquiera de las Reivindicaciones 1 a 6, caracterizada por que la vacuna combinada comprende un transportador, excipiente o vehículo farmacéuticamente o veterinariamente aceptable.

8. La vacuna combinada de cualquiera de las Reivindicaciones 1 a 7, caracterizada por que la composición o vacuna no tiene adyuvante.

Control of canine parvovirus infection in breeding kennels :

Study of the efficacy of a high titer attenuated canine parvovirus vaccine

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SUMMARY

The aim of this study, which was conducted in two infected breeding kennels, is to assess the efficacy of a high titer attenuated canine parvovirus vaccine in 4- to 8-week-old pups and, in parallel, to determine the extent of the reduction in the critical period that this vaccine enables us to obtain. At the start of the intervention, the 126 pups in the two breeding kennels constituted a sensitive study population - 108 (85.71 %) had non-immunizing HI antibody titers (≤ 64). A fortnight after a single injection with the vaccine, the rate of immunity was 91.27 % : 115 presented HI antibody titers ≥ 128 . The overall postvaccination seroconversion rate was 81.48 % for the 108 pups which had prevaccination titers of ≤ 64 . The referential seroconversion rates of 95 %, 50 % and 0 % corresponded to HI antibody titers of < 32 , 64 and ≥ 128 respectively. With reference to traditional vaccines, these results enable the critical period to be reduced from 30 to 10 days, at a seroconversion rate of 95 %, and from 20 to 0 day(s), at a seroconversion rate of 50 %. The age groups to be vaccinated (4 to 8 weeks) correspond to the age groups most affected by a previous epizootic (6 to 8 weeks), with an anticipation period of 15 days (4 and 5 weeks). The pups, aged from 4 to 5 weeks old, which had HI antibody titers of ≤ 64 responded well to vaccination (vaccine efficacy 100 %).

KEY-WORDS : canine parvovirose infection - vaccination - high titer attenuated vaccine - critical period.

RÉSUMÉ

Maîtrise de la parvovirose canine en élevage : étude de l'efficacité d'un vaccin atténué à haut titre viral. Par C. WILLEM, S. LATOUR et A. LACHERETZ.

L'objectif de ce travail, réalisé dans deux élevages infectés, est d'évaluer chez des chiots âgés de 4 à 8 semaines l'efficacité d'un vaccin antiparvovirus atténué à haut titre viral et parallèlement de préciser en durée la réduction de la période critique qu'il permet d'obtenir. Au début de l'intervention, les 126 chiots des deux élevages étudiés constituaient une population sensible : 108 (85,71 %) avaient un titre IHA non protecteur (≤ 64). Quinze jours après l'injection du vaccin, le taux de couverture immunitaire est de 91,27 % : 115 chiots présentent un titre IHA ≥ 128 . Le taux de séroconversion après vaccination est de 81,48 % pour les 108 chiots dont le titre pré-vaccinal était ≤ 64 . Les taux de conversions sérologiques de référence 95 %, 50 %, et 0 % correspondent respectivement à des titres IHA < 32 , 64 et ≥ 128 . Par référence aux vaccins classiques, ces résultats correspondent à une réduction de la période critique de 30 à 10 jours, au point 95 % de séroconversion et de 20 à 0 jour(s), au point 50 % de séroconversion. Les classes d'âge à vacciner (4 à 8 semaines) correspondent aux classes d'âge les plus touchées par une précédente épizootie (6 à 8 semaines) avec une période d'anticipation de 15 jours (4 et 5 semaines). Les chiots, âgés de 4 et 5 semaines, dont les titres IHA étaient ≤ 64 ont répondu efficacement à la vaccination (100 %).

MOTS-CLÉS : parvovirose canine - vaccination - vaccin atténué à haut titre viral - période critique.

Introduction

Although initially observed in all age groups, canine parvovirus infection is now mainly seen in pups of less than five months old [8]. This confinement of the infection to the first few months of life bears witness not only to the widespread use and efficacy of vaccination, but also, in young pups, to a physiological limit, traditionally known by the name "critical period" [7]. Indeed, this period corresponds to the final phase of elimination of maternally derived antibodies, when their titers are not high enough to confer immunity, but are sufficient to inhibit vaccinal antigens.

Using traditional vaccines within the framework of canine parvovirus infection, the critical period persists for around

four weeks [9]. This critical period occurs somewhere between the age of 1 to 5 months, depending on the pups in question and more particularly their initial rate of maternally derived antibody titers [8]. The aim of this study, which was carried out in infected breeding kennels, is to assess the efficacy of a high titer attenuated canine parvovirus vaccine* in 4- to 8-week-old pups and, in parallel, to determine the extent of the reduction in the critical period that this vaccine enables us to obtain.

* PRIMODOG® Merial.

1. Materials and methods

A) PRESENTATION OF THE BREEDING KENNELS

The vaccine trials were carried out in two breeding kennels whose zootechnical references are set out in tables I and II. At the time of the intervention, both of these kennels were affected by a fatal parvovirus epizootic, which concerned mainly 6- to 8-week-old pups.

B) VACCINES USED

The following vaccines were used for the trials :

- **Primadog®** : modified living canine parvovirus vaccine - Cornell 780916 strain - minimum infectivity titer per 1 ml dose $10^{5.5}$ TCID₅₀ on a feline cell line, i.e. $10^{7.8}$ TCID₅₀ on a canine cell line [4].

- **Triviroxax®** : attenuated living vaccine combining canine distemper, canine adenovirus type-2 (CAV₂) and canine parvovirus valencies. The parvovirus valency is represented by the Cornell 780916 strain - minimum infectivity titer per 1 ml dose 10^3 TCID₅₀ on a feline cell line, i.e. 10^5 TCID₅₀ on a canine cell line [4].

- **Biviroxax®** : attenuated living vaccine combining canine distemper and canine adenovirus type-2 (CAV₂).

C) VACCINATION PROTOCOLS

There was no regular vaccination protocol in place at the kennels studied. Accordingly, the adults were systematically

vaccinated with Triviroxax® by the subcutaneous route : the aim was to obtain or re-establish a satisfactory and uniform rate of immunity against distemper, hepatitis and parvovirus in these animals.

126 pups, 48 from the first kennel and 78 from the second, were given an initial injection of one dose of Primadog®. The pups were between 4 to 8 weeks old (Table III). A fortnight after the initial injection, the pups received a second vaccination, this time of Biviroxax® (distemper and adenovirus type-2 (CAV₂)) and Primadog® (parvovirus).

D) INDIVIDUAL MONITORING OF PUPS

In order to monitor the efficacy of the vaccination over time, the pups were first identified using an electronic microchip injected into their neck (Indexel® - Merial). After vaccination, each pup was observed to check for any local and/or general postvaccinal reactions.

In order to determine the pre- and postvaccination antibody titers for the pups, blood samples were taken from their jugular vein (2ml) upon initial vaccination as well as a fortnight later, just before the second vaccination. The paired sera were frozen and analyzed at the same time. The serological effects of the second vaccination are not tested.

E) TITRATION OF ANTIBODIES

Canine parvovirus antibody titers were tested on serum dilution pairs using the hemagglutination-inhibition technique [2]. The titer determined corresponds to the inverse

	No. breeding stock		Pup production and breeding		
	Bitches	Dogs	Births/month	Age / weaning	Age / sale
Kennel n°1	100	20	80	8 (w)	12 (w)
Kennel n°2	200	80	50	6 (w)	8 (w)

TABLE I. — No of breeding stock. Pup production and breeding methods (w = weeks).

	Litter	Feed	Maternity
Kennel n°1	Clay	Abattoir waste. Dry food on weaning.	Yes
Kennel n°2	Straw on cement floor	Crushed turkey bones. Cereals.	Yes

TABLE II. — Keep and feeding of breeding stock.

Age of pups	4 (w)	5 (w)	6 (w)	7 (w)	8 (w)	Total
Kennel n°1	9	5	15	10	9	48
Kennel n°2	0	5	65	8	0	78
Total	9	10	80	18	9	126

TABLE III. — Breakdown of the 126 pups subject to full protocol (w = weeks).

function of the maximum dilution for which inhibition can still be observed.

F) SANITARY MEASURES

Sanitary measures were implemented in parallel to the vaccination protocol, including :

- daily collection and disposal of feces
- daily cleaning of animal units using pressurized steam, followed by disinfecting with bleach
- steam cleaning of the birth areas, followed by disinfecting with bleach and a depopulation period of one fortnight
- compliance with the principle of forward flow in the administration of daily care, from the least contaminated areas (birth areas) to the most infected areas
- installation of a foot bath at the entrance to birth areas
- quarantine for all newly-admitted dogs.

G) KENNEL MANAGEMENT

All vaccination and sanitary interventions are assessed for each kennel with reference to a "kennel register" which lists births, arrivals, sales, medical interventions (including vermifuge operations) and any changes in protocol.

H) STATISTICAL ANALYSES

Results were analyzed using the χ^2 method, when necessary modified by the Yates correction.

2. Results

A) KENNEL PERFORMANCE

1. Kennel n° 1

Six months after the start of the intervention, the population of pups had returned to its initial level (50 to 80 newborn pups per month). The morbidity and mortality rates (100 % and 50 % respectively) initially observed in pups of 6 to 8 weeks old had both fallen to 0 %.

2. Kennel n° 2

Five months after the start of the intervention, the production of pups had returned to its normal level (the same level

as before the epizootic). The morbidity and mortality rates (100 % and 93 % respectively) initially observed in pups of 6 to 8 weeks old had both fallen to below 3 %.

However, at the same time, an outbreak of gastroenteritis was noted in 5- to 6-week-old pups which had not been vaccinated or which had only been vaccinated one to two days previously. For the 50 pups concerned, the morbidity and mortality rates were 20 % and 10 % respectively. No parvovirus was detected in the feces or intestines of the dead animals.

B) SAFETY

No local and/or general reactions were observed in the pups in the five minutes following vaccination.

C) SEROLOGICAL TITERS

1. Kennel n° 1

1.1. Prevaccination titers

Table IV shows the distribution of prevaccination HI antibody titers for the 48 pups included in the study. With reference to a commonly acknowledged immunizing HI antibody titer of 80 [9, 11], these pups can be broken down into two sub-groups :

- A first sub-group of 32 pups (66.66 %), with HI antibody titers of less than or equal to 64, constituting a group of animals which were, a priori, susceptible to possible infection.

- A second sub-group of 16 pups (33.33 %), with HI antibody titers of greater than or equal to 128, constituting a group of animals which were, a priori, resistant to possible infection.

Table IV also details the distribution of serological titers in relation to the age of pups upon initial vaccination. The pups of 4 to 5 weeks old showed a significant initial deficit (≤ 32) in relation to the older pups which had titers varying from 16 to 1024. For 8-week old pups, the titers were greater than or equal to 64.

1.2. Postvaccination titers

Table V shows the breakdown in HI antibody titers for the 48 pups, 15 days after a single injection with the high titer vaccine :

Age of pups	Prevaccination HI antibody titer								Total
	8	16	32	64	128	256	512	1024	
4 weeks		7	2						9
5 weeks	1	4							5
6 weeks			5	5	2	2	1		15
7 weeks		3	3	1	1	2			10
8 weeks				1	1	5	1	1	9
Total	1	14	10	7	4	9	2	1	48
Seroconversion (rate(%))	1/1 100	14/14 100	9/10 90	4/7 57,14	0/4 0	1/9 11,11	0/2 0	0/1 0	29/48 60,41

TABLE IV. — Distribution of prevaccination HI antibody titers in relation to the age of pups upon initial vaccination and evolution in the seroconversion rate in relation to the prevaccination HI antibody titer. Kennel n° 1.

- 45 pups (93.75 %) had HI antibody titers of greater than or equal to 128 and therefore were, a priori, resistant to possible infection.

- 3 pups (6.25 %) had HI antibody titers of less than or equal to 64 and therefore were, a priori, susceptible to possible infection.

The lowest postvaccination HI antibody titers (≤ 64) were observed in 8-week old pups.

