EXPERIMENTAL AND FIELD STUDIES ON INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

by

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DECLARATION

I declare that the work presented in this thesis has been carried out by me. The mycoplasmology, pathology and electron microscopy were done in conjunction with Dr. E.M. Allan, Department of Veterinary Pathology, who also prepared the vaccine described in Chapter 4 (Materials and Methods).

Some of the material in this thesis has already been published in the following paper:-

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SUMMARY

The aim of this study was to reproduce IBK in conventional calves following instillation of Moraxella bovis. This, combined with a system of scoring the severity of lesions, would allow investigations into the factors involved in the aetiology, treatment and prevention of the disease.

A review of the literature revealed marked discrepancies between descriptions of clinical signs during the early stages of the disease, which reflects the present confusion on its aetiology. Although M.bovis is implicated by the majority of authors, the ocular lesions produced by this bacterium alone have been mild and potentiating factors, such as ultraviolet irradiation, have been required to reproduce the disease consistently. In a series of experimental infections, the disease has now been reproduced in the eyes of 15, two to three month old calves infected by simple instillation into the lower conjunctival sac of haemolytic strains of M.bovis. Other recognised predisposing factors were minimised or absent. Two strains produced similar degrees of morbidity but marked differences in pathogenicity were noted and these were reflected in the clinical scoring system. The lesions were similar to those seen in field outbreaks of IBK. However, complete corneal perforation did not occur and profuse purulent ocular discharges were not a feature.

The two strains were compared in transmission experiments using groups of five calves maintained under

identical conditions in which only one eye was artificially challenged. M.bovis was successfully reisolated from 17 out of the 18 non-challenged eyes. Transmission of infection was similar with both strains although the less pathogenic strain took longer to become established. The lesions produced by each strain were similar in severity to those produced by artificial challenge. The more pathogenic strain produced lesions in six out of nine naturally infected eyes while the less pathogenic strain produced lesions in four out of nine eyes with minor signs of irritation in four other eyes.

Two routes of administration of two antibiotics were used in treatment of the disease, (i) subconjunctival injection of short acting 5% oxytetracycline hydrochloride and (ii) topical application of a benzathine cloxacillin based ointment, formulated for prolonged retention in the eye. Both treatments resulted in decreased isolation rates of M.bovis and an overall diminuation of signs of ocular irritation. Benzathine cloxacillin treatment was apparently more successful in eradicating infection than subconjunctival oxytetracycline but the results are not entirely comparable due to differences in the severities of the lesions treated.

Following a period of negative isolations, treated calves were subjected to homologous or heterologous reinfection using a pathogenic strain of M.bovis (GS). In calves subjected to heterologous reinfection, M.bovis became established and mild lesions developed in all five artificially challenged left eyes although the right eyes

were resistant to natural challenge. Homologous reinfection resulted in establishment of infection in only two artificially infected, left eyes and the development of lesions in one: the right eyes were completely resistant to natural challenge.

High doses of corticosteroids were administered to one group of calves following resolution of clinical signs which resulted in increased isolations of M.bovis over a period of five days without the development of ocular irritation or signs of IBK.

Fifteen, two to three month old calves were used to study the effectiveness of a formalin killed, whole cell vaccine against homologous challenge. Ten calves were given two doses of vaccine with a 28 day interval and all calves were challenged in the right eye fifteen days after the second vaccination. Marked variations were noted in the serological response to vaccination with poor correlation between the development of serum or lachrymal antibodies and protection against the development of lesions. The control calves were more severely affected than the vaccinated calves in both artificially and naturally challenged eyes although severe lesions developed in half of the artificially challenged right eyes of vaccinated group. Infection became established in all of the left eyes of both groups following natural transmission although the control group became infected more rapidly than the vaccinated group, persistence of infection was similar in both groups.

Fimbriated strains of M.bovis were shown to adhere diffusely to fresh bovine corneas in vitro but no adherence was found using heat killed bacteria. Prior incubation of the bacteria with rabbit anti M.bovis serum did not prevent the bacteria from adhering but produced agglutination of the bacteria into clumps. Histological examination of lesions demonstrated the presence of M.bovis within the corneal stroma with infiltration predominantly by neutrophils.

The effectiveness of fly control in the prevention of IBK in a suckler beef herd was studied using fenvalerate impregnated ear tags. There were no differences between the groups in the proportion of calves developing IBK or the severity of disease produced. Weight gains from turnout to weaning were not found to differ between the two groups or between IBK free and IBK affected calves.

GLOSSARY, ABBREVIATIONS AND NOMENCLATURE

Glossary of clinical terms

Blepharospasm	Spasm resulting in closure of the eyelids.
Conjunctivitis	Inflammation of the conjunctivae.
Descemetocoele	Erosion of corneal stroma to level of Descemet's membrane.
Epiphora	Overflow of tears
Hypopyon	Collection of pus in the anterior chamber of the globe.
Iridospasm	Spasm of the iris resulting in closure of the pupil.
Keratitis	Inflammation of the cornea.
Keratoconus	Protrusion of central area of cornea.
Panophthalmitis	Inflammation of all of the structures within the globe of the eye.
Synechia (anterior)	Adhesion of the iris to the cornea.

Microbiology

The names of the bacteria are those used in Bergey's Manual of Determinative Bacteriology (21A). In the interests of brevity, the sub genus classifications were not used hence Moraxella (Moraxella) bovis was

abbreviated to Moraxella bovis. To avoid confusion, Mycoplasma spp. was contracted to Myco.spp.

Abbreviations used in text

BAP	Blood agar plates
CFU	Colony forming units
DID	Double immunodiffusion
ELISA	Enzyme linked immunosorbent assay
FAT	Fluorescent antibody test
GDP	Gel diffusion precipitation
IHA	Indirect haemagglutination
i.m.	Intra muscular
i.v.	Intra venous
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
RID	Radial immuno diffusion
SEM	Scanning electron microscope
s.c.	Sub cutaneous
TEM	Transmission electron microscope
UV	Ultra violet

Pharmaceutical preparations

Therapeutic agents are referred to by their generic name.

References

In the reference section, the contractions for the various journals quoted are those given in Serial Sciences for the Biosis Data Base, published by the Biosciences Information Service, Philadelphia.

CHAPTER 1

GENERAL REVIEW OF LITERATURE

CHAPTER 1

GENERAL REVIEW OF LITERATURE

Historical introduction

The earliest reference to an ocular disease similar to infectious bovine keratoconjunctivitis (IBK) was by Billings (24) who described outbreaks of ocular disease in groups of feedlot cattle in Nebraska, naming the disease "keratitis contagiosa". The problem has subsequently been reported from all of the major cattle rearing areas of the world (18) although there is some evidence that in certain regions it has only been recently introduced, notably New Zealand (87) and Nigeria (82). Descriptions of the disease have been published under several synonyms; Mitter (137) named the disease "contagious ophthalmia" while Allen (4) (despite having noted the presence of a conjunctivitis in his description of the lesions) used the term "infectious keratitis". The disease has also been known by the colloquial names of "pink-eye" in the USA (46,214) and New Zealand (87), "blight" in Australia (198) and "New Forest eye" in Great Britain (62,74,75,134). These terms have been superceded by the more descriptive bovine infectious keratoconjunctivitis (BIK) (17,33,50,51) and infectious bovine keratoconjunctivitis (14,21,39,49,72,91,98,117,126, 134,142,158,180,199) the latter now being the most commonly used and widely accepted term.

Production effects, prevalence, economic impact

Although IBK is rarely fatal it can result in serious economic losses. Analysis of growth performance in an American beef herd revealed that, when weaned at 205 days, Hereford bull and heifer calves which were free from lesions of IBK during a single examination in late July had gained 17 and 18 kg more, respectively, than comparable calves with lesions (214). Moreover, in bull calves significant differences were found between animals bilaterally affected when compared to those with lesions in one eye only. The same authors (214) also calculated that, by one year of age, the IBK free bulls had gained 31 kg more than those which were affected although the difference between affected and non-affected heifers had decreased to 8 kg, which probably reflected their less intensive feeding system. In a similar study, also in America (117), it was calculated that, at weaning, there was an average suppression of 11 lbs (5 kg) per head in calves with unilateral lesions and 35 lbs (16 kg) per head in calves with bilateