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Virulence, immunogenicity and reactivation of seven bovine herpesvirus 1.1 strains: clinical and virological aspects

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Specific pathogen-free calves were inoculated intranasally with one of seven strains of bovine herpesvirus 1.1 (BHV1.1) to identify a highly virulent strain for use in vaccination-challenge experiments. The calves were monitored clinically and virologically. Clear differences in virulence between the strains were observed. The Iowa strain was the most virulent; the four calves infected with the strain had the most severe clinical signs; two of them died and viraemia was detected in three of them. To evaluate the immunogenicity of the seven strains all the calves were challenged 16 weeks later with the Iowa strain. The calves of a control group showed the typical signs of a BHV1 infection, whereas all the other calves were protected against disease and shed little or no virus. Hence, the differences in virulence were not associated with differences in immunogenicity. After the calves had been treated with dexamethasone, differences were observed between the strains in the amount of virus that was excreted.

BOVINE herpesvirus 1 (BHV1) is an important pathogen of cattle. Infectious bovine rhinotracheitis (IBR) is the most common sign of a BHV1 infection (Gibbs and Rweyemamu 1977). Infectious pustular vulvovaginitis and infectious pustular balanoposthitis are also caused by BHV1, but are less severe than IBR. The outcome of a BHV1 infection can vary from subclinical (Van Oirschot and others 1993) to a systemic infection in neonatal calves, sometimes resulting in death (Higgins and Edwards 1986).

BHV1 strains are classified into several genotypes (Misra and others 1983, Metzler and others 1985). Strains of the BHV1.1 genotype are isolated predominantly from the respiratory tract and strains of the BHV1.2b genotype from the genital tract, but both genotypes can cause infections of both tracts. Differences in virulence have been observed between strains of the different genotypes (Msolla and others 1983a, Edwards and others 1991).

The virulence and immunogenicity of seven strains within the BHV1.1 genotype were compared in order to identify a highly virulent strain for use in challenge experiments and to select a highly immunogenic strain with low virulence as a parental strain for future vaccines. The establishment of latency was also studied because it may be an important characteristic for future vaccines. Latent virus can be reactivated by stress or by treatment with dexamethasone (Rock and others 1992) and as a result the virus can be excreted. Studies of the differences between the strains in the extent of excretion of virus after treatment with dexamethasone were also made.

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Materials and methods

Virus strains and cells

Embryonic bovine tracheal cells (EBTr) were used for virus isolation and titration assays and for neutralising antibody tests. The cells were cultured with Earle's minimal essential medium with 2 to 10 per cent BHV1-negative calf serum from the institute's specific pathogen-free (SPF) herd, and 0·2 per cent of an antibiotic stock solution containing 27,000 iu penicillin-G, 27 mg streptomycin, 7000 iu nystatin and 7 mg kanamycin/ml.

Seven strains of BHV1.1 were selected for the experiment: strain Lam and strain Harberink were isolated from outbreaks of IBR in the Netherlands in 1972 and 1987, respectively; strain Iowa has been described as causing lesions of the reproductive tract and early embryonic death (Miller and Van Der Maaten 1984); BHV1 strain 108 has caused fatal multisystemic infections of newborn calves (Mechor and others 1987); the ED1 strain has a genotype that is predominant in Great Britain (Edwards and others 1990); and the seventh strain of BHV1 (Espuna) was isolated in 1988 from an outbreak of IBR in central Spain. All the strains were plaque purified three times. Virus was grown on EBTr cells to obtain stocks with titres of 10^8 $TCID_{50}$/ml or more. The stocks were stored at –70°C until use. Restriction enzyme analysis was used to verify the genotypes. The viruses and cells were free from bovine viral diarrhoea virus.

Experimental design

Thirty-two calves were delivered by caesarean section, all within one week. They were deprived of colostrum and kept in identically conditioned stables with a filtered air supply. Everyone entering the stables changed clothes and put on a surgical mask, cap and gloves to reduce microbiological contamination. Because colostrum-deprived calves are very susceptible to neonatal infec-

tions, all the calves were treated with antibiotics until the start of the experiment. At the start of the experiment two control calves, one calf infected with the Lam strain and one calf infected with the Iowa strain continued to be treated, because they had omphalophlebitis and arthritis. The calves were fed milk replacer twice daily for six weeks and then gradually weaned on to a diet of calf concentrates and grass pellets.

The calves were allotted to eight groups of four calves. At approximately two weeks of age the calves of seven of the groups were infected, each with a different strain. Each calf received a total of 8×10^7 $TCID_{50}$ by intranasal spray. The eighth group served as uninfected controls. Sixteen weeks after infection, all the calves were challenged intranasally with the most virulent strain, the route of infection and the dose being the same as when they were first infected. Six weeks after the challenge all the calves were treated intramuscularly with dexamethasone, 0·1 mg/kg bodyweight, for five consecutive days to reactivate any putatively latent BHV1.

Clinical observations

The calves were examined before infection, on the day of infection, daily for 10 days, and 13, 15 and 17 days after the infection, after the challenge and after the start of the dexamethasone treatment. The calves' respiratory rate, heart rate and rectal temperature were recorded, and their behaviour, appetite, cough and stridor and the conditions of their nasal, ocular and oral mucosae were also recorded in order to evaluate objectively the differences in the severity of disease caused by the infections with the different strains. A clinical score was obtained as described by Kaashoek and others (1994).

Virological and serological examinations

Samples for virological examination were collected on the days when the calves were examined clinically. The nasal, ocular and vaginal swabs were eluted in 3 ml sample medium (Hanks' minimal essential medium containing 2 per cent of the antibiotic stock and 2 per cent SPF calf serum). The calves' prepuces were rinsed with 50 ml sample medium. After clarifying the samples, two lots of one ml of each sample were stored at –70°C. Serum samples for virus isolation and neutralising antibody tests were stored undiluted. One ml of each sample was tested on monolayers of EBTr cells for the presence of virus. The samples positive in BHV1 isolation tests were titrated in microtitre plates. The virus titres were calculated and expressed as $\log_{10}$ $TCID_{50}$/ml of nasal, ocular or genital fluid or serum.

Serum samples collected before infection and on days 10, 20 and 35 after infection, on the day of challenge and regularly thereafter were tested in serial two-fold dilutions in a 24-hour BHV1 neutralisation test (Kaashoek and others 1994).

DNA isolation and restriction enzyme analysis

DNA was isolated as described by Van Oirschot and others (1995) and used for restriction enzyme analysis. Restriction enzyme digests were made under the conditions recommended by the manufacturers (GibcoBRL) and the DNA fragments were separated on a 0·5 per cent agarose gel as described by Van Oirschot and others (1995). As a molecular weight marker the 1 kb DNA ladder of GibcoBRL was used. The gels were stained with ethidium bromide and photographed under short-wave ultraviolet light.

Post mortem examinations

A gross pathological examination was made of the calves that died during the experiment. Samples for virus isolation were collected from the nasal and pharyngeal mucosae, trachea, tonsils, major lymph nodes of the respiratory tract, spleen, liver, kidneys and adrenals. From these tissues, 10 per cent weight/volume suspensions were made in sample medium. After clarification, 1 ml of each sample was tested for virus on monolayers of EBTr cells. Standard bacteriological examinations were made of the same tissues.

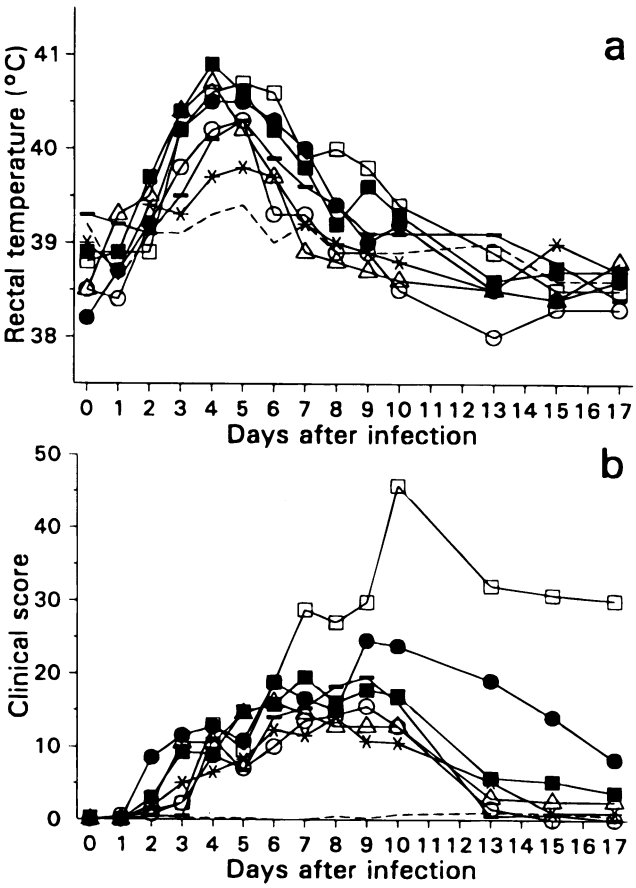

FIG 1: Rectal temperature (a) and clinical score (b) after infection. Groups of four calves were infected intranasally with one of seven BHV1.1 strains; Lam (*), Harberink (○), Iowa (□), 108 (△), Cooper-1 (–), ED1 (●) or Espuna (■). The calves of the control group (– – –) were not infected. Each line indicates the average values for a group. Two of the calves infected with Iowa died on day 10, after which the average of the two remaining calves is shown

Statistical methods

The results were evaluated statistically by analysis of variance. The least significant difference for P=0·05 was determined. Comparisons were made within the groups from day to day, and comparisons were made between the groups. The statistical evaluations were made using Statistix (Analytical Software). The term 'significant' in the text mean statistically significant (P≤0·05).

Results

Clinical observations

The respiratory rates and the heart rates after the infection varied widely from day to day, and no obvious effect of the BHV1 infection was observed. On day two the rectal temperatures of the infected calves started to increase (Fig 1a). The highest temperatures were recorded on days four and five. Fever (≥40°C) was recorded for four or more days in the calves infected with the Iowa, the ED1 and the Espuna strains and for two to three days in the other infected calves.

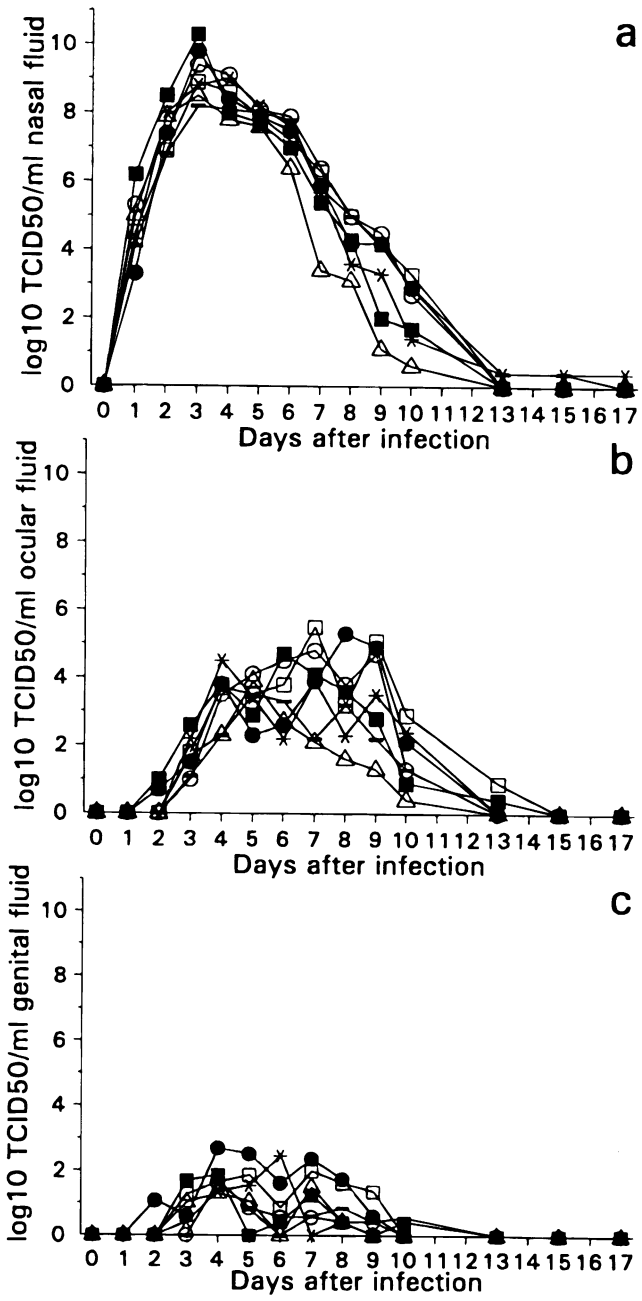

FIG 2: Nasal (a), ocular (b) and genital (c) virus shedding after infection. Key as in Fig 1

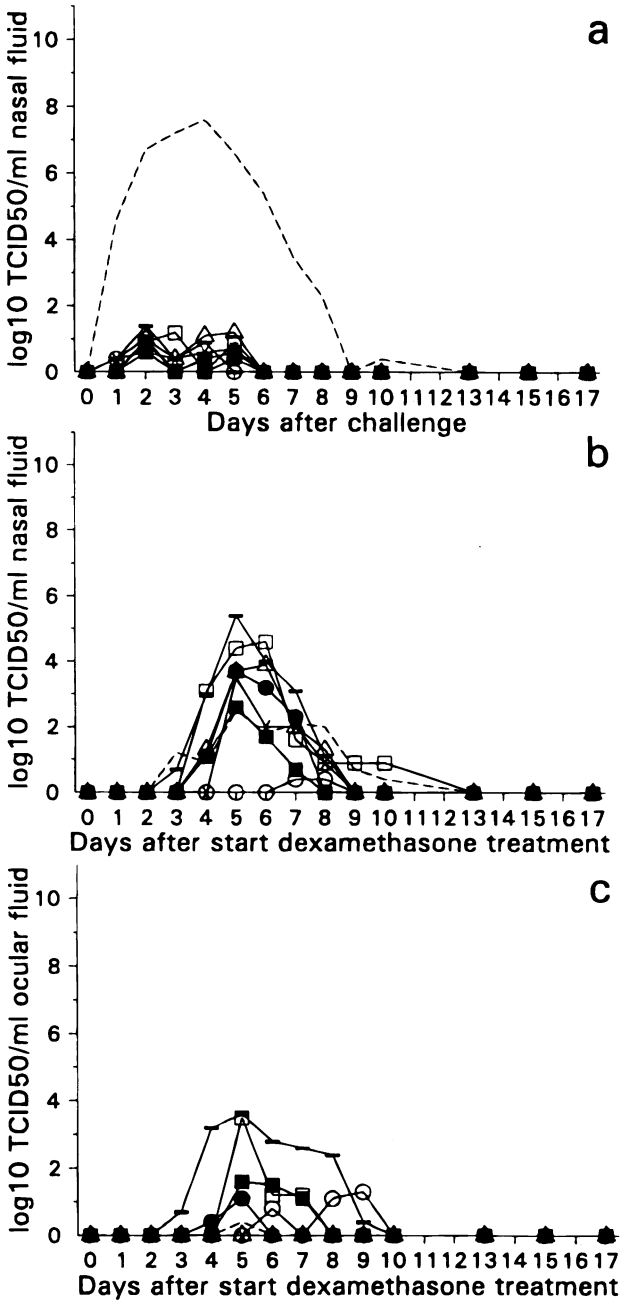

FIG 3: Nasal (a) virus shedding after challenge, and the nasal (b) and ocular (c) virus shedding after dexamethasone treatment. Key as in Fig 1

The calves infected with the Iowa, the EDI and the Espuna strains showed severe signs of illness; apathy, loss of appetite, rhinitis, nasal discharge, lesions of the nasal mucosae and nasal stridor were often observed. Milder signs of illness were observed in the other infected calves. In all the groups coughing, either spontaneous or during the handling of the calves, was rarely heard. On day 10, two of the calves infected with the Iowa strain died. The two remaining calves of this group were very sick, but recovered quickly thereafter. Two calves infected with the EDI strain were also very sick, and they recovered more slowly. As a result, the calves infected with the Iowa or the EDI strain had very high clinical scores (Fig 1b). The clinical scores for the other groups were lower. The average total clinical scores for the calves infected with the Lam or the Harberink strain were significantly lower than the scores for the calves infected with the Iowa or the EDI strain.

At challenge, all the calves were infected with the Iowa strain, because this strain was considered to be the most virulent of the seven strains. The respiratory rate and the heart rate of the 18-week-old calves were more uniform but, again, no effect of the infection could be observed. The rectal temperatures of the control calves started to increase after day two and were highest (41·1°C) on day four. The fever lasted for three days. The calves of the control group lost their appetite, were apathetic and had lesions of the nasal mucosa. The clinical signs were less severe than at the first infection; the average daily clinical score was always less than 15 and the total clinical score in the 18-week-old calves was significantly lower than in the two-week-old calves that were infected with the Iowa strain. The other calves remained healthy and fever was never recorded.

After treatment with dexamethasone, a slight fever was recorded for one or two days in one calf infected with the Cooper-1 strain and one calf infected with the EDI strain. No other clinical signs of disease were observed.

Virological findings

No virus was isolated either from the samples collected before the infection or from the control calves before the challenge. Virus was isolated from the noses of most of the infected calves for a mean (sd) period of 9·8 (0·4) days, but for 8·8 (1·0) days from the calves infected with strain 108 (Fig 2a). The total amount of virus shed by the calves infected with strain 108 was significantly less than the total amount of virus shed by the other calves. The calves infected with the Harberink strain shed virus for the longest period and shed the largest amounts. Ocular virus shedding started on day two for the calves infected with the ED1 or the Espuna strain and on day three for the calves infected with one of the other strains (Fig 2b). Again, the calves infected with strain 108 shed less virus for a shorter period than the other calves, but the difference was not significant. Virus was isolated from the genital mucosae between day two and day 10 from all the infected calves, except from one of the calves infected with strain 108. Small amounts of virus were isolated from the genital mucosae of the calves infected with the Harberink strain, strain 108 or the Cooper-1 strain. Larger amounts of virus were isolated from the genitals of the other infected calves (Fig 2c).

Virus was isolated from the serum samples of one calf infected with the Espuna strain and three calves infected with the Iowa strain. These calves had very small amounts of virus in their serum for one to three days, but virus was isolated from day four to day nine after infection, at titres up to 10^4 $TCID_{50}$/ml serum on day five from one of the calves infected with the Iowa strain. This calf died on day 10. Virus was not isolated from any of the serum samples from the other calves.

After challenge, the control calves shed virus from their noses from day one to day eight (Fig 3a). In the other groups, small amounts of virus were isolated from the noses of some of the calves in each group. Virus was isolated from only three ocular samples from the control calves. No virus was isolated from any of the other ocular, genital and serum samples collected after challenge. The shedding of the challenge virus was reduced equally in all the groups previously infected with one of the seven strains.

Treatment with dexamethasone induced the excretion of virus from the noses and the eyes of the calves by day three after the start of the dexamethasone treatment, with peaks on days five and six (Fig 3b and 3c). One control calf, one of the calves infected with the Lam strain, two of the calves infected with the Harberink strain and two of the calves infected with the Espuna strain did not shed virus from their nose or eyes after the dexamethasone treatment. Significantly more virus was isolated from the calves infected with the Cooper-1 strain than from the calves infected with the Harberink strain. No virus could be isolated from the genital and serum samples collected from any of the calves after their treatment with dexamethasone.

Virus typing

In a few cases the viruses isolated after the infection, after the challenge and after the treatment with dexamethasone were analysed by DNA restriction enzyme analysis. With the restriction enzyme used (EcoRI) the patterns of the DNA fragments of the viruses isolated after infection were indistinguishable from the patterns of the original strains (Fig 4, lanes 1 to 9). The patterns of the viruses isolated after the challenge were indistinguishable from the pattern of the challenge strain Iowa (Fig 4, Lanes 10 to 12). The patterns of the EcoRI fragments of the viruses isolated after the treatment with dexamethasone were indistinguishable from the patterns of the viruses used for the first infection (Fig 4, Lanes 13 to 18).

Antibody response

Before the start of the experiment, none of the calves had BHV1 neutralising antibodies. On day 20 after infection all the infected calves had BHV1 neutralising antibodies (Fig 5) and there were no significant differences between the average titres of the seven groups.

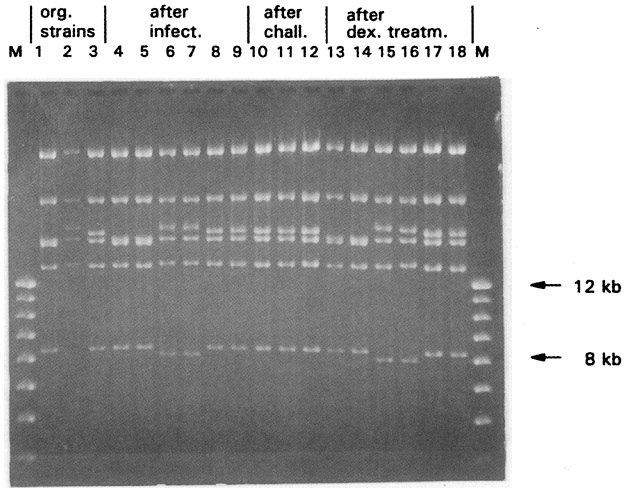

FIG 4: Restriction enzyme analysis of the DNA from a selection of BHV1.1 isolates after infection, challenge or dexamethasone treatment. The viral DNAs have been digested with the restriction enzyme EcoRI, separated on a 0·5 per cent agarose gel and stained with ethidium bromide. Lane M: Molecular weight marker. Lanes 1, 2 and 3: DNA of the original strains, Lam, Harberink and Iowa, respectively. Lanes 4 to 9: DNA from six isolates after the first infection. Lanes 4 and 5: infected with Lam; lanes 6 and 7: infected with Harberink; and lanes 8 and 9: infected with Iowa. Lanes 10 to 12: DNA from three isolates after challenge with Iowa. Lane 10: first infected with Lam; lane 11: first infected with Harberink; and lane 12: first infected with Iowa. Lanes 13 to 18: DNA from six isolates after dexamethasone treatment. Lanes 13 and 14: first infected with Lam; lanes 15 and 16: first infected with Harberink; and lanes 17 and 18: first infected with Iowa. kb = kilo base pairs

On the day of challenge, the control calves did not have BHV1 neutralising antibodies. The neutralising antibody titres of the other calves on the day of challenge were as high as the titres on day 35 after infection and there were no significant differences between the titres of the different groups. After challenge, BHV1 neutralising antibodies were induced in all the control calves. Four-fold or higher increases of the antibody titres were detected in most of the other calves. In nine of the calves the antibody titres did not increase after challenge, although the virus replicated in the noses of six of these calves. In eight of the calves there were significant increases in the antibody titres without detectable virus replication.

At the start of the treatment with dexamethasone there were no significant differences between the neutralising antibody titres of the seven groups of infected calves; the titres in the control calves were significantly lower. After the dexamethasone treatment, four-fold or higher increases in the neutralising antibody titres were detected in 17 of the 24 calves that excreted virus. Six of the calves did not excrete virus, although in two of them the antibody titres increased four-fold. At the end of the experiment, the BHV1 neutralising antibody titres of the calves infected with the Harberink strain were significantly lower than the titres of the other infected calves. The titres in the calves infected with the Cooper-1 strain were the highest.

Pathological findings

Two of the calves infected with the Iowa strain died on day 10 after infection. They had severe inflammation of the tonsils, nasal-, pharyngeal- and tracheal mucosae, and degeneration of the adrenal glands, kidneys and liver. BHV1 was isolated from these organs. The lungs were normal and pneumonia was not observed. *Staphylococcus aureus* and *Proteus vulgaris* were isolated from the nasal mucosae of these calves.

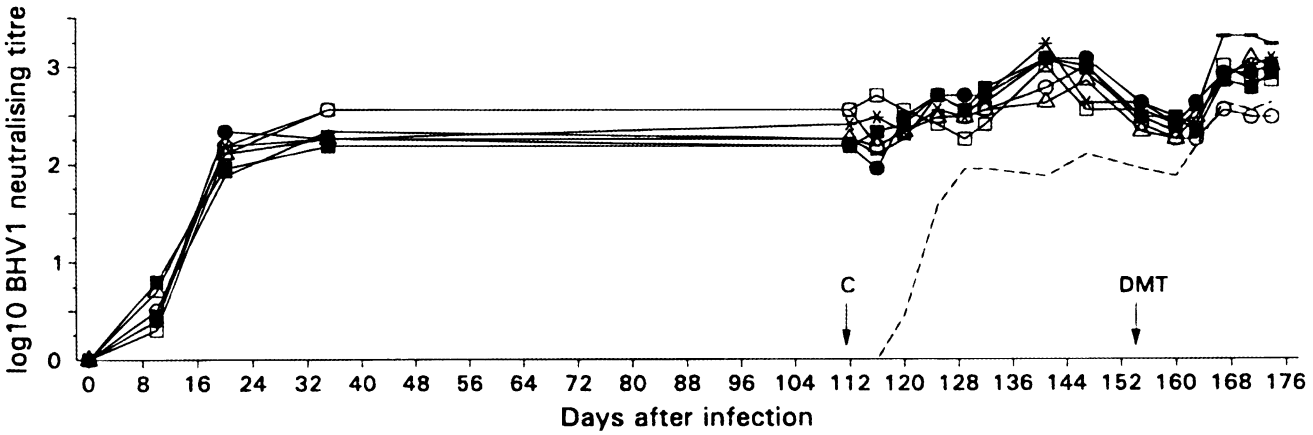

FIG 5: BHV1 neutralising antibody response of the calves. Calves were infected on day 0, the arrows indicate the day of challenge (C) and the start of the dexamethasone treatment (DMT). Key as in Fig 1

Discussion

The virulence of seven strains of BHV1.1 was assessed in order to find a highly virulent strain for BHV1 vaccination-challenge experiments. Only small differences were expected between the virulence of the seven strains, so a sensitive test system was required. Because young calves are more severely affected by BHV1 infections than older cattle (Msolla and others 1983b), the virulence of the seven strains was studied in two-week-old calves.

Secondary infections can worsen the outcome of a BHV1 infection. A strain of BHV1 given at the same dose and by the same route of infection induced more severe disease in conventionally raised calves than in specific pathogen-free calves (Kaashoek and others 1994, 1995). Specific pathogen-free calves were therefore used to exclude any possible differences in the microbiological status of the calves.

Clear differences in virulence were observed between the strains. From the clinical observations, it was concluded that the calves infected with the Iowa or the ED1 strain were most severely affected. Two of the four calves infected with the Iowa strain died and two of the four calves infected with the ED1 strain were severely ill for a long time. The total clinical scores for the calves infected with the Iowa and the ED1 strains were much higher than the total scores for the calves infected with the other strains. The rectal temperatures also indicated the differences in virulence; the more virulent strains induced fever for a longer period than the less virulent strains.

All the seven strains used in the experiment were strains of BHV1.1. Edwards and others (1991) did not find any differences in virulence between four strains of BHV1.1, possibly because the four strains all came from the United Kingdom and had been isolated within a period of two years (Edwards and others 1990). The strains studied in this experiment originated from five countries on two continents and had been isolated over a period of more than 15 years. They were therefore less likely to be related, and small genetic differences may explain the differences in virulence.

The virulence of some of the strains has been described. Mechor and others (1987) used strain 108 to infect 48-hour-old calves. In that experiment all the seronegative calves died from the infection, whereas in this experiment strain 108 caused only moderately severe disease. This difference may have been due to the differences in the age of the calves at the time of infection, and to the different routes of infection. Forman and others (1982) compared intranasal instillation and exposure to an aerosol of the Colorado-1 strain in calves. Lesions of the lower respiratory tract were more severe in the calves exposed to the aerosol and more virus was isolated from these calves than from the calves infected intranasally.

In addition to the clinical score and rectal temperature, attempts were made to find more objective measures of the severity of the disease caused by BHV1. The respiratory rate and heart rate of the calves were recorded and serum iron, zinc and haemoglobin concentrations, haematocrit and total leucocyte count were determined (data not shown). These measurements were very variable in the two-week-old calves, and the BHV1 infections had no significant effect on them. As a result, it was not possible to use them to compare the differences in virulence of the different strains.

The shedding of virus from the nose was not correlated with virulence. Virus was isolated for the same period and at the same titres from the calves infected with Iowa, a very virulent strain, as from the calves infected with Harberink, one of the least virulent strains. The calves infected with strain 108 shed the least virus, although this strain was not the least virulent. There were no significant differences between the seven groups of calves in the amounts of virus isolated from the ocular and genital mucosae. It is possible that the genitals were infected either directly or indirectly by a route outside the calf. Nevertheless, virus was isolated from the genitals of the very young calves but not from the genitals of the 18-week-old calves. This observation could support an age dependent spread of the virus through the body, most likely through the blood stream. Viraemia was also observed only in the very young calves and not in the older calves. Viraemia was observed less often than infection of the genitals, however, possibly because the viraemia is at a low level or short-lived. In the authors' experiment the calves were sampled only once a day. Virus was isolated for several days from the serum of the calves infected with the most virulent strain, Iowa. Brenner and others (1989) isolated the virus from blood samples taken four days after a BHV1 infection, although the infection induced only a mild clinical effect on the calves. Virus was isolated from many of the organs of the two calves that died after being infected with the Iowa strain, and this could also indicate viraemia. Generalised infections are more commonly reported (Higgins and Edwards 1986, Mechor and others 1987), usually in very young calves. The occurrence of viraemia probably depends on the virulence of the strain and the age of the calves.