1.3. Comparison of pre- and postvaccination titers

Traditionally, seroconversion is defined as an increase in antibody titers of at least four serum dilutions. Moreover, this reference is commonly used for the HI antibody technique [1, 6, 9]. On this basis, Table IV and V show the seroconversion rates obtained for the 48 pups in the first kennel in relation to their initial HI antibody titers and in relation to their age.

Among the 48 pups, 29 (60.41 %) were seroconverted : 28 of the 32 pups which had initial titers of ≤ 64 , i.e. 87.5 % and 1 of the 16 pups which had initial titers of ≥ 128 , i.e. 6.25 % (Table IV). The seroconversion rate was 100 % (14/14) for 4- to 5-week-old pups, 56 % (14/25) for 6- to 7-week-old pups and 11.11 % (1/9) for 8-week-old pups (Table V).

2. Kennel n° 2

2.1. Prevaccination titers

Table VI shows the distribution of prevaccination HI antibody titers for the 78 pups in this kennel :

- 76 (97.5 %) had initial HI antibody titers of less than or equal to 64 and, a priori, were susceptible to possible infection.

- 2 (2.50 %) had initial HI antibody titers of greater than or equal to 128 and, a priori, were resistant to possible infection.

Accordingly, the pups in kennel n° 2 constituted a uniform and susceptible population. This population differed significantly (with a 5 % risk) from that of kennel n° 1.

2.2. Postvaccination titers

Table VII shows the postvaccination HI antibody titers obtained 15 days after a single injection with the high titer vaccine :

- 70 pups (89.75 %) had HI antibody titers ≥ 128 ,
- 8 pups (10.25 %) had HI antibody titers ≤ 64 .

These results do not differ significantly (with a 5 % risk) from those obtained for kennel n° 1.

Age	Seroconversion	Postvaccination HI antibody titer								
		16	32	64	128	256	512	1024	2048	4096
4 w	9/9 (100,00%)					1	1	4	3	
5 w	5/5 (100,00%)						1	4		
6 w	7/15 (46,67%)				1	3	5	3		3
7 w	7/10 (70,00%)				1	1	3	4		1
8 w	1/9 (11,11%)	1		2	2	3		1		
T	29/48 (60,41%)	1	0	2	4	8	10	16	3	4

TABLE V. — Evolution in the seroconversion rate and distribution of postvaccination HI antibody titers in relation to the age of pups upon initial vaccination. Kennel n° 1.

Age of pups	Prevaccination HI antibody titer									
	8	16	32	64	128	256	512	1024	≥ 2048	Total
5 weeks		2	2	1						5
6 weeks	6	26	21	10			1		1	65
7 weeks		4	3	1						8
Total	6	32	26	12			1		1	78
Seroconversion (rate (%))	6/6 100	29/32 90,62	19/26 73,07	6/12 50,00			0/1		0/1	60/78 76,92

TABLE VI. — Distribution of prevaccination HI antibody titers in relation to the age of pups upon initial vaccination and evolution in the seroconversion rate in relation to the prevaccination HI antibody titer. Kennel n° 2.

Age	Seroconversion	Postvaccination HI antibody titer								
		16	32	64	128	256	512	1024	2048	≥ 4096
5 w	5/5 (100%)								2	3
6 w	48/65 (73,84%)	3	3	1	4	11	12	14	7	10
7 w	7/8 (87,50%)		1						2	3
T	60/78 (76,92%)	3	4	1	4	11	12	16	11	16

TABLE VII. — Evolution in the seroconversion rate and distribution of postvaccination HI antibody titers in relation to the age of pups in weeks (w) upon initial vaccination. Kennel n° 2.

2.3. Comparison of pre- and postvaccination titers

The seroconversion rates obtained for the 78 pups in kennel n° 2 do not differ significantly (with a 5 % risk) from those observed for kennel n° 1 (Table VI). The seroconversion rates were 90 % and 50 % respectively for initial titers of 16 and 64. In total, 60 of the 78 pups (76.92 %) were seroconverted : 60 of the 76 pups which had initial titers of ≤ 64 , i.e. 78.94 % and neither of the 2 pups which had initial titers of ≥ 128 , i.e. 0 %.

The seroconversion rates in relation to the age of pups are relatively uniform, with rates of 100 % (5 weeks), 73.84 % (6 weeks) and 87.5 % (7 weeks) respectively (Table VII).

3. Discussion

A) ZOOTECHNICAL REFERENCES

The fatal epizootic of canine parvovirus which had decimated mainly 6- to 8-week-old pups resulted in the total shut-down of activities at both kennels studied. In both kennels, the medical and sanitary interventions proposed enabled a return to the referential zootechnical and economic performance levels in six months.

B) SAFETY OF THE VACCINE

No local and/or general reactions were observed in the 126 pups in the minute following single vaccination with the high titer vaccine. These results enable us to conclude on the high level of safety of the vaccine used in an infected environment and comply with the data contained in the French marketing authorization (AMM) files (AMM 673 495.0 dated 13/03/1996 and AMM 699 496.7 dated 13/03/1996).

C) GENERAL VACCINE ACTIVITY

At the start of the intervention, the 126 pups in the two kennels constituted a particularly susceptible population. 108 (85.71 %) had non-immunizing maternally derived antibody

titers (≤ 64) and 18 (14.29 %) had titers in excess of the referential threshold (≥ 128).

A fortnight after single injection with the high titer vaccine, 115 pups presented titers ≥ 128 (91.27 %) and 11 (8.73 %) were, a priori, still susceptible to infection (≤ 64). The acquired and/or residual immunity rate alone is enough to explain the end to the epizootic in both kennels (the Charles NICOLLE law).

The contribution made by the vaccine to this evolution is represented by an overall seroconversion rate of 70.63 % (89 pups out of 126), including 81.48 % for those pups which had prevaccination titers of ≤ 64 (88/108) and 5.55 % for those pups which had prevaccination titers of ≥ 128 (1/18) (Table VIII).

D) INTERFERENCE WITH MATERNALLY DERIVED ANTIBODIES

Traditional canine parvovirus vaccines are known to be extremely susceptible to active immunization by maternally derived antibodies. They combine seroconversion rates of 95 %, 50 % and 0 % respectively for HI antibody titers of less than 10, around 20 and greater than or equal to 80 [3].

Analysis of Table VIII which shows the seroconversion rates obtained in the 126 pups in relation to their initial antibody titers, enables the references 95 %, 50 % and 0 % respectively to be placed at titers of less than 32, 64 and ≥ 128 . These results correspond to a gain of two dilutions in relation to traditional vaccines, and, in parallel, a considerable decrease in the critical period.

E) DURATION OF THE CRITICAL PERIOD

Using a theoretical a half-life time for maternally derived antibodies of 10 days [5, 11], the gain of two dilutions obtained for seroconversion rates of 95 % and 50 % respectively corresponds to a reduction of 20 days in the critical period.

For an average pup with an HI antibody titer of 1024 at birth [11], the critical period obtained with a traditional vac-

Age (weeks)	Prevaccination HI antibody titer									SC/W (rate(%))
	8	16	32	64	128	256	512	1024	≥ 2048	
4 w		7	2							9/9 (100)
5 w	1	6	2	1						10/10 (100)
6 w	6	26	26	15	2	2	2		1	55/80 (68,75)
7 w		7	6	2	1	2				14/18 (77,77)
8 w				1	1	5	1	1		1/9 (11,11)
SC/HI	7/7	43/46	28/36	10/19	0/4	1/9	0/3	0/1	0/1	89/126
(rate(%))	100	93,47	77,77	52,63	0	11,11	0	0	0	70,63

TABLE VIII. — Distribution of prevaccination HI antibody titers in relation to the age of pups and evolutions in seroconversion rate in relation to the prevaccination HI antibody titer (SC/HI) and to the age of pups (SC/Weeks) . Kennel n°s 1 and 2.

	Age of pup in days								
	0	10	20	30	40	50	60	70	80
HI antibody titer	1024	512	256	128	64	32	16	8	0
Critical period (1)									
Critical period (2)									

TABLE IX. — Theoretical evolution in maternally derived immunization in relation to the initial HI antibody titer acquired at birth (1024). Highlighting of the duration of the critical period using a traditional (1) or a high titer vaccine (2).

cine is located between the 40th and 70th day (or between the 6th and 10th week), i.e. between titers of 64 (protection threshold) and 8 (95 % seroconversion threshold). This gives a critical period of thirty days (Table IX).

With reference to the results obtained with the high titer vaccine, the critical period is located between titers of 64 (protection threshold) and 32 (95 % seroconversion threshold), i.e. between the 40th and 50th day. This gives a reduced critical period of 10 days (Table IX).

More particularly, the critical period at a seroconversion rate of 50 % is 20 days for a traditional vaccine and almost non-existent for a high titer vaccine.

F) AGE UPON INITIAL VACCINATION

Given the gradual decrease in maternally derived antibodies, potential seroconversion rates increase with the age of pups. For an initial reference titer of ≤ 1024 at birth, vaccination with high titer vaccines may validly be envisaged as of the age of 6 weeks (seroconversion rate of ≥ 50 %).

When necessary, a booster may be administered 10 to 20 days later in order to ensure the seroconversion of pups with an initial antibody titer at birth of more than 1024, from one to two dilutions. These conclusions correspond to the instructions for use of the vaccine tested (AMM 673 495.0 and AMM 699 496.7), as well as to the protocol followed in the study (second injection 15 days after the first).

Nonetheless, in the kennels studied which have known an epizootic, the breakdown of prevaccination antibody titers in relation to age groups (Table VIII), shows an inverse distribution, since 6- to 8-week-old pups had higher titers than some of the 4- to 5-week-old pups. For the latter, the seroconversion rate was 100 % (19/19) (Table VIII).

These results confirm that :

- for initial vaccinations, it is more important to consider the prevaccination antibody titer than the age of the animal,
- in order to determine the ideal time for vaccination when the prevaccination titer of the pups is unknown, the age groups most affected by the last epizootic must be considered and those pups at risk should be vaccinated 10 to 20 days in advance,
- pups of less than 6 weeks old, i.e. 4- to 5-week-old pups, show a good response to vaccination (excluded from AMM), when the initial titer is less than or equal to 64.

Conclusion

When combined with appropriate sanitary measures, the systematic use of a high titer attenuated canine parvovirus vaccine in two infected kennels enabled us to obtain a protection rate of more than 90 % of the endangered pups and, in parallel, to curb the parvovirus epizootic afflicting both kennels.

These results are based on the ability of the vaccine used to confer active immunity, even in the presence of residual maternally-derived antibodies. The referential serological conversion rates of 95 % and 50 % correspond to initial HI antibody titers of less than 32 and 64 respectively, and the latter antibody titer is also equivalent to the protection threshold. In parallel, the "critical period", which posed a major obstacle in the use of traditional vaccines against canine parvovirus, was practically eliminated.

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Product Sheet

Canine parvovirus (ATCC® VR-2016™)

Please read this FIRST



Storage Temp.
-70°C or colder



Biosafety Level
2

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: Canine parvovirus (ATCC® VR-2016™)

American Type Culture Collection
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800.638.6597 or 703.365.2700
Fax: 703.365.2750
Email: Tech@atcc.org

Or contact your local distributor



Description

Strain: Cornell-780916-115

Classification: *Parvoviridae*, *Parvovirus*, Feline panleukopenia virus

Original Source: Intestinal contents of beagle pups with severe enteritis

Depositor: Cornell Res. Fndn., Inc.



Batch-Specific Information

Refer to the Certificate of Analysis for batch-specific test results.



Propagation

Propagation Host:

Dog kidney (DK) cells from SPF beagle pups.

Effect on Host:

CPE, presence of virus monitored by increase in hemagglutinin (HA) titer and/or positive immunofluorescence.

Growth Conditions

Recommendations for Infection: Plant cells 6 hours in advance and infect when cultures are 40-50% confluent.

Incubation: Incubate infected DK cell culture for 3-5 days at 35°C in 100% atmospheric air, until CPE is well advanced through 90% of the culture.



Comments

Reconstitute freeze-dried vials to 1 mL with distilled water. Reduced virulence variant obtained by multiple passages at 33°C as per depositor. Virus agglutinates pig and rhesus monkey erythrocytes. Can be tittered using A-72 (ATCC® CRL-1542™) cells. NOTE: This material is cited in a U.S. and/or other patent applications and may not be used to infringe the patent claims.



References

References and other information relating to this product are available online at www.atcc.org.



Key Abbreviations

°C, Degrees Celsius

CPE, Cytopathic effect

SPF, Specific pathogen free



Biosafety Level: 2

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

ATCC Warranty

The viability of ATCC® products is warranted for 30 days from the date of shipment, and is valid only if the product is stored and cultured according to the information included on this product information sheet. ATCC lists the media formulation that has been found to be effective for this strain. While other, unspecified media may also produce satisfactory results, a change in media or the absence of an additive from the ATCC recommended media may affect recovery, growth and/or function of this strain. If an alternative medium formulation is used, the ATCC warranty for viability is no longer valid.

Disclaimers

This product is intended for laboratory research purposes only. It is not intended for use in humans. While ATCC uses reasonable efforts to include accurate and up-to-date information on this product sheet, ATCC makes no warranties or representations as to its accuracy. Citations from scientific literature and patents are provided for informational purposes only. ATCC does not warrant that such information has been



Product Sheet

Canine parvovirus (ATCC® VR-2016™)

confirmed to be accurate.

This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of strains on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of cultures.

Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org

Additional information on this culture is available on the ATCC web site at www.atcc.org.

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Please read this FIRST

	Storage Temp. -70°C or colder
.....	
	Biosafety Level 2

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: Canine parvovirus (ATCC® VR-2016™)

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Or contact your local distributor

Señora Directora

NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Referencia: Expediente No. NC2018/0003460
Asunto: Solicitud de patente para: VACUNAS DE PARTÍCULAS TIPO VIRUS (VLP) CONTRA EL PARVOVIRUS CANINO (CPV) Y SUS USOS
Solicitante: MERIAL INC.
Fecha de solicitud: 28 de marzo de 2018

RESPUESTA A PRIMER EXAMEN DE PATENTABILIDAD

ANDRÉS RINCÓN USCÁTEGUI, identificado como aparece al pie de mi firma, en mi condición de apoderado de la sociedad solicitante de la patente de la referencia, doy respuesta al Oficio No. 371, notificado por fijación en lista el 01 de marzo de 2019.

I. MODIFICACIONES AL CAPÍTULO REIVINDICATORIO

En respuesta al examen de patentabilidad aportó un Capítulo Reivindicatorio modificado conformado por 8 reivindicaciones en las que se han incluido las siguientes modificaciones respecto del Capítulo Reivindicatorio radicado el 01 de junio de 2018:

- Se eliminaron las antiguas Reivindicaciones 8 y 10 a 16.
- Se incluyeron las características técnicas reclamadas en la antigua Reivindicación 7 en la Reivindicación 1.
- Así mismo, se redactó la Reivindicación 1 de manera que el objeto reclamado sea clara para una persona versada en la materia y se indicó, que de acuerdo con las enseñanzas del Párrafo [00120], la composición comprende una cantidad eficaz de un parvovirus canino vivo modificado (CPV MLV) y de partículas de tipo virus de parvovirus canino (CPV VLP), componentes que permiten superar el efecto neutralizante de neutralizar los anticuerpos derivados por vía materna (MDA) (Capítulo Descriptivo, Párrafos [0006] y [0033]).

- Adicionalmente, se modificó la Reivindicación 1 para indicar que el parvovirus vivo modificado (CPV MLV) corresponde a la cepa Cornell 780916, la cual es el ingrediente activo del CPV MLV empleado en el Ejemplo 5 de la presente invención: PRIMODOG®, como lo demuestran los Anexos 1 a 3, a fin de evitar referencias a nombres comerciales en el Capítulo Reivindicatorio. Dicha cepa ha sido depositada en el *American Type Culture Collect* (<https://www.atcc.org/products/all/VR-2016.aspx#generalinformation>) y está disponible al público (ver Anexo 4).
- Todas las reivindicaciones, así como sus relaciones de dependencia fueron reenumeradas de acuerdo a las modificaciones arriba detalladas.

Me permito resaltar que estas modificaciones se ajustan completamente a lo establecido por el Artículo 34 de la Decisión 486 de 2000, ya que no constituyen una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial.

Por otro lado, a pesar de eliminar materia del Capítulo Reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud.

II. EN CUANTO A LA SUPUESTA FALTA DE CLARIDAD Y CONCISIÓN

En primer lugar, considero como superadas las objeciones de claridad referentes a las antiguas Reivindicaciones 8, 15 y 16, teniendo en cuenta que éstas fueron completamente eliminadas del Capítulo Reivindicatorio modificado adjunto, tal y como lo señalé en el numeral I de este memorial. Así mismo, considero como superada la objeción respecto de la definición de las CPV VLP reclamadas en la Reivindicación 1, teniendo en cuenta que las características técnicas esenciales de la antigua Reivindicación 7 fueron incluidas en la Reivindicación 1, las cuales indican que las CPV VLP corresponden a la SEQ ID NO.: 1, 3, 4, 6, 8, 9 o 10 o fragmentos de las mismas.

En cuanto a la objeción respecto de la expresión "*fragmentos de los mismos*" referente a las secuencias definidas para las CPV VLPs, deseo señalar que dicha expresión es válida en el contexto de la Reivindicación 1, considerando que las CPV VLPs se refieren a partículas

virales que eliciten una respuesta inmunogénica. De esta manera, polipéptidos producidos por fragmentos de dichas secuencias también podrían generar el efecto de acuerdo con la presente invención y por tanto realizaciones de este tipo deben ser protegidas por la presente solicitud. Así mismo, preciso que la SEQ ID NO. 6 corresponde a un polipéptido VP2 de la cápside de CPV, por lo tanto, corresponde a una partícula de tipo virus, como las arriba mencionadas.

Por otro lado, el Examinador señala que la Reivindicación 1 no es clara dado que se refiere a un “*un componente de un virus vivo modificado (MLV)*”, sin definir su secuencia polipeptídica.

Respecto de lo anterior, señalo que el Capítulo Reivindicatorio modificado adjunto indica que la cepa de virus vivo modificada corresponde a la cepa Cornell 780916, que es el ingrediente activo de la vacuna comercial PRIMODOG® empleada como CPV MLV en el Ejemplo 5 de la presente solicitud (Capítulo Descriptivo, página 66, Tabla 6), como lo constatan los Anexos 1 a 3 de la siguiente manera:

- Anexo 1: Correspondiente al artículo de referencia: De Cramer, K. G. M., Stylianides, E., & Van Vuuren, M. (2011). *Efficacy of vaccination at 4 and 6 weeks in the control of canine parvovirus*. Veterinary microbiology, 149(1-2), 126-132; el cual indica:

*“La vacuna temprana administrada fue una vacuna viva no adyuvada modificada **que contiene la cepa Cornell 780916-115** de parvovirus con un título viral de 107.8 DICT50 (PRIMODOG Merial, Lyon, Francia, para cachorros según lo determinado en la línea celular canina).”* - De Cramer et al. (2010), p. 128, col. izq. [texto traducido]¹

- Anexo 2: Correspondiente al artículo de referencia: Willem, C., Latour, S., & Lacheretz, A. (2001). *Control of canine parvovirus infection in breeding kennels: Study of the efficacy of*. Revue Méd. Vét, 152(5), 373-378.; el cual indica:

*“**Primadog®**: vacuna viva modificada contra el parvovirus canino - **cepa Cornell 780916** - título de infectividad mínimo por dosis de 1 ml 105.5 TCID50 en una línea de células*

¹ Versión original: “The early vaccine administered was a non-adjuvanted, modified-live vaccine **containing parvovirus strain Cornell 780916-115 strain** with a viral titre of 107.8 TCID50 (PRIMODOG Merial, Lyon, France, for puppies as determined on canine cell line).”

felinas, es decir, 107.8 TCID50 en una línea de células caninas” – Willem et al. (2010), p. 374, col. izq. [texto traducido]²

- Anexo 3: Correspondiente al artículo de referencia: Grellet, A., Feugier, A., Chastant-Maillard, S., Carrez, B., Boucraut-Baralon, C., Casseleux, G., & Grandjean, D. (2012). Validation of a fecal scoring scale in puppies during the weaning period. Preventive veterinary medicine, 106(3-4), 315-323.; el cual indica:

*“Los cachorros fueron vacunados a las 5, 6 y 7 semanas de edad con una vacuna viva sin adyuvante modificada **que contiene la cepa parvovirus Cornell 780916-115** con un título viral de 105.5 DICT50 (**Primodog®**, Merial, Lyon, Francia).” – Grellet et al. (2012), p. 318, col. izq. [texto traducido]³*

Con base en lo anterior, indico que el soporte de la modificación arriba detallada se encuentra implícito en la mención a la formulación PRIMODOG® (Capítulo Descriptivo, Ejemplo 5), cuyo ingrediente activo es el virus vivo modificado empleado de la cepa Cornell 780916. De esta manera, la persona versada en la materia apreciará que PRIMODOG® se refiere a una vacuna de virus vivo modificado formulada por Merial en la que se emplea la cepa arriba indicada como ingrediente activo.

Adicionalmente, deseo poner de presente que dicha cepa se encuentra depositada en la institución *American Type Culture Collect* (ATCC) (ver <https://www.atcc.org/products/all/VR-2016.aspx#generalinformation>) reconocida como Autoridad Depositaria Internacional (ADI) de acuerdo con el Tratado de Budapest. Adjunto a este memorial presento el Anexo 4 correspondiente a la Hoja de Producto emitida por la ATCC para la cepa en cuestión. En vista de lo anterior, amablemente solicito al Examinador tener la presente objeción por superada, considerando que el CPV MLV empleado es de acceso público y corresponde al ingrediente activo de la formulación comercial PRIMODOG® evaluada en el Ejemplo 5 de la presente solicitud.

² Versión original: “**Primadog®**: modified living canine parvovirus vaccine - **Cornell 780916 strain** - minimum infectivity titer per 1 ml dose 105.5 TCID50 on a feline cell line, i.e. 107.8 TCID50 on a canine cell line”

³ Versión original: “Puppies were vaccinated at 5, 6 and 7 weeks of age with a non-adjuvant, modified-live vaccine **containing parvovirus Cornell 780916-115 strain** with a viral titer of 105.5 TCID50 (**Primodog®**, Merial, Lyon, France).”

Considerando lo anterior y particularmente, teniendo en cuenta que la vacuna reclamada ha sido definida en términos de la cepa específica de CPV MLV empleada y que la secuencia polipeptídica específica de las CPV VLPs ha sido incluida en la Reivindicación 1, amablemente solicito al Examinador considerar como superada la objeción de falta de claridad respecto de las Reivindicaciones 3 a 6, toda vez que las características técnicas esenciales de la presente invención fueron definidas en la reivindicación independiente.

Adicionalmente, en cuanto al conector “*por que*”, deseo llamar la atención del Examinador sobre las enseñanzas de la Real Academia de la Lengua⁴ en donde se especifica que el uso de la preposición “*por*” y la conjunción subordinante “*que*”, se emplea con verbos “*que rigen un complemento introducido por la preposición por y llevan además una oración subordinada introducida por la conjunción que*”, como es el caso de los verbos “*optar por*”, “*preocuparse por*” y “*caracterizado por*”, como se emplea en el Capítulo Reivindicatorio modificado adjunto. Con base en lo anterior, amablemente solicito al Examinador tener como superada la objeción arriba detallada, considerando que la conjunción “*porque*” indica una relación de causalidad la cual es imprecisa para definir el objeto de una invención, teniendo en cuenta que la materia reclamada no se caracteriza por una causa, sino por [características adicionales] que la definen.

En cuanto a las expresiones objetadas por el Examinador en las Reivindicaciones 1 a 2 como referentes a resultados a alcanzar, deseo señalar que *la Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad* (GESPMU) indica en la sección 2.6.5.8.⁵ de dicho documento, que los resultados a alcanzar son permitidos en una reivindicación siempre que la invención esté definida por sus características técnicas esenciales. En el presente caso, las expresiones referidas por el Examinador, hacen referencia la vacuna de la Reivindicación 1, cuyos componentes han sido definidos de manera suficiente. Por lo tanto, las expresiones señaladas por el Examinador no pretenden definir el objeto reclamado en la presente invención, sino que por el contrario lo delimitan, toda vez que quedarían excluidas del alcance de esta solicitud vacunas que no puedan superar el efecto neutralizante de los anticuerpos derivados por vía materna o que no provean inmunidad con una sola dosis.

⁴ <http://www.rae.es/consultas/porque-porque-por-que-por-que>

⁵ Superintendencia de Industria y Comercio (2014) *GUÍA PARA EXAMEN DE SOLICITUDES DE PATENTE DE INVENCION Y MODELO DE UTILIDAD* (pp. 79). Bogotá, Colombia.

En conclusión, considero que el Capítulo Reivindicatorio modificado adjunto cumple con todos los requisitos de claridad y concisión, ajustándose así a los lineamientos del Artículo 30 de la Decisión 486, ya que cualquier persona del oficio normalmente versada en la materia está en completa capacidad de reproducir la invención sin ambigüedad alguna.

III. EN CUANTO AL CAPÍTULO DESCRIPTIVO

El Examinador indica que el Capítulo Descriptivo no divulga la invención de manera suficiente y argumenta que la modalidad en la que *“la composición o vacuna no tiene adyuvante”* no está descrita de manera tal que la persona normalmente versada en la materia pueda ejecutar o reproducir la realización preferida mencionada.

Sobre este particular, deseo indicar que la característica técnica demarcada en la Reivindicación 8 (antigua Reivindicación 9) corresponde a una limitación negativa que significa que la composición está libre de adyuvantes, lo cual no requeriría de experimentación adicional o un esfuerzo inventivo significativo para abstenerse de incluir un adyuvante en una composición de vacuna. Particularmente, un adyuvante es un agente farmacéutico o inmunológico que potencia o dirige la acción del ingrediente principal de una composición, por lo tanto, éste es un agente adicional que puede o no estar incluido en la composición sin alterar el efecto técnico proporcionado por la misma, considerando que el efecto inmunogénico de la vacuna está dado por las VLPs y MLV que la componen y que fueron definidos en la Reivindicación 1 del Capítulo Reivindicatorio modificado.

Adicionalmente, deseo señalar que, contrario a lo indicado por el Examinador, modalidades de la vacuna en donde no se agrega un agente adyuvante adicional cuentan con debido soporte en el Capítulo Descriptivo como se indica en los Párrafos [0038], [0080], [0083] y [0119], por lo tanto, una objeción referente a falta de soporte respecto de una composición en la cual no se agrega un adyuvante es discutible.

Por otro lado, deseo señalar que la persona normalmente versada en la materia entiende fácilmente lo que significa tener una vacuna a la cual no se agrega un adyuvante. Por ejemplo, De Cramer et al. (2011) en la página 128 indican que la primera vacuna administrada fue una vacuna modificada que no contenía un adyuvante, así mismo Grellet

et al. (2012) indica en la página 318 que los cachorros fueron vacunados con una vacuna viva modificada sin adyuvante.

IV. ESTADO DE LA TÉCNICA

El Examinador indica que los siguientes documentos son relevantes respecto de la patentabilidad del objeto de la solicitud.

No.	Documento	Título	Fecha de Publicación
D1	Azevedo M P, et al.	<i>“Human rotavirus virus-like particle vaccines evaluated in a neonatal gnotobiotic pig model of human rotavirus disease</i> ”	13 Enero 2011

V. EN CUANTO A LA SUPUESTA FALTA DE NIVEL INVENTIVO

El Examinador señala que el objeto de la solicitud carece de altura inventiva en vista de las enseñanzas de D1. Particularmente, el Examinador indica que dicho documento revela combinaciones de vacunas vivas atenuadas con vacunas VLP e indica que la combinación de este tipo de vacunas es más efectiva que sus componentes por separado. Adicionalmente, argumenta que la única diferencia entre dicha invención y el objeto de la presente solicitud es que éste último involucra además un componente MLV, lo cual no confiere un efecto técnico distintivo según el Examinador.

Respecto de lo anterior, deseo señalar que D1 evaluó el uso de vacunas de partículas similares a rotavirus humanos como un enfoque alternativo a las vacunas orales de rotavirus atenuadas en vivo (D1, Resumen). El diseño experimental de D1 evaluó varios regímenes de refuerzo principal que utilizaron rotavirus humano atenuado y VLP.

Respecto de lo anterior, deseo indicar que D1 no enseña (i) una vacuna contra el CPV o (ii) una vacuna combinada que comprenda virus vivos modificados y VLPs. Por lo tanto, una persona normalmente versada en la técnica no se vería motivada a modificar el objeto de D1 incluyendo las características técnicas de la presente invención y esperar obtener una vacuna eficaz en contra de CPV, con una expectativa razonable de éxito. En primer lugar, D1 no brinda enseñanzas que siquiera sugieran una vacuna contra CPV o una vacuna

combinada. En segundo lugar, indico que la acción de entes biológicos es impredecible y por tanto el experto en la materia no podría inferir el objeto de la presente invención únicamente basado en lo divulgado en D1. De acuerdo con lo anterior, señalo que las enseñanzas de D1 relacionadas con el rotavirus humano no arrojan ninguna luz sobre la probabilidad de éxito de una vacuna de combinación de MLV y VLPs en contra de CPV, lo anterior, en vista de que el arte previo citado no enseña ni sugiere de ninguna manera las secuencias particulares de VLP o MLV empleadas en la presente invención ni la combinación de dichos péptidos en una única composición de vacuna.

Además, deseo resaltar que D1 menciona que:

“Los altos niveles de anticuerpos pasivos de Rotavirus circulantes indujeron una supresión significativa del efector intestinal de IgA e IgG, las células de memoria B y los títulos de anticuerpos en cerdos que recibieron la vacuna **AttHRV-VLP-ISCOM**.” - D1, página 174, col. Derecha. [texto traducido]⁶

Lo anterior evidencia que una vacuna que combina un virus atenuado vivo (AttHRV) y VLPs no logra superar la barrera planteada por los MDA de los cerdos inyectados con suero hiperinmune (solución que imita los títulos de anticuerpos maternos en mujeres). Por lo tanto, la inmunización contra la infección por Rotavirus se suprimió en cierto porcentaje (D1, página 174, col. derecha), lo que indica que la vacuna descrita en D1 no fue suficiente para evitar el bloqueo de los MDA. Con respecto a lo anterior, D1 sugiere que las vacunas no replicantes son posiblemente más efectivas en presencia de altos niveles de anticuerpos maternos (D1, página 174, col. derecha) y que estudios adicionales que evalúen diferentes VLP (2/4/6/7 VLPs) de Rotavirus se debe realizar para proporcionar una vacuna capaz de evitar el bloqueo por MDA.

A la luz de lo anteriormente mencionado, una persona normalmente versada en la materia no se vería motivada a combinar en una misma composición VLPs junto con MLV, a fin de obtener una vacuna que pueda eludir la neutralización de la vacuna por MDA residuales, debido a que las enseñanzas de D1 evidencian que una vacuna de este tipo presentó altos porcentajes de inhibición en presencia de MDA.

⁶ Versión original: “High levels of passive circulating RV antibodies induced significant suppression of intestinal IgA and IgG effector, memory B cells and antibody titers in pigs receiving the AttHRV-VLP-ISCOM vaccine.”

Por el contrario, la presente invención destaca en las Figuras 12, 13 y las Tablas 6, 13 que el título de anticuerpos anti-CPV fue mayor en los tratamientos en los que se emplearon composiciones de vacuna VPL + MLV de acuerdo a la presente invención, en comparación con vacunas que solo presentan MLV, al evaluar la eficacia de la vacuna en presencia de títulos de MDA caninos normales. Lo anterior, claramente resalta que la combinación particular de un MLV como el definido en la Reivindicación 1 de la presente invención, junto con las VLPs de secuencia SEQ ID NO.: 1, 3, 4, 6, 8, 9 ó 10, brindan una composición de vacuna efectiva para proporcionar inmunización en presencia de niveles de MDA inalterados después de que los cachorros adquieren inmunidad pasiva, evitando así la neutralización de la vacuna por MDA y resolviendo un problema técnico de la medicina veterinaria canina.

En vista de lo anterior, señalo que el objeto de la presente invención **implicaría ir en contra de las enseñanzas de la técnica anterior citada**, las cuales sugieren que las vacunas que solo comprenden VLP deberían ser más eficientes que una en la que MLV y VLP se encuentren presentes en la misma composición de vacuna. A la luz de lo anterior, el alto título de anticuerpos anti-CPV recuperado de los tratamientos que emplean la vacuna de acuerdo con la presente invención es un resultado sorprendente (ver Ejemplo 5, Párrafos [00190] a [00193]), que no podría haberse anticipado con base en el arte previo citado, dado que implica ir en contra de las enseñanzas del mismo. Particularmente, teniendo en cuenta que la presente invención provee un efecto sinérgico que permite al animal desarrollar una inmunidad protectora contra CPV, incluso si el animal tiene MDA circulantes contra la CPV, lo cual es un logro significativo en el campo de las vacunas contra el CPV, puesto que superar la interferencia a la vacuna causada por los MDA ha desafiado a científicos de esta área del conocimiento durante mucho tiempo. Como resultado del trabajo de los inventores, los animales vacunados con la composición de la presente invención ahora tienen un inicio más temprano de inmunidad protectora contra el CPV.

De esta manera, señalo que los hallazgos de los inventores y los resultados que se derivan de ellos no pudieron haber sido predichos razonablemente por un experto en la materia, ya sea por su propio conocimiento o basado en las enseñanzas de D1, como lo señalé anteriormente, teniendo en cuenta además que la naturaleza y secuencias específicas de

los componentes de la invención no habrían podido ser inferidos con base en las enseñanzas de D1, las cuales ni siquiera están dirigidas a CPV. En vista de todo lo anterior, amablemente solicito al Examinador reconocer la altura inventiva de la presente invención.

VI. PETICIÓN

1. Puesto que se ha dado respuesta a todas las objeciones formuladas por la Dirección de Nuevas Creaciones, solicito proceder al otorgamiento de la solicitud de patente.
2. De manera subsidiaria, y en caso de que su Despacho considere que la modificación introducida al Capítulo Reivindicatorio amerita revisión adicional, solicito un tercer examen de patentabilidad de conformidad con lo dispuesto en el Artículo 2 de la Resolución 3719 de 02 de febrero de 2016, para lo cual realizo el pago de tasa correspondiente.

VII. ANEXOS

1. Capítulo Reivindicatorio modificado conformado por 9 reivindicaciones.
2. Recibo de pago en línea por concepto de examen de patentabilidad adicional.
3. Anexo 1 – De Cramer et al. (2010)
4. Anexo 2 – Willem et al. (2001)
5. Anexo 3 – Grellet et al. (2012)
6. Anexo 4 – Hoja de producto cepa Cornell 780916 - ATCC

Señora Directora,

ANDRÉS RINCÓN USCÁTEGUI

C.C. 79.780.910 de Bogotá

T.P. 114.908



Validation of a fecal scoring scale in puppies during the weaning period

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Breed size

ABSTRACT

In puppies weaning is a high risk period. Fecal changes are frequent and can be signs of infection by digestive pathogens (bacteria, viruses, parasites) and indicators of nutritional and environmental stress. The aim of this study was to define a pathological fecal score for weaning puppies, and to study the impact on that score of two intestinal viruses (canine parvovirus type 2 and canine coronavirus). For this, the quality of stools was evaluated on 154 puppies between 4 and 8 weeks of age (100 from small breeds and 54 from large breeds). The scoring was performed immediately after a spontaneous defecation based on a 13-point scale (from 1; liquid to 13; dry and hard feces). Fecal samples were frozen for further viral analysis. Each puppy was weighed once a week during the study period. The fecal score regarded as pathological was the highest score associated with a significant reduction in average daily gain (ADG). Fecal samples were checked by semi-quantitative PCR or RT-PCR for canine parvovirus type 2 and canine coronavirus identification, respectively. The quality of feces was affected by both age and breed size. In small breeds, the ADG was significantly reduced under a fecal score of 6 and 7 for puppies at 4–5 and 6–8 weeks of age, respectively. In large breeds, the ADG was significantly reduced under a fecal score of 5 whatever the age of the puppy. Whereas a high viral load of canine parvovirus type 2 significantly impacted feces quality, no effect was recorded for canine coronavirus. This study provides an objective threshold for evaluation of fecal quality in weaning puppies. It also emphasizes the importance to be given to age and breed size in that evaluation.

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1. Introduction

In dogs, feces quality can be influenced by own characteristics of the dog (breed size and age), digestive pathogens (bacteria, viruses, and parasites), diet (change without transition, type and quality of food), lifestyle and environment (stress) (Weber et al., 2002, 2003; Sokolow et al.,

2005; Hernot et al., 2006; Stavisky et al., 2011). Various fecal scales have been proposed to evaluate feces quality in adult dogs (Meyer et al., 1999; Rolfe et al., 2002a,b; Propst et al., 2003; Hernot et al., 2006; Allenspach et al., 2007). These scales are divided in 4–10 points, with lower grades corresponding to either dry feces or diarrhea, and with an optimal fecal score varying from 2 to 7.5 (Rolfe et al., 2002a,b; Hernot et al., 2005; Allenspach et al., 2007). Digestive health is particularly challenged in puppies around the weaning period. Weaning is associated with major changes in food intake, together with profound environmental and social modifications. Besides these major alterations in digestive balance, the

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Table 1
Fecal scale for the evaluation of the quality of feces in weaning puppies.

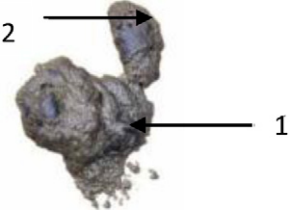
Fecal score	Visual aspect of the feces	Description of the feces
1		Feces completely liquid
2		Liquid feces associated with soft feces (soft feces do not represent the main quantity of feces)
3		Liquid feces associated with soft feces (soft feces represent the main quantity of feces)
4		Pasty feces with no shape
5		Pasty unformed feces visualization of the apparition of a cylindrical shaped (1) which has not kept its shape due to the high humidity
6		Feces mainly unformed (1) but with a part which is formed (2)
7		Formed stools but very soft. Cylindrical shaped feces without any stria observed.
8		Formed stools but very soft. Cylindrical shaped feces with presence of stria (1)

Table 1 (Continued)

Fecal score	Visual aspect of the feces	Description of the feces
9		Formed stools but very soft. Cylindrical shaped feces separated in pellets
10		Formed, drier but not hard feces. Cylindrical shaped feces, slightly tacky to the touch, separated in pellets
11		Formed, drier but not hard feces. Cylindrical shaped feces, dry appearance, separated in pellets, can be easily crushed out of shape
12		Formed, drier but not hard feces. Cylindrical shaped feces, dry appearance, separated in pellets, can be crushed out of shape with a moderate difficulty
13		Formed, dry and hard feces

weaning period is associated with a high incidence of some gastro-intestinal pathogens. All these factors can affect feces quality and reduce the average daily gain (ADG). Canine parvovirus type 2 (CPV2) is frequently isolated in puppies from shelters (Marshall et al., 1984; Vanparijs et al., 1991; Naylor et al., 2001) and was reported to have a major impact on feces quality at weaning (Schulz et al., 2008). Canine coronavirus (CCV) is also frequently identified in the feces of puppies but its role in the etiology of acute diarrhea remains controversial (Tennant et al., 1993; Yesilbag et al., 2004).

Since weaning is a high risk period for puppies, a tool for precise follow-up of digestive health would be most useful. Scoring systems used in adult dogs cannot be applied to young puppies because of a physiological lower fecal quality prior to the weaning period (Giffard et al., 2004). No study has defined an abnormal fecal score during the weaning period. The objectives of this study were thus to determine in puppies a threshold in fecal score that could be regarded as pathological, based on its correlation with a drop average daily gain, and to evaluate the association between pathological scores and digestive infection by CPV2 or CCV.

2. Material and methods

2.1. Animals

The study was conducted from December 2009 to June 2010 in the North of France in one large breeding kennel housing 330 purebred dogs (280 females, 50 males) of 16 different breeds.

Each pregnant bitch was housed singly for the 2 weeks preceding the expected delivery date. All puppies stayed with their dam in heated whelping boxes (surface: 2 m²) from birth to approximately 8 weeks of age, when they were sold. They were fed a dry expanded complete diet balanced for growing dogs (food composition: moisture 8%, protein 30%, crude fat 22%, crude fiber 1.8%, ash 6.9%, and metabolizable energy 4251 kcal/kg). The daily ration was calculated based on the number of puppies in the litter and on breed size. Half the quantity was distributed early in the day and half in the end of the day. For young puppies 4–6 weeks of age, the dry diet was rehydrated with water just before distribution.

Each puppy was treated with a single dose of diclazuril (Vecoxan®; Janssen Animal Health; Issy-Les-Moulineaux, France, 2.5 mg/kg, per os) at 4 and 7 weeks of age and

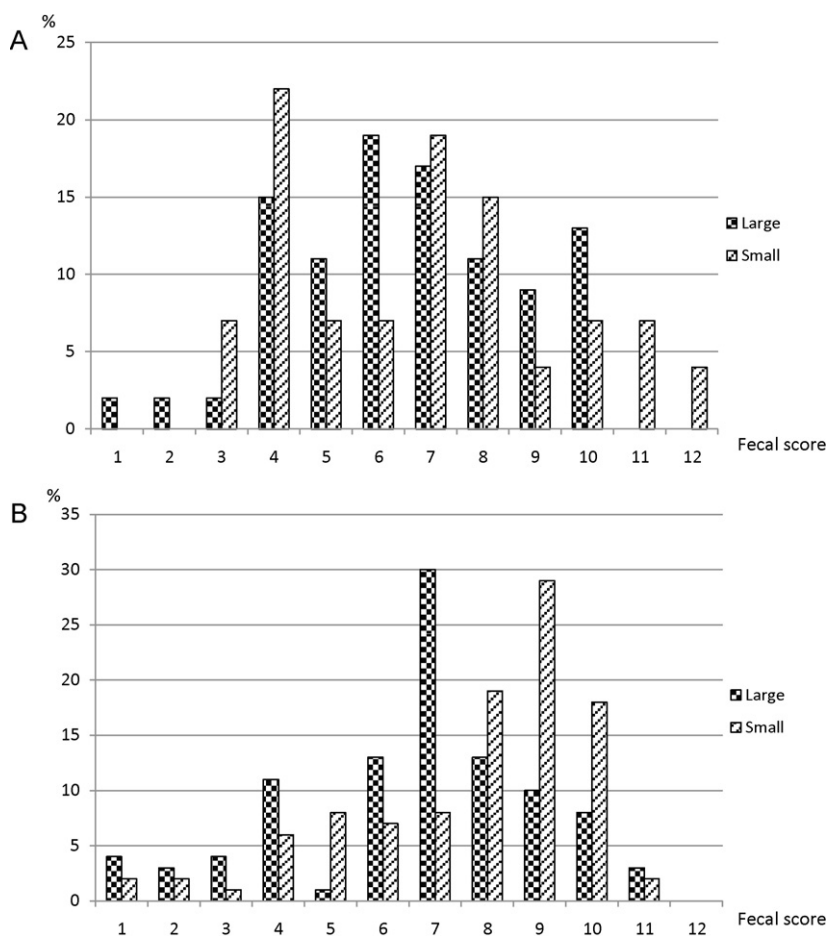


Fig. 1. Feces quality in puppies of 4–5 (Fig. 2A) and 6–8 (Fig. 2B) weeks of age depending on breed size ($n = 278$ feces).

with fenbendazole (Panacur®, Intervet; Beaucouzé, France, 50 mg/kg, per os, q 24 h) for 3 consecutive days at 2, 4, 6 and 8 weeks of age. Puppies were vaccinated at 5, 6 and 7 weeks of age with a non-adjuvant, modified-live vaccine containing parvovirus Cornell 780916-115 strain with a viral titer of $10^{5.5}$ TCID₅₀ (Primodog®, Merial, Lyon, France).

154 puppies (75 males and 79 females, from 46 litters) between 4 and 8 weeks of age were included in the study. Depending on the mean adult body weight of their respective breed, two groups of dogs were considered: small breed dogs including breeds with a mean adult body weight <10 kg (West Highland White Terrier, Bichon Frise, Poodle, Shih Tzu, Lhasa apso) and large breed dogs including breeds with a mean adult body weight >25 kg (German Shepherd, Golden retriever, Labrador retriever). 100 small breed puppies and 54 large breed puppies were enrolled. Within one litter, puppies were identified by wool collars of various colors.

2.2. Average daily gain

Puppies between 4 and 8 weeks of age were weighed every 7 days using calibrated electronic scales. The average

daily gain (ADG) was calculated for each puppy every week between 4 and 8 weeks of age as: $(\text{weight of week } (n) - \text{weight of week } (n - 1)) / 7$.

2.3. Feces sampling and scoring

All stool samples were scored by a single operator (AG) using a 13-point scale, based on the texture and shape of the feces (from liquid to hard and dry) (Table 1). A rectal swab was performed immediately after sampling for CPV2 and CCV detection. The swab was stored at -20°C until qPCR or qRT-PCR.

2.4. CCV and CPV2 detection

Total nucleic acids were extracted from the rectal swabs using a commercial kit (Nucleospin, Macherey Nagel; Hoerd, France), as recommended by the supplier and eluted in 400 μL . The analyses were performed by real-time polymerase chain reaction (PCR) or reverse-transcriptase PCR, for CPV2 and CCV detection, respectively, in a 7900 HT sequence detection system (Applied Biosystems, Villebon sur Yvette, France). Five microliters of eluate was used for PCR and absence of PCR inhibitors was checked for

Table 2

Viral load in rectal swab for canine parvovirus type 2 (CPV2) and canine coronavirus (CCV).

Viral load level	CPV2	CCV
1	<10 ^{4.2} copies	<10 ^{4.2} copies
2	<10 ^{5.7} copies	<10 ^{5.3} copies
3	<10 ^{7.7} copies	<10 ^{7.3} copies
4	<10 ^{10.3} copies	<10 ^{9.3} copies
5	>10 ^{10.3} copies	>10 ^{9.3} copies

each eluate. The detection threshold of the CPV2 real-time PCR (i.e. the lower number of genomic copies detected in 95% of runs) is 4 copies of genomic DNA VP2 template. The quantification threshold is 200 copies. These were determined by probit analysis as recommended by European Pharmacopeia for PCR assays assessment. The detection and quantification thresholds for canine coronavirus detection are, respectively, 36 and 200 copies of genomic RNA. Results from duplicate analysis (mean of the two results) were expressed semi-quantitatively as 5 viral load levels (Table 2). For CPV2, viral load levels have been determined in asymptomatic non-vaccinated, asymptomatic vaccinated or diseased dogs with confirmed parvovirus (histopathologic exam and/or positive testing using antigen assays). High and very high viral loads as defined in Table 2 are typical loads in dogs with parvovirus and correlated to positive antigen testing and detection of wild-type strains of virus, i.e. CPV2a, b or c (unpublished results). In vaccinated dogs the CPV2 viral loads are mostly low or very low but sometimes may be medium especially when the dog has been vaccinated a few days before sampling with high-titer vaccines. Whatever the vaccine used we neither obtained high viral loads of vaccine strain even in the days after vaccination (unpublished results). As far as CCV is concerned, the viral load levels have been arbitrary defined on the basis of several hundreds of tests in asymptomatic or diarrheic puppies from different breeds.

2.5. Statistical analysis

The effects of age, size of the breed and interaction between them were evaluated with the Catmod procedure of SAS (SAS Institute, Cary, NC, USA). Two ages (4–5 weeks and 6–8 weeks) were combined with two sizes (small and large breeds) in 2 × 2 factorial designs.

For determination of a threshold value for pathological fecal scores, a comparison of weight gain above and below of each fecal score was performed with a variance analysis (PROC GLM, SAS).

The effect of age, and breed size on the average daily gain was evaluated by variance analysis (PROC GLM, SAS).

The effects of CPV2 and CCV on the pathological feces and the interaction between them were evaluated using the Catmod procedure of SAS. Two levels of CPV2 (levels 1–3 vs 4–5) were combined with two levels of CCV (levels 1–3 vs 4–5) in 2 × 2 factorial designs. A *p* value <0.05 was considered statistically significant.

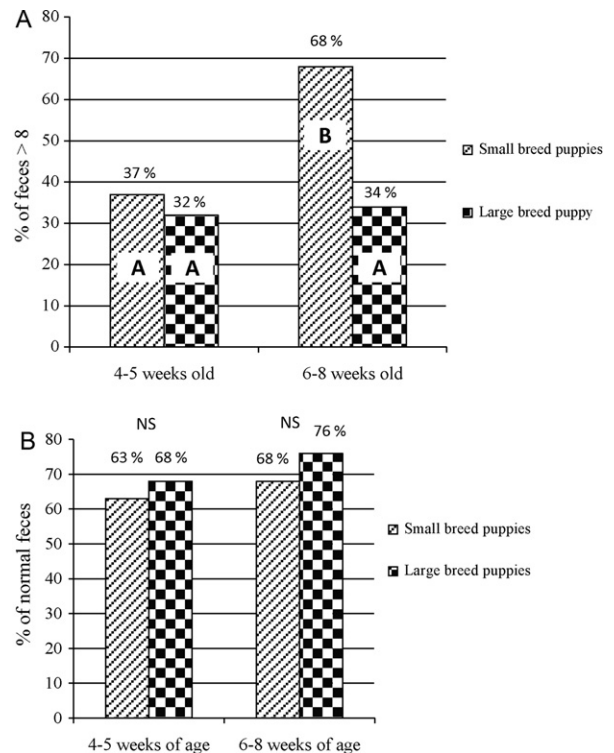


Fig. 2. Percentage of puppies with a fecal score > 8 (A) and with normal feces (using the new scale) (B) depending on breed size and age of puppies (*n* = 278 feces). NS: non significant; and A, B: categories with different letters were significantly different (*P* < 0.05).

3. Results

A spontaneous defecation was not observed from each puppy during each observation session. 74 feces samples (27 from small breeds and 47 from large breeds) and 204 feces samples (133 from small breeds and 71 from large breeds) were collected.

3.1. Effect of age and breed size on feces quality

Over the whole study, a median fecal score of 8 was obtained (ranging from 1 to 11). The proportion of feces with a score ≥ 8 was affected by age (*p* = 0.023) and breed size (*p* = 0.006). A significant interaction (*p* = 0.047) between age and breed size was recorded. Fecal scores of small breed puppies improved with age whereas no such effect was observed in large breed puppies (Figs. 1 and 2A). In view of this difference in effect of breed size on fecal score, further analyses were conducted separately for the two groups of puppies (small and large breeds).

3.2. Determination of a threshold value for pathological fecal score

The ADG was affected by breed size but not by age. As expected, significantly lower ADGs were observed in small breeds compared to large breeds, whatever the period (*P* < 0.001). No significant difference in ADG was observed

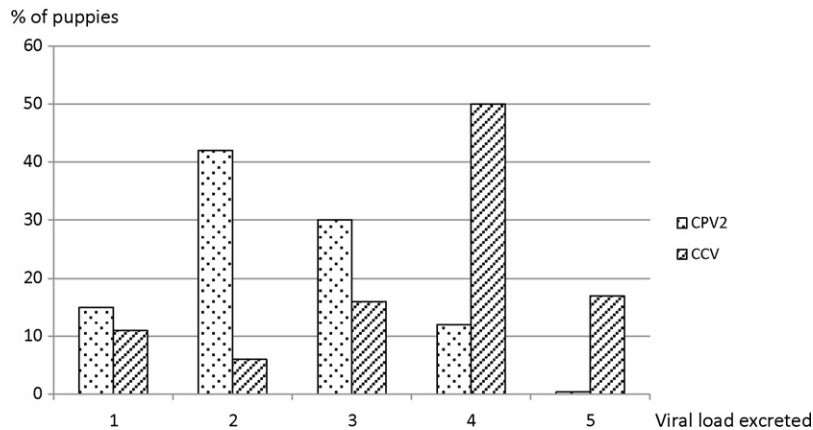


Fig. 3. Parvovirus type 2 and coronavirus loads excreted by puppies between 4 and 8 weeks of age ($n = 264$ feces).

the same management, food distribution and environmental pathogens. This made it possible to study the effect of breed size on the fecal score. Large breed puppies had more frequently soft and unformed feces. Such an effect of breed size has already been reported in adult dogs. In large breed dogs, such as German shepherds or Great Danes, the fecal moisture content is higher, soft stools are more frequent and the number of defecations is higher than in small breeds (Weber et al., 2001, 2002, 2003; Hernot et al., 2006). This difference may be due to a lower mineral absorption and/or to higher fermentative activity reflecting higher intestinal colic permeability and a longer colic transit time, respectively (Herschel et al., 1981; Kirkwood, 1985; Meyer et al., 1993, 1999; Rolfe et al., 2002a; Weber et al., 2004).

Distinct pathological thresholds were determined in our study for small and large breeds (Table 3). The proposed definition of abnormal fecal score, based on a reduction in ADG, makes it possible to balance the effects of age and breed size (Fig. 2). The relationship between infection by enteropathogens and abnormal fecal scores,

whatever the age and breed size of puppies, can then be investigated.

Fecal score thresholds were based on in ADG, but in a second time, the scoring scale was validated in evaluating the association between fecal score and the load of two intestinal viruses, CPV2 and CCV. Both viruses were isolated from all feces samples collected. Earlier serological and virological studies had demonstrated that puppies and dogs from kennels and animal shelters are particularly exposed to these infections (Rimmelzwaan et al., 1991; Tennant et al., 1993; Bandai et al., 1999; Naylor et al., 2001; Yesilbag et al., 2004; Schulz et al., 2008). The high prevalence observed here reflects the high contagiousity of these two viruses. Whereas viruses identified in the feces may sign the excretion of a wild viral strain, the low level of CPV2 detected in 87.3% of the feces may also result from vaccination. Puppies in our study were vaccinated at 5, 6, and 7 weeks of age with a non-adjuvant, modified live-vaccine containing a type 2 parvovirus strain. The use of such vaccines has been associated with fecal excretion of

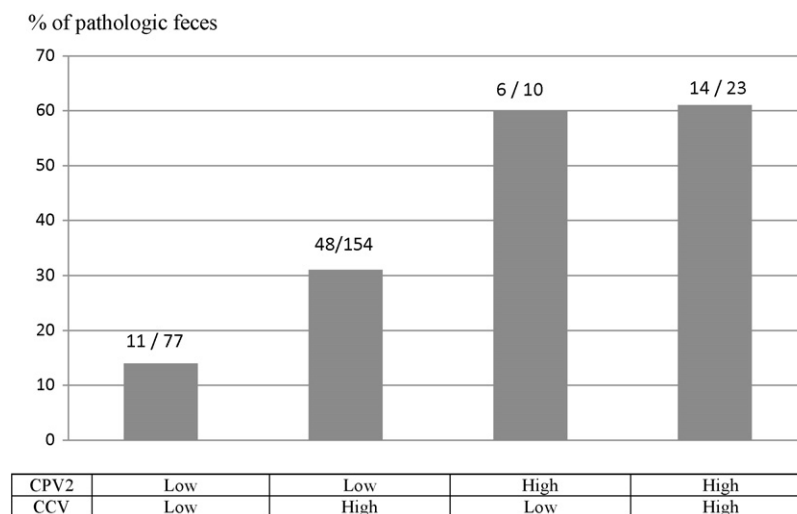


Fig. 4. Relationship between viral load (CCV and CPV2) and abnormal feces on puppies between 4 and 8 weeks of age ($n = 264$ feces). x/X ; x = number of pathological feces; and X = total number of feces in the group.

the vaccinal virus, the excretion remaining nevertheless markedly lower than in the case of a natural infection (Carmichael et al., 1981). The PCR assay used in this study has been shown to detect viruses in the feces of low-grade shedding animals (Desario et al., 2005; Schmitz et al., 2009).

In this work, a deterioration of the fecal score was recorded in dogs with high amounts of fecal CPV2. This virus is well known to cause weaning diarrhea (Potgieter et al., 1981; Carman and Povey, 1982; Schulz et al., 2008). So the fecal score proposed in this study may be of use in epidemiological studies designed to evaluate the effect of various enteropathogens on the quality of feces.

Interestingly, none of the puppies suffered from bloody diarrhea. Our study thus demonstrated that puppies may excrete high viral loads and yet present no systemic signs (anorexia, prostration). These healthy looking animals may however become major sources of virus for other animals. Whereas we confirmed the impact of CPV2 on fecal quality, we did not evidence any influence of CCV infection on the fecal score, whatever the amount excreted (Fig. 4). This virus is a common pathogen in dogs, but its actual role in acute dog diarrhea remains controversial (Tennant et al., 1993; Yesilbag et al., 2004). An interaction between CCV and CPV2 (aggravation of clinical signs in dogs infected by both viruses) was observed in 8 week old puppies under experimental condition (Appel, 1988). This interaction was not observed in our study.

5. Conclusion

Based on this study, a new objective fecal score, specific for puppies during the weaning period, could be defined. Thresholds below which feces are to be considered as pathological vary, according to age and breed size. This tool for management of digestive health may be of major interest to evaluate the impact of various infectious and environmental risk factors of digestive alterations in puppies during the weaning period.

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Research article

Efficacy of vaccination at 4 and 6 weeks in the control of canine parvovirus

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ABSTRACT

Seroconversion after early vaccination at four weeks against canine parvovirus (CPV) using a high antigen titre vaccine was evaluated in 121 puppies from three breeds of dogs housed in kennels representative of the private practitioner's environment. The trial included 52 German shepherd pups, 25 Rottweiler pups and 44 Boerboel pups. From each group 11, 4, and 18 puppies acted as control dogs, respectively. Depending on the different groups, puppies were vaccinated at 4, 6, 9 and 12 weeks. The experimental group differed from the control group in that they received the high titre vaccine at 4 weeks of age, whereas the control group was not vaccinated at 4 weeks. Blood was collected from all pups prior to vaccination to measure maternally derived colostral antibody. The results indicated that vaccination at 4 weeks of age in pups with high maternally derived antibody levels, results in seroconversion rates that may lead to a reduction in the window of susceptibility with respect to CPV infection. The implications of the findings with respect to dogs in heavily contaminated environments are discussed.

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1. Introduction

Parvoviral enteritis in dogs is a highly contagious disease that occurs worldwide but is still evolving (Shackelton et al., 2005; Truyen et al., 2000). Since the initial parvovirus enteritis pandemic following the emergence of canine parvovirus type 2 (CPV-2) (Hoelzer and Parrish, 2010), three variants, namely CPV-2a, 2b and 2c completely replaced the original type 2 virus (Buonavoglia et al., 2000). Certain species of wildlife have not been spared during that pandemic and the following periods (Mech et al., 2008). Despite advances in biotechnology and recommended vaccination protocols by academia and professional veterinary associations, clinical manifestation of CPV infection remains a serious problem in recently sold pups and in breeding kennels.

Early studies following the emergence of CPV demonstrated significant interference by maternally derived antibody (MDA) to vaccination of puppies (Gooding and Robinson, 1982; Waner et al., 1996). Additionally, the recommendations of vaccine manufacturers have aided to advance the practice of accepting six weeks as the earliest age of vaccination against CPV. MDA accounts for the passive immunity that the pups acquire from the dam and is very useful at an early age, but makes implementation of standard vaccination schedules difficult (Chappuis, 1998). Concerns regarding the influence of MDA have also prompted general opposition by practitioners to earlier vaccination. Trials have reported the possibility to overcome residual MDA and to induce immunity post-vaccination (Chappuis, 1998) and serological response to be titre-related in puppies (Burtonboy et al., 1991), indicating that concerns regarding MDA are not necessarily applicable under all circumstances.

The age of first vaccination against CPV and MDA interference to CPV vaccination in pups have been

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discussed in numerous publications (Burtonboy et al., 1991; Carmichael et al., 1983; Chappuis, 1998; Day, 2007; Hoare et al., 1997; Iida et al., 1990; Moore, 1983; Pollock and Carmichael, 1982; Pratelli et al., 2000; Willem et al., 2001). It is known that colostral transfer accounts for approximately 90% of the MDA (Burtonboy et al., 1991; Pollock and Carmichael, 1982). MDA declines with a half-life of 9.7 days but this is variable (Pollock and Carmichael, 1982). In the absence of inhibitory MDA, pups and kittens are capable of mounting a protective immune response at a very young age (Chappuis, 1998; Day, 2007). The use of a similar high titre vaccine has been studied but with few cases at four weeks of age (Willem et al., 2001).

The magnitude of the MDA in the pup is of significance. Maternal antibodies with a haemagglutination inhibition (HI) titre of $\geq 1:20$ are able to interfere with an active immune response after vaccine administration, but such titres do not prevent infection with a virulent virus. In contrast, titres of $\geq 1:64$ are considered fully protective against both infection and disease. Such MDA titres, equivalent to 2–4 maternal antibody half-lives (about 2–5 weeks), may prevent successful immunisation and leave pups susceptible to infection (Pollock and Carmichael, 1982). This interference accounts for a window of susceptibility of the pups to CPV infections and failure of the vaccine to protect. The window of interference was reported to be between the ages of 40 and 69 days in one study (Iida et al., 1990).

The decline in MDA may also be influenced by whether the experimental group studied is subjected to exposure to virulent virus or not. One study demonstrated that MDA titres in pups declined more rapidly following challenge than before (Macartney et al., 1988), with authors postulating that this rapid decline of antibody was due to its sequestration by virus after the initial phase of viral replication in the lymphoid tissues. The more rapid decline of MDA, which could occur in endemically infected premises, may complicate immunisation programmes based on the isolation and segregation of puppies in anticipation of a predicted decline in MDA before vaccination (Macartney et al., 1988). It thus follows that it may be prudent to vaccinate pups in infected breeding kennels at four weeks of age, rather than the standard six weeks in order to shorten the window of susceptibility. Factors that may affect the magnitude of the post-vaccinal immune responses to CPV vaccines are the vaccine virus titre, the degree of virus attenuation, the antigenic properties of the vaccine strain and the route of administration (Martella et al., 2005).

Canine parvovirus infection is the most common cause of enteritis in young dogs in animal rescue centres (Wells and Hepper, 1999). There is sometimes a perception among practitioners that only pups are susceptible to CPV-induced clinical disease. Outbreaks of CPV-associated enteritis and mortalities have been described in adult dogs (Decaro et al., 2008), but the incidence of true CPV-induced disease in older dogs is probably very low. Practitioners are often left confused by the multitude of published articles and comparative advertising material by the pharmaceutical industry. Many practitioners work in areas where CPV infection is rare, whereas others,

despite consistent vaccinations, attend regularly to confirmed CPV cases. The former group practices in areas where there are few breeders and where vaccination compliance is high. These areas are often more affluent and practitioners are likely to perceive all vaccination programmes to be protective for puppies under all circumstances. Current recommended vaccination programmes are followed and early vaccination protocols are frowned upon. In addition, some owners and veterinarians erroneously hold the view that infectious diseases such as parvovirus infection can be controlled by frequent vaccination alone (Carmichael, 1999). In contrast, practitioners acting as consultants to large-scale breeders are well aware of the significance of CPV infections as a constant threat to susceptible dogs resulting in high morbidity and mortality.

It has been speculated that certain breeds are at higher risk for CPV infection than others. In one study Rottweilers, American Pit Bull Terriers, Doberman Pinschers and German shepherd dogs (GSDs) were at increased risk and Toy Poodles and Cocker Spaniels were at decreased risk for developing CPV-induced enteritis, compared with that for mixed-breed dogs (Houston et al., 1996). In another study Doberman Pinschers, Rottweilers and English Springer Spaniels had a significantly increased risk of CPV enteritis (Glickman et al., 1985). In another study evaluating the factors influencing the antibody response of dogs vaccinated against rabies, it was found that animal size, age, and breed strongly influenced results (Kennedy et al., 2007). The latter study also alluded to genetic susceptibility linked to haplotypes. In studies designed to determine vaccine efficacy, the choice of breed and size may therefore be of significance.

The aim of this study was to assess the efficacy of a high titre attenuated CPV vaccine in pups from four weeks of age under kennel conditions in South Africa. The primary objective was to record seroconversion rates achieved two weeks after vaccination in the presence of high maternally derived antibody levels.

2. Materials and methods

2.1. Experimental design

The study was designed to assess the ability of a high titre vaccine to stimulate seroconversion in puppies from 3 separate breeding establishments under natural husbandry conditions and with varying levels of maternal antibody.

2.2. Experimental animals

The study was conducted in three breeds, namely GSD, Rottweiler and Boerboel. Each breed belonged to a separate breeder. The number of bitches selected from each breeder included nine GSD bitches, four Rottweiler bitches and eight Boerboel bitches. A total of 52 GSD, 25 Rottweiler and 44 Boerboel pups were included of which 11, 4 and 18 were used as controls, respectively. All the pups were pedigree stock and registered with their respective breed associations.

The Boerboel breeder ran a medium-sized kennel with approximately 11 breeding bitches on site. No specific outbreaks of disease were encountered during the preceding three years. The GSD breeder managed a large breeding colony that experienced an outbreak of CPV infection 2 years before the trial despite an intensive vaccination programme. In excess of thirty breeding bitches were kept on the same premises resulting in large numbers of susceptible pups at any given time. Many adult working dogs left and entered the premises continuously. The Rottweiler breeder had a large breeding station with numerous breeds on the same premises. This breeder had sporadic mortality in neonates due to gastro-enteritis and septicemia.

All breeding bitches were identified with either legible tattoo numbers or microchip transponders. Additional information such as treatments for illness, deworming, vaccine batch numbers, whelping dates, litter size, weight-gain and pup survival until 4 weeks of age were recorded. At 4 weeks of age, a transponder was inserted in all the pups.

2.3. Housing and nutrition

The experimental animals were housed at the respective breeders in kennels with outside runs and fed a standard commercial pelleted ration.

2.4. Blood collection

Blood was collected for serology by jugular or cephalic venipuncture using a sterile syringe and needle. Blood was centrifuged and the serum transferred to glass serum tubes. Serum samples were kept frozen until the end of the trial and tested simultaneously. Serum was collected from all puppies at 4, 6, 9 and 12 weeks, and where possible at 16 weeks.

2.5. Vaccines and vaccinations

The early vaccine administered was a non-adjuvanted, modified-live vaccine containing parvovirus strain Cornell 780916-115 strain with a viral titre of $10^{7.8}$ TCID₅₀ (PRIMODOG Merial, Lyon, France, for puppies as determined on canine cell line). Inoculations were performed at the breeding establishments or at one designated private veterinary hospital. The vaccines used and the vaccination schedules are set out in Table 1.

Control dogs included puppies from the respective litters. They did not receive the initial vaccination at 4 weeks of age but were vaccinated from 6 weeks. One pup

did not receive any vaccination and acted as a sentinel. All the dams in the study were vaccinated with EURICAN DHPPi2-LR[®], Merial, France, within the preceding 6 months before parturition but not later than 30 days before the expected date of parturition.

2.6. Serology

Serum antibodies were detected by means of the haemagglutination-inhibition (HI) technique (Carmichael et al., 1980), with minor modifications. Sera were diluted 1:2 in phosphate buffered saline (pH 7.4) and heat inactivated at 56 °C for 30 min. Duplicate serial twofold dilutions of the sera were made in a barbital-acetate buffer (pH 6.2). To the first dilution series, 8 HA units of a field strain of CPV-2 isolated in South Africa was added, while the second set of serum dilutions contained buffer and therefore served as a control for non-specific inhibition. After the addition of the antigen, the plates were held at room temperature for 90 min. This was followed by the addition of 1% porcine red blood cells (prepared in barbital-acetate buffer). Plates were placed at 4 °C overnight and the titre was read as the highest dilution at which 50% inhibition occurred. A positive control serum with a known antibody titre was included with each batch of tests. Seroconversion was confirmed when antibody titres showed a numerical increase of fourfold or more. Puppies with HI titres of 1:64–1:80 can be infected by parvovirus (Chappuis, 1998). For the purpose of this study an HI titre of ≥ 128 was regarded as protective.

[®]PRIMODOG is a registered trademark of Merial in France and elsewhere.

2.7. Analysis of the results

Statistical analyses were done using SIGMASTAT[®] and SIGMAPLOT[®] software. The level of significance was set at $P \leq 0.05$. Seroconversion rates were compared using a standard *t*-test or the Mann–Whitney Rank Sum Test.

3. Results

The seroconversion rate after the 4-week vaccination in the Rottweiler group was 80% (17/21) and was statistically significant when compared to the control group ($P = 0.013$, Mann–Whitney Rank Sum Test). Only 4 puppies were non-responders, but by the second vaccination all pups had seroconverted. None of the vaccinates ever had a titre less than 128. In the control group no pups seroconverted at six weeks (Fig. 1).

The seroconversion rate after the 4-week vaccination in the Boerboel group was 62% (16/26) and was statistically significant when compared to the control group ($P = < 0.001$). Ten puppies were non-responders, but by the second vaccination two more seroconverted. By the third vaccination only three puppies had not responded, but all pups in the group had seroconverted by the fourth vaccination. Three of the pups had a titre of less than 128 for a maximum period of 3 weeks between the ages of 9–12 weeks. In the control group, 1/18 seroconverted uneventfully (Fig. 2).

Table 1
Vaccines used and vaccination schedules.

Age at vaccination	Vaccine used
4 weeks	PRIMODOG [®] (Merial, France) monovalent canine parvovirus vaccine
6 weeks	EURICAN [®] DA2PPi2 (Merial, France)
9 weeks	EURICAN [®] DA2PPi2 (Merial, France)
12 weeks	EURICAN [®] DA2PPi2 + LR (Merial, France)

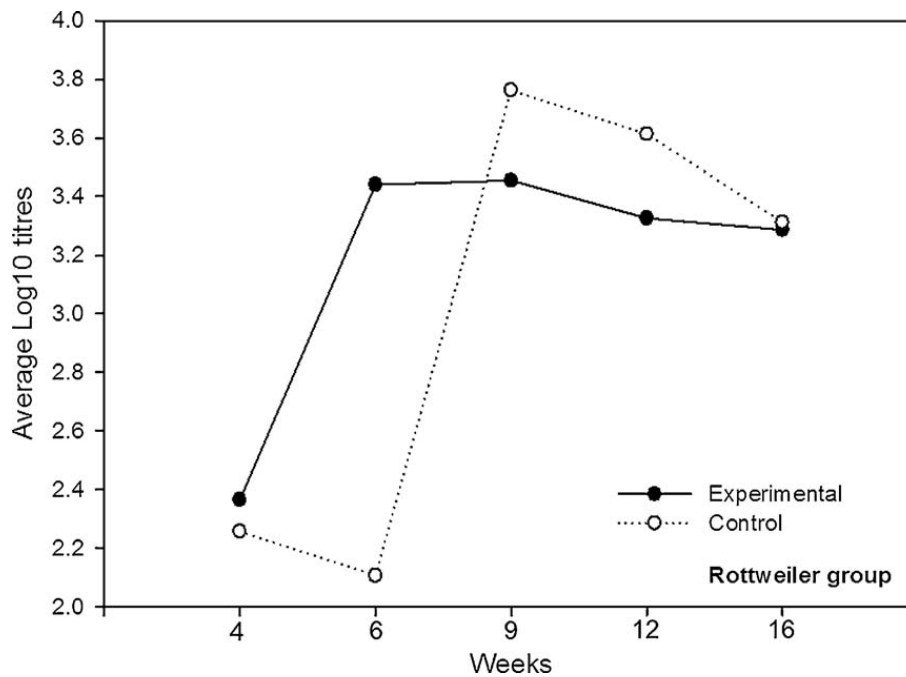


Fig. 1. Comparative log-transformed HI titres within the Rottweiler group.

