

# Serological response in cattle immunized with inactivated oil and Algel adjuvant vaccines against infectious bovine rhinotracheitis

G. Kamaraj, S.K. Rana, V.A. Srinivasan

Research and Development Centre, Indian Immunologicals Ltd. Gachibowli, Hyderabad, India

## SUMMARY

Infectious bovine rhinotracheitis (IBR) virus was grown in Madin Darby bovine kidney (MDBK) cell line using a roller culture system for its large-scale production. Optimum multiplicity of infection (MOI) of 1:750 was found to give consistent virus yield. To determine the appropriate payload, three batches of antigen with virus titres ranging from  $10^{8.37}$  to  $10^{6.37}$  TCID<sub>50</sub> per ml were used to prepare experimental inactivated IBR oil adjuvant vaccine. Beta-propiolactone (BPL) was used as inactivant. The vaccine formulation using inactivated BHV-1 virus antigen with a pre-inactivation titer of  $10^{8.37}$  TCID<sub>50</sub> per dose elicited better sero-conversion in cattle calves as evidenced from the mean log SN titre of 1.02. To choose the appropriate adjuvant, two batches of vaccine each containing aluminum hydroxide gel (Algel) and Montanide oil respectively were tested in calves. Two groups of 16 calves each were inoculated with Algel and oil adjuvant vaccine respectively twice at four weeks to test the immunogenicity. Adequate titres of vaccine induced anti BHV-1 antibodies could be demonstrated both by ELISA and MNT up to 180 days post vaccination in both the groups.

**KEY WORDS:** Bovine herpesvirus-1, Multiplicity of infection, Inactivated vaccine, IBR, Neutralizing antibody

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## INTRODUCTION

Bovine herpesvirus-1 (BHV-1), also known as Infectious Bovine Rhinotracheitis (IBR) virus, is one of the emerging diseases of cattle and buffalo associated with respiratory, enteric, ocular, nervous systems and genital infections (Gibbs and Rweyemamu, 1977). In adults, BHV-1 may cause only mild clinical signs or reduced milk production or reduced fertility and abortions. In young cattle, less than one year of age, BHV-1 may be involved in bovine respiratory disease (BRD) complex or Shipping fever (Yates, 1982). Following infection, the virus establishes a latent

state in the ganglia that innervate the infected region (Wyler *et al.*, 1989). Under conditions such as stress or treatment with corticosteroid, the latent virus can be reactivated and intermittently re-excreted at the original site of infection. Once infected, animals must be regarded as lifelong carriers and potential shedders.

In India, more than 45 per cent of apparently healthy breeding bulls were reported to be seropositive for IBR and around 50 per cent of the semen produced by seropositive bulls tested positive for IBR virus (Deka *et al.*, 2005). Hence, it is needless to emphasize the need for an affordable and potent vaccine for effective control of IBR. Animals which received live, attenuated vaccine may shed the virus in nasal secretion, urine, semen and milk thereby acting as a source of infection to susceptible animals (McKercher and Crenshaw, 1971; Roberts and Carter, 1974). Additionally attenuated vaccines were reported to induce a post vaccination reaction (Roberts

Corresponding author

V.A. Srinivasan

Research Director

Indian Immunologicals Ltd.

Gachibowli, Hyderabad, India, Pin: 500 032

E-mail: srini@indimmune.com

and Carter, 1974; Robinson *et al.*, 1961) and abortion (Robinson *et al.*, 1961). Inactivated IBR vaccine therefore offers the best alternative to attenuated vaccine. However, the effectiveness of vaccines inactivated especially with formalin is debatable (Kolar *et al.*, 1973; Schipper and Kelling, 1975). Several immunogenicity trials on inactivated IBR vaccine (Liao *et al.*, 1992; Strube *et al.*, 1994; Petzhold *et al.*, 2001) indicated that the most potent formulation requires an optimum payload of inactivated viral antigen per dose of vaccine so as to induce adequate protection. There is a need to try and develop economical processes to reduce the vaccine cost. The present study describes the methods for producing BPL inactivated, oil and Algel adjuvant IBR vaccines in a roller culture system, and evaluates the immune response in animal experiment.

## MATERIALS AND METHODS

### *Cells*

Madin Darby Bovine Kidney (MDBK) cells obtained from ATCC, USA were used for the bulk propagation of BHV-1 virus.

### *Virus*

BHV-1 virus, isolated from bovine semen was obtained from the Biotechnology Laboratory, National Dairy Development Board, Anand, India. The virus isolate was adapted to the MDBK cell line and the virus-infected cell culture supernatant was used as the source of viral antigen in vaccine.

### *Medium*

Eagle's modified minimum essential medium (MEM Glasgow) supplemented with 10% and 0.5% adult bovine serum (Selbourne, Australia) was used for cell and virus growth respectively.

### *Multiplicity of infection (MOI) standardization*

BHV-1 virus was grown in MDBK cell line and MOI (virus-to-cell ratio) studies were conducted to optimize the virus yields using various virus-cell ratios *viz.* 1:10, 1:50, 1:100, 1:200, 1:500, 1:750 and 1:1000. The cell culture supernatants were sampled for 4 days post infection once in 24 hours to determine the virus yields.

### *Production of IBR antigen*

Based on the MOI study, BHV-1 viral antigen production was standardized in a roller culture system. Roller culture bottles (Corning, USA) with 850 cm<sup>2</sup> surface area were used to grow the MDBK cells. For MDBK cell growth, Eagle's MEM supplemented with 10% adult bovine serum at 0.3 ml per cm<sup>2</sup> was used. The virus inoculum with a MOI of 1:750 was used to infect the confluent MDBK monolayer. Eagle's MEM supplemented with 0.5% serum at 0.25 ml per cm<sup>2</sup> was used for virus propagation. Twenty lots were prepared with a lot size of 20 x 850 cm<sup>2</sup> roller bottles. Infected culture bottles showing more than 90% cytopathic effect (CPE) were subjected to two rounds of freeze-thaw cycles before harvesting the virus-infected fluids. Virus harvests from each lot were pooled and clarified by centrifugation at 2500 x rpm for 15 minutes. A sample from pooled virus harvest was taken to determine the infective virus titre.

### *Inactivation of the virus*

The BHV-1 virus in the clarified culture supernatant was inactivated with BPL (Ferak, Berlin GmbH: NMR 98.5%) @ 1:500 (BPL: cell culture supernatant) at 37°C for 90 minutes and the pH was adjusted to 7.2 to 7.4.

### *Viral infectivity assay*

Eight serial tenfold dilutions of the virus were prepared and the viral infectivity titre was determined in microtitre plates using the MDBK cell line. The highest dilution of the virus showing CPE was recorded and the virus infectivity titre expressed as log<sub>10</sub> TCID<sub>50</sub>/ml was calculated essentially following the method described by Reed and Munch (1938).

### *Amplification*

Virus amplification test was carried out using 2 x 150 cm<sup>2</sup> tissue culture flasks with confluent MDBK cell monolayer. After removal of spent media 10 ml of inactivated virus concentrate was inoculated into each flask. After adsorption at 37°C for 30 minutes, 30 ml of Eagle's MEM supplemented with 0.5% adult bovine serum was added to each flask and incubated at 37°C for 4 days. Then the culture supernatant was harvested and inoculated into fresh tissue culture flasks containing MDBK cell monolayer. The tissue culture

flasks were observed daily for CPE for 4 days. Lack of discernable CPE after three consequent passages was taken as an indication of complete virus inactivation.

#### *Enzyme-linked immunosorbent assay*

The Chekit-trachitest (Idexx laboratories, Switzerland) serum-screening enzyme immunoassay (EIA) kit was used to detect anti BHV-1 antibodies.

#### *Microserum neutralization test (MNT)*

The MNT was carried out essentially following the OIE protocol (OIE, 2004). The vaccine-induced BHV-1 virus neutralizing antibody titre in sera samples was estimated by the method. Twofold serial diluted sera samples were mixed with a constant dose of 100 TCID<sub>50</sub> virus to set up the neutralization reaction. MDBK cells were used as an indicator to detect unneutralized virus. The presence or absence of CPE was recorded after 72 h of incubation.

#### *Vaccine formulation and vaccination*

To determine the optimum payload of viral antigen eliciting a satisfactory immune response, 3 inactivated IBR (IBRTB-1/05 termed as Group-1; IBRTB-2/05 termed as Group-2; and IBRTB-3/05 termed as Group-3) vaccines were formulated using the antigen which had a pre-inactivation virus titre of 10<sup>8.37</sup> TCID<sub>50</sub>/ml, 10<sup>7.37</sup> TCID<sub>50</sub>/ml and 10<sup>6.37</sup> TCID<sub>50</sub>/ml respectively. Each final vaccine dose was adjusted to 2 ml volume after adjuvanting with an equal volume of Montanide oil (ISA 50V, Seppic, France).

Crossbred cattle calves (10-12 months old) were used in the vaccination experiments. Calves were found to be seronegative for BHV-1 antibodies. Each trial blend of 3 vaccine formulations (IBRTB-1/05, IBRTB-2/05 and IBRTB-3/05) was administered by deep intramuscular route to the cattle that belonged to Group 1, Group 2 and Group 3 respectively. Each group consisted of 5 cattle calves. Another group (Group 4) of 3 cattle calves were included as unvaccinated controls which were not inoculated with either adjuvant or PBS. After 21 days, serum antibody against BHV-1 was assessed by MNT and ELSA for all the groups.

Based on the results of the above experiment, another experiment was carried out to choose the right adjuvant eliciting a satisfactory immune re-

sponse. Two formulations of inactivated IBR vaccine, adjuvanted with oil (IBRTB-4/05) and aluminium hydroxide gel (CZ Veterinaria, Spain) (IBRTB-5/05) respectively were prepared using the antigen which had a pre-inactivation titer of 10<sup>8.37</sup> TCID<sub>50</sub>/ml. Montanide oil and aluminium hydroxide gel was used at a concentration of 50% and 10 mg per dose respectively. Comparison was made between Oil (IBRTB-4/05) and Algel (IBRTB-5/05) vaccines in 2 groups of 16 cattle calves each. Another group of 6 cattle calves were included as unvaccinated controls. Two ml dose of Oil and Algel adjuvant vaccines was administered by intramuscular and subcutaneous route to animals in Groups 5 and 6, respectively on '0' day with a booster dose on day 28 by the same route. Serum antibody assay to determine anti-BHV-1 antibodies was carried out both for the vaccinated and control animals on days 0 and 28 after first vaccination and on days 30, 60, 90, 120 and 150 after booster vaccination by ELISA and MNT.

#### *Safety*

Before carrying out the above two experiments, twice the dose of each of the vaccine formulations was administered to two susceptible cattle calves and observed for local as well as systemic reaction for a period of 7 days. The rectal temperature of these animals from 3 days before vaccination until 7 days post inoculation was also recorded.

## **RESULTS**

*Standardization of MOI:* Different virus-to-cell ratios were tried for infecting cell cultures to determine the optimum MOI for maximizing IBR virus yields. Maximum virus yield was obtained with a MOI of 1:500 and 1:750. However, virus yield was not directly proportional to the MOI as evident from the fact that higher MOI did not result in higher virus yield. More than 90 % CPE was achieved within 72 hours post infection with MOI of 1:500 and 1:750. The virus titres were found to be in the range of 1X10<sup>8.23</sup> to 1X10<sup>8.51</sup> TCID<sub>50</sub>/ml after triplicate testing.

#### *Production of viral antigen*

A MOI of 1:750 as determined from the above experiment was used to produce 20 lots of BHV-1

virus in MDBK cell line using a roller culture system. The pooled and clarified virus culture supernatant which had an infective titre of  $10^{8.37}$  TCID<sub>50</sub>/ml at 72 h post-infection was used for production of experimental batches of BHV-1 vaccine.

#### Formulation and testing of vaccines

To determine the optimum viral antigen payload in the vaccine required for adequate sero-conversion in cattle, 3 different vaccine formulations (IBRTB-1/05, IBRTB-2/05 and IBRTB-3/05) in oil were prepared with an antigen which had a pre-inactivation virus titre of  $10^{8.37}$  TCID<sub>50</sub>/ml,  $10^{7.37}$  TCID<sub>50</sub>/ml and  $10^{6.37}$  TCID<sub>50</sub>/ml/dose respectively. When tested for virus amplification in tissue culture the inactivated virus concentrates were not found to contain live viruses even after three consecutive passages in MDBK cell monolayer. Thereafter the vaccine antigen was blended with adjuvant.

The vaccine formulations were safety tested in calves by inoculating twice the dose of each of the vaccine formulations. No local or systemic adverse reactions were observed.

Subsequently each vaccine formulation (IBRTB-1/05, IBRTB-2/05 and IBRTB-3/05) was administered to calves that formed Group 1, Group 2 and Group 3 respectively. Vaccine-induced seroconversion in the vaccinated animals was determined on day 21 post vaccination (DPV) by MNT and ELISA (Table 1). In Group 1, seroconversion was recorded in all 5 animals both by ELISA and MNT. The mean MNT titre in the sera ranged from 0.9 to 1.2 with a mean titre of 1.02. In Group 2, all 5 animals were declared positive for IBR antibodies by ELISA and the mean MNT titre in the sera was 0.72. One of the animals in Group 2 failed to seroconvert. In Group 3, all 5 animals were positive for IBR antibodies by ELISA and the mean MNT titre was recorded as 0.24. However, one of the animals in Group 2 was declared negative for IBR antibodies by MNT. Hence, the vaccine batch, i.e. IBRTB-1/05 formulated with an antigen which had a pre-inactivation virus titre of  $10^{8.37}$  TCID<sub>50</sub>/dose, induced higher seroconversions than other vaccine batches formulated with antigen that had pre-inactivation titres of  $10^{7.37}$  TCID<sub>50</sub>/dose and  $10^{6.37}$

TABLE 1 - Immune response determined by micro-neutralization test and ELISA in cattle calves vaccinated with inactivated IBR oil vaccine with different pay loads of IBR antigen.

Trial blend number (Group of animals)	IBR antigen pay load (pre-inactivation virus titre in log <sub>10</sub> TCID <sub>50</sub> /dose)	Animal ID No.	Antibody titre on 21 DPV		
			Log antibody titre in MNT	Mean	Detection of antibody by ELISA
IBR TB-01/05 (Group-1)	8.37	226	0.9	Mean 1.02	Positive
		227	1.2		Positive
		229	0.9		Positive
		232	0.9		Positive
		233	1.2		Positive
IBR TB-02/05 (Group-2)	7.37	234	1.2	Mean 0.72	Positive
		237	0.9		Positive
		238	<0.3		Positive
		239	0.6		Positive
		263	0.9		Positive
IBR TB-03/05 (Group-3)	6.37	245	0.3	Mean 0.24	Positive
		246	0.6		Positive
		247	<0.3		Positive
		248	0.3		Positive
		262	<0.3		Negative
Un-vaccinated control (Group-4)	No viral antigen	251	<0.3	Mean 0	Negative
		254	<0.3		Negative
		256	<0.3		Negative

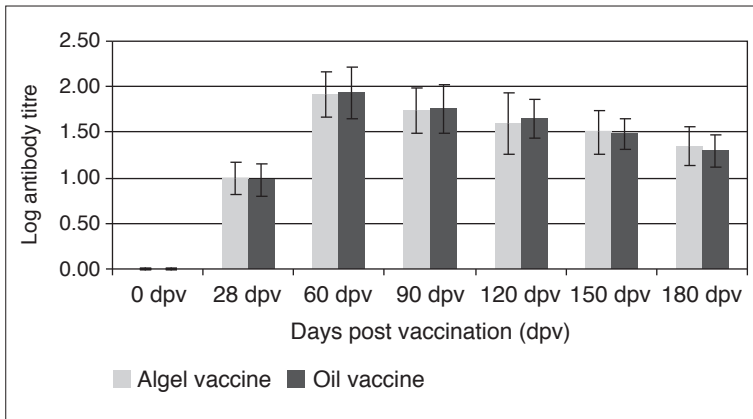


FIGURE 1 - Comparison of sero-conversion in cattle calves vaccinated with inactivated tissue culture Algel and oil adjuvant IBR vaccines.

TCID<sub>50</sub>/dose, respectively. No seroconversion could be seen in any of the animals in the unvaccinated control group by ELISA and MNT (Table 1).

On the basis of the data obtained from the above study, two more formulations of Algel and oil vaccine were prepared using antigen which had an antigenic pay load of 10<sup>8.37</sup> TCID<sub>50</sub>/dose. Both the vaccine formulations were found to be safe when tested in seronegative calves. They were tested for immunogenicity in 16 seronegative calves and the antibody response was assayed by MNT and ELISA with the sera collected on days 28, 60, 90, 120, 150 and 180 post vaccination. All the animals in the vaccinated group turned positive for BHV-1 antibodies by ELISA and remained seropositive until the end of the experiment (180 DPV). The unvaccinated control group had no antibody titres. The MNT results are shown in Figure 1.

On day 28 post primary vaccination, seroconversion was observed in all the animals in Groups 5 and 6 when tested by MNT. In Group 5, the antibody titre ranged from 0.6 to 1.2 (Mean 0.99). Whereas, in Group 6, the antibody titres were 0.6 to 1.2 (Mean 0.98). On day 30 post booster vaccination (*i.e.* 60 DPV), a sharp rise in antibody titre was recorded in all the animals of both the groups. In Group 5, the antibody titre ranged from 1.5 to 2.4 (Mean 1.91). In Group 6, the lowest and highest antibody titres were 1.5 and 2.1 respectively (Mean 1.93). Subsequently, a gradual decline in antibody titre was recorded in both groups of animals. In Group 5, 90<sup>th</sup> day antibody titre ranged from 1.2 to 2.1 (Mean 1.74). In Group 6, the lowest and height antibody titres were 1.2

and 2.1 respectively (Mean 1.76). In Group 5, the antibody titres on 120, 150 and 180 DPV were in the range of 0.9 to 2.1 (Mean 1.59), 1.2 to 2.1 (Mean 1.50) and 0.9 to 1.8 (Mean 1.35) respectively. Similarly, in Group 6, the antibody titres on 120, 150 and 180 DPV were in the range of 1.2 to 1.8 (Mean 1.65), 1.2 to 1.8 (Mean 1.48) and 0.9 to 1.5 (Mean 1.29) respectively. Seroconversion was not seen in any of the animals of the unvaccinated control group by either ELISA or MNT.

## DISCUSSION

BHV-1 incidence in cattle and buffaloes is reported to be high in India (Deka *et al.*, 2005). An appropriate disease control strategy is not available. Hence an attempt was made to develop a suitable vaccine for effectively controlling IBR. In this study, BHV-1 virus isolated from a field outbreak in India was used to prepare experimental vaccine batches. This virus isolate has never been used as a candidate strain for any commercial vaccine production. The MOI studies conducted before preparing the experimental vaccine batches using different virus-to-cell ratios clearly indicated that the maximum virus yields such as 10<sup>8.23</sup> and 10<sup>8.51</sup> TCID<sub>50</sub> per ml could be obtained with a MOI of 1:500 and 1:750 respectively. The present study showed that high MOI did not result in rapid onset of CPE which is in contrast to the findings of Nadel (1958). However, our findings are comparable with those of Lesko *et al.* (1993) where a MOI of 0.1 PFU per cell yielded 10<sup>8.33</sup>-10<sup>8.50</sup> TCID<sub>50</sub>/ml in a microcarrier culture system whereas the same MOI yielded only

$10^{7.3}$ - $10^{7.60}$  TCID<sub>50</sub>/ml in stationary culture. The observation in the present study indicated that high virus yield is of vital importance for production of economical vaccine for BHV-1. Our results indicated that MOI of 1:500 and 1:750 yielded high virus titres of  $>10^{8.00}$  TCID<sub>50</sub> per ml in a roller culture system at 72 hours post infection and this titre seems to be comparable with the findings of Lesko *et al.* (1993).

Animal studies using oil adjuvant vaccines formulated with three different antigen payloads indicated that the highest antibody titre could be achieved by a single dose of vaccine formulated with an antigen payload of  $10^{8.37}$  TCID<sub>50</sub>/dose. Other commercially available inactivated vaccines for IBR capable of eliciting adequate neutralizing antibody response contain not less than  $7.7 \log_{10}$  TCID<sub>50</sub>/dose (Patel, 2004). Based on the success with the initial experiment, we formulated adjuvant IBR vaccines with an antigen which had a pre-inactivation virus titre of  $10^{8.37}$  TCID<sub>50</sub>/dose using oil and Algel preparation for further studies.

Various factors known to affect the efficacy of inactivated BHV-1 vaccines include the type of inactivating agent and the virus antigen mass per dose of the vaccine (Kaashoek *et al.*, 1995; Strube *et al.*, 1996). Liao *et al.* (1992) reported that formalin-inactivated, DEAE-Dextran adjuvanted vaccine induced higher antibody response than formalin-inactivated, aluminium phosphate adjuvanted multivalent inactivated vaccine composed of IBR, bovine viral diarrhoea (BVD) and parainfluenza-3 viral antigens in that an IBR virus titre  $10^{7.0}$  TCID<sub>50</sub>/ml was used prior to inactivation. Petzhold *et al.* (2001) used  $10^{7.25}$  TCID<sub>50</sub>/ml virus and prepared water-in-oil type emulsion after inactivating the virus with binary ethyleneimine (BEI). However, in the present study, BPL was used as an inactivating agent. The results indicated that antigen payloads containing pre-inactivation virus titres above  $10^{8.0}$  TCID<sub>50</sub>/dose can induce a good antibody response. Inactivated BHV-1 vaccine does not cause latency and has no risk of reversion to virulence including the potential to cause abortions (Patel, 2005).

The selection of most appropriate and safe adjuvants, which can enhance immunity to different antigens, remains as an important area of research. The use of potent adjuvant is essential to confer solid immunity for a longer period of time,

which will also reduce the number of vaccinations. Our results have shown that good antibody response was induced both by inactivated Oil IBR and Algel IBR vaccine on 28 DPV and a booster vaccination on 28 DPV resulted in an anamnestic response. Antibody titres declined gradually on 180 DPV in both groups of animals and the titers elicited by either IBR Algel or IBR Oil adjuvant vaccines did not show significant variation on different post vaccination periods until 180 days (Figure 1). This finding is in agreement with an earlier report (Kilari *et al.*, 2000), where IBR oil adjuvant vaccine was described to induce good antibody response on 21 DPV.

The present study indicated that a safe and effective tissue culture inactivated Algel/oil adjuvant vaccine could be produced for IBR by incorporating a payload of antigen with a pre-inactivation virus titre of  $10^{8.37}$  TCID<sub>50</sub>/dose which confers immunity at least for 180 DPV by administering two doses of vaccine at 28 days interval. Since the titres declined on 180 DPV, six monthly vaccinations are likely to maintain good titres.

## REFERENCES

- DEKA D., RAMNEEK N.K., OBEROI M.S. (2005). Detection of bovine herpesvirus-1 infection in breeding bull semen by virus isolation and polymerase chain reaction. *Rev. Sci. Tech. Off. Int. Epiz.* **24** (31), 1085-1094.
- GIBBS E.P.J., RWEYEMAMU M.M. (1977). Bovine herpesviruses. Part I. Bovine herpes virus 1. Commonwealth Bureau of Animal Health. *Vet. Bull.* **47**, 317-343.
- KAASHOEK M.J., MOERMAN A., MADIC J.A., WEERDMEESTER K., MARIS VELDHUIS M.A., FAM RIJSEWIJK (1995). An inactivated vaccine based on a glycoprotein E-negative strain of bovine herpesvirus-1 induces protective immunity and allows serological differentiation. *Vaccine.* **13**, 342-346.
- KILARI S., SINGH S.N., BUCHE A.V., SAWARKAR S., GHOSH S. (2000). Evaluation of immunogenicity of infectious bovine rhinotracheitis vaccine in Indian tropical climate. *Indian Vet. J.* **77**, 185-188.
- KOLAR J.R. JR. SHECHEMEISTER I.L., STRACK L.E. (1973). Field experiments with formalin killed

- virus vaccine against infectious bovine rhinotracheitis, bovine viral diarrhoea and parainfluenza-3. *Am. J. Vet. Res.* **34**, 1469-1471.
- LESKO J., VEBER P., HRDA M., FEKETEOVA. (1993). Large scale production of infectious bovine rhinotracheitis virus in cell culture on micro-carriers. *Acta Virol.* **37**, 73-78.
- LIAO Y.K., LU Y.S., CHIU S.Y., LEE T.L., LIN D.T., LIN D.F., KWANG M.J., TSAI H.J. (1992). The development of multivalent inactivated vaccines composed of infectious bovine rhinotracheitis, bovine viral diarrhoea and parainfluenza-3 viral antigens. *J. Chinese Soc. Vet. Sci.* **18** (1), 47-52.
- MCKERCHER D.G., CRENSHAW (1971). Comparative efficacy of intranasally and parenterally administered infectious bovine rhinotracheitis vaccines. *J. Am. Vet. Med. Assoc.* **159**, 1362-1369.
- NADEL M.K. (1958). Growth curves of infectious bovine rhinotracheitis (IBR) as assayed in tissue culture. *Bacteriological Proceedings.* 58-60.
- OIE. (2004). Manual of diagnostic tests and vaccines for terrestrial animals, 5th edition. Part I. 2004. Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis. *Off. Int. Epiz.*, Paris.
- PATEL J.R. (2004). Evaluation of a quadrivalent inactivated vaccine for the protection of cattle against diseases due to viral infections. *J. South African Vet. Assoc.* **75** (3), 137-146.
- PATEL J.R. (2005). Relative efficacy of inactivated bovine herpesvirus-1 (BHV-1) vaccines. *Vaccine.* **23**, 4054-4061.
- PETZOLD S.A., RECKZIEGEL P.E., PRADO J.A.P., TEIXEIRA J.C., WALD V.B., ESTEVES P.A., SPILKI F.R., ROEHE P.M. (2001) Neutralizing antibodies to bovine herpesviruses types 1 (BHV-1) and 5 (BHV-5) induced by an experimental, oil-adjuvanted, BHV-1 vaccine. *Brazilian J. of Vet. Res. and Animal Sci.* **38**, 184-187.
- REED L.J., MUENCH H. (1938). A simple method of estimating 50 percent end point. *Am. J. Hyg.* **37**, 493.
- ROBERTS A.W., CARTER G.R. (1974). Infectious bovine rhinotracheitis virus recovered from the milk of a cow with mastitis. *J. Am. Vet. Med. Assoc.* **164**, 413.
- ROBINSON V.B., NEWBERNE J.W., MITCHELL F.E. (1961). Vaccination of pregnant cattle with infectious bovine rhinotracheitis vaccine. *Vet. Med.* **56**, 437-440.
- SCHIPPER I. A., KELLING C.L. (1975). Evaluation of inactivated infectious bovine rhinotracheitis vaccines. *Canadian J. Comp. Med.* **39**, 402-405.
- STRUBE W., AUER S., ABAR B., BERGLE R.D., BLOCK W., HEINEN E., KRETZDORN D., RODENBACH C., SCHMEER N., TRENTI F. (1994). Proceedings 18th world Buiatrics congress: 26<sup>th</sup> congress of the Italian Association of Buiatrics, Bologna, Italy, August 29-September 2, 1994. Volume **1**, 761-764.
- STRUBE W., AUER S., BLOCK W., HEINEN E., KRETZDORN D., RODENBACH C. (1996). A gE deleted infectious bovine rhinotracheitis marker vaccine for use in improved bovine herpesvirus-1 control programs. *Vet. Microbiol.* **53**, 181-189.
- WYLER R., ENGELS M., SCCHWYZER M. (1989). Infectious bovine rhinotracheitis/vulvovaginitis (BHV-1). In: Wittmann, G., ed. Herpesvirus diseases of cattle, horses and pigs. Kluwer Academic Publishers, Boston, p. 1.
- YATES W.D.G. (1982). A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Canadian J. Comp. Med.* **46**, 225-263.