The Iowa strain was considered to be the most virulent, and it was therefore used to challenge all the calves to evaluate the immunogenicity of the different strains. In contrast with their differences in virulence, all the seven strains were equally immunogenic. After the challenge, no clinical disease was observed and virus shedding was greatly reduced, whereas the control calves became ill and shed a lot of virus. The age-dependent susceptibility to BHV1 observed by Msolla and others (1983b) for the Strichen strain was also observed for the Iowa strain. After infection with the Iowa strain, the 18-week-old calves had fever for a shorter period and their total clinical score was significantly lower than in the two-week-old calves; furthermore, significantly less virus was isolated from the nasal and ocular mucosae of the older calves and no virus was isolated from their genitals or sera.

Restriction enzyme analysis of a few isolates after the challenge showed that the Iowa challenge virus was shed. The challenge did not reactivate the virus used for the first infection.

After the treatment with dexamethasone, significant differences

were observed between the groups in the amounts of virus excreted, although all the groups, except the control group, had been infected and challenged with the same amount of virus. These differences were not related to the differences in virulence. The titres of BHV1 neutralising antibody at the start of the dexamethasone treatment were similar for all the twice–infected groups, and this titre therefore apparently had no influence on the excretion of BHV1, as has been suggested by Pastoret and others (1984).

A few isolates obtained after the treatment with dexamethasone were examined by DNA restriction enzyme analysis and found to be the strain used for the first infection. This was not an unexpected finding, because the strain used for the first infection replicated to much higher titres in the nasal mucosae than the Iowa strain after challenge.

On the basis of the results of this experiment, the Iowa strain has been selected for future challenge experiments, and the Lam strain has been selected as the parental strain for vaccine development (Van Engelenburg and others 1994). It was not possible to identify parameters other than the rectal temperature and the clinical score to measure the severity of the disease caused by an infection with BHV1.

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Short Communications

Occurrence of resistance to anthelmintics in sheep in Paraná State, Brazil

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IN the past few years, the resistance of gastrointestinal nematodes to anthelmintics has become common and this can be a major problem in countries where *Haemonchus contortus* is endemic. In countries of the southern hemisphere, surveys have shown a high incidence of resistance to the benzimidazoles, imidazothiazoles and avermectin families in New Zealand (McKenna 1990), Australia (Eagleson and others 1992), South Africa (van Wyk and van Schalkwyk 1990) and South America (Nari 1987).

In the southern Brazilian State of Rio Grande do Sul, there have been reports of *Haemonchus, Trichostrongylus* and *Nematodirus* species resistant to benzimidazoles, rafoxanide and ivermectin (Amaral 1985, Echevarria and Trindade 1989, Echevarria 1995). In a recent survey, multiple resistance was found in Argentina, Brazil, Paraguay and Uruguay and the percentage of resistance to levamisole was: 32, 84, 68 and 70 per cent; to benzimidazole: 40, 90, 73 and 86 per cent and to ivermectin: 6, 13, 73 and 1·2 per cent, respectively (Nari 1995).

In Parana State, intensive sheep breeding has been developed in recent years through government sponsored sheep farm development. Sheep are maintained under intensive or semi-intensive systems at high density and with a suppressive anthelmintic programme. Acute clinical haemonchosis with a high percentage of mortality are not uncommon. The present study was undertaken to determine if these cases were associated with anthelmintic resistance.

Six sheep flocks with a history of apparent anthelmintic failure were selected for the trial. The farms were located in the following regions of Paraná State: eastern – Castro and Campo Largo;

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Virulence, immunogenicity and reactivation of seven bovine herpesvirus 1.1 strains: clinical and virological aspects

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