The seroconversion rate after the 4-week vaccination in the GSD group was 90% (35/39) and was statistically significant when compared to the control group ($P = <0.001$). Four puppies did not seroconvert. These 4 pups remained non-responders after the second and third vaccinations and their 16 weeks titres were not determined. None of the pups ever had a titre of less than 128. In the control group, 1/11 seroconverted uneventfully (Fig. 3).

The combined results for all the puppies showed that 80% (69/86) of the pups in the experimental groups seroconverted after the first vaccination at four weeks.

In the control group 2 of 27 pups seroconverted uneventfully. Three of the pups in the experimental group had a titre less than 128 for a maximum period of 3 weeks between the ages of 9–12 weeks.

4. Discussion

The efficacy of a high titred CPV-2 vaccine was studied by monitoring antibody titres following a series of vaccinations. It is evident from the CPV titres at 4 weeks of age prior to vaccination that transfer of colostral

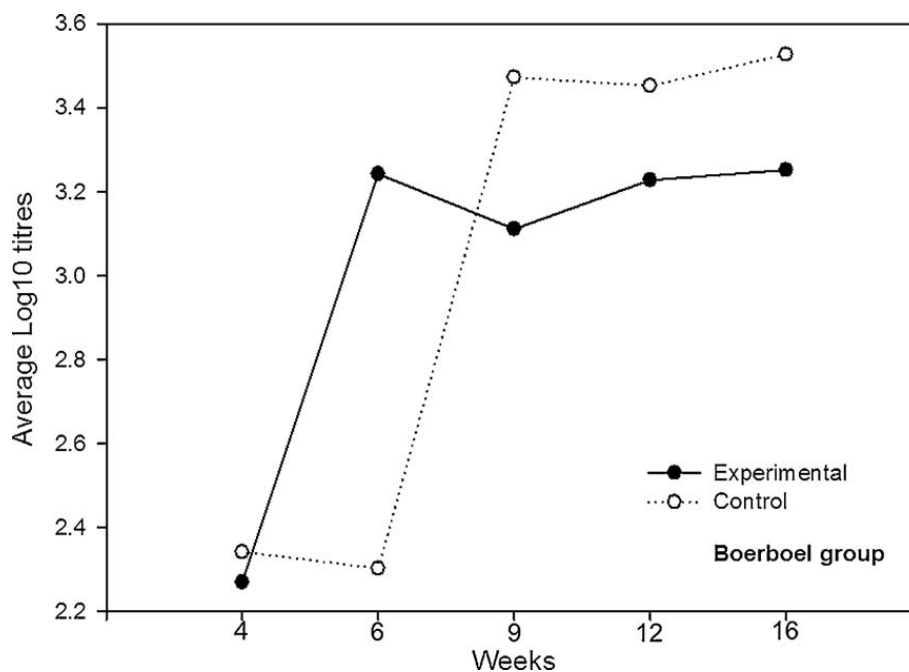


Fig. 2. Comparative log-transformed HI titres within the Boerboel group.

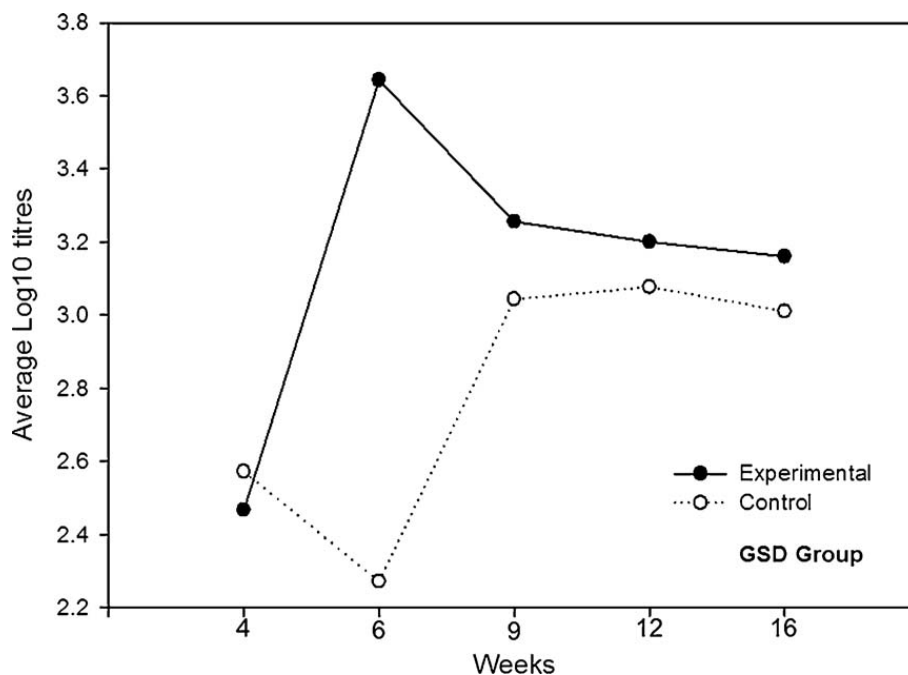


Fig. 3. Comparative log-transformed HI titres within the German Shepherd Dog group.

antibodies was satisfactory in all the pups. The MDA titres ranged from 64 to 2048 with an average of 256. Despite high levels of MDA at 4 weeks, 80% of pups that received the 4-week vaccination seroconverted. In contrast, only 2 pups that did not receive the 4-week vaccination did not seroconvert at six weeks. These control pups showed a sharper increase in titres than the 4-week vaccinates as measured at nine weeks of age. Their titres ranged from 256 to 8192 but generally averaged 2048 and above and remained protected. These results are in contrast to another study where a similar vaccine was used at 4–8 weeks of age but good seroconversion rates were only achieved in the group with MDA titres below 128 (Willem et al., 2001). All the pups in the experiment maintained high serum CPV titres until the last bleed. This suggested that under the particular circumstances specific to these dogs, vaccination beyond 12 weeks would have been of little or no value.

The Boerboel group performed markedly poorer than the Rottweiler and GSD groups. This breed is a larger breed than the Rottweiler and GSD breeds, and it remains speculative whether size influenced the seroconversion rates as reported for rabies vaccination (Kennedy et al., 2007).

During the trial two pups were hospitalised with signs of lethargy, depression, dehydration, gastroenteritis, vomiting and died despite intensive therapy. Electron microscopic examination of the faeces did not reveal the presence of enteric viruses. *Toxocara* spp. worm eggs and coccidial oocysts were found in abundance with the aid of faecal flotations in these pups and its littermates. One pup in the GSD group that did not receive the 4-week vaccination, presented with severe haemorrhagic gastroenteritis at 9 weeks of age and died after 2 days of treatment in hospital. Histological examination of sections of the small intestines was suggestive of CPV but viral isolation was not performed.

It is not clear why some pups in the control groups seroconverted between the ages of 4–6 weeks without having been vaccinated. Although histopathological examination in one animal suggested that wild type CPV virus was active during the trial, confirmation by means of electron microscopy or viral isolation was not attempted.

It must be borne in mind that some vaccine efficacy studies are performed under conditions similar to field conditions and not under gnotobiotic conditions. Given the worldwide distribution and the endemic nature of CPV infection in many areas, it may be difficult to assess whether the immunity was boosted by natural exposure during the test period in field trials, thus leading to the erroneous conclusion that the primary vaccination is the sole source of the antibody status of the experimental animal in question. This was illustrated in a study (Bohm et al., 2004) where natural CPV boosting was strongly suspected. The dogs had significantly higher titres three years after their primary vaccination than two weeks after it and three unvaccinated dogs had acquired protective antibody levels uneventfully.

This study revealed the shortcomings of a field trial. Trials conducted in commercial breeding stations and large breeding concerns have continuous movement of both animals and people on their premises. These cannot be considered closed isolated kennels. The implication is that the introduction of problems like verminosis, coccidiosis, CPV and other causes of gastro-enteritis cannot be adequately controlled. Notwithstanding these constraints, a field trial should be considered valuable as it mimics real life conditions under which the researched product will be used in practice. A field trial of this nature may be equated with post marketing surveillance with the difference that there is more reliable data collection and feedback.

Companion animals visiting private veterinary clinics or hospitals may be healthy at the time of presentation (as is the case with admissions for routine surgery), or be

compromised by injury, disease or immunosuppressive therapy. Any private clinic or hospital is considered a high-risk environment for nosocomial infections. This is due to constant movement of animals and sharing of entrances in spite of disinfection efforts and isolation of infectious patients in separate wards. The “effective quarantine” of such animals in the standard private clinics may be questioned. Many of these animals hospitalised have either an incomplete or unknown vaccination history, placing them at risk for infectious disease. Vaccination at the time of hospitalisation of dogs with incomplete or unknown vaccination histories therefore seems reasonable.

It has been documented that compromised immune function in dogs affects their response to vaccinations (Strasser et al., 2003). This does not, however, imply that the newly hospitalised patient may not be vaccinated to reduce risk. Vaccination pre- and post-surgery neither induces severe immuno-suppression, nor potentiates the severity of concurrent disease, and does not cause inapparent infections to become clinically apparent (Miyamoto et al., 1995). They also recorded comparatively good production of serum antibody titres, including antibody against CPV. Human transplant patients that are severely immune-suppressed were still able to mount protective antibody levels against influenza virus albeit lower than normal controls (Cavdar et al., 2003). Similarly corticosteroid-treated mice could mount protective immune responses following vaccination against normally fatal *Aspergillus fumigatus* infections (Ito and Lyons, 2002).

With regards to the risk period between vaccination and exposure to virulent virus, it has been shown for canine distemper that dogs given a single vaccination only hours before being placed in a contaminated environment did benefit (Larson and Schultz, 2006). The value of immediate vaccination of possibly unvaccinated dogs in a high risk environment is also supported by another study (Carmichael et al., 1983) where parenteral vaccination of sero-negative dogs resulted in HI antibody titres as early as post-vaccination day 2. Dogs mounted a significant immune response in as little as seven days post vaccination (Miyamoto et al., 1995) and maximal titres occurred within 1 week in a recent trial performed by Minke et al. (unpublished results).

The utilization of a modified-live virus (MLV) CPV vaccine with a titre of 10^7 TCID₅₀/dose was effective even when HI titres of pups were $\geq 1:80$ (Buonavoglia et al., 1992). They speculated that high titre MLV vaccines might be superior to others. In independent studies, other researchers concluded that seroconversion rates attained in their studies despite high MDA were dose related (Burtonboy et al., 1991; Hoare et al., 1997; Martella et al., 2005). Nevertheless, the titre in a vaccine may be meaningless if the minimum protective dose is not known (Appel, 1999), suggesting that over-attenuated virus may have a high titre in tissue culture but may not be adequately immunogenic. The use of an MLV CPV-2b vaccine with a relatively low virus titre administered parenterally proved to be highly effective in overcoming the obstacle of MDA (Pratelli et al., 2000).

The findings of our study showed that the use of a high titre CPV vaccine in pups at four weeks of age under field

conditions in South Africa, and in the presence of high MDA, resulted in significant seroconversion rates of 80% in puppies with quantitative values higher or equal to the known minimum protective antibody levels against CPV. Earlier vaccination than the accepted norm did indeed bridge the window of susceptibility in most vaccinates. This makes the use of such a vaccine very suitable in breeding kennels. Reduction, but not complete elimination of CPV-induced disease in large breeding kennels or in highly contaminated environments is a realistic expectation using this approach.

Conflict of interest

It is declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vetmic.2010.11.004](https://doi.org/10.1016/j.vetmic.2010.11.004).

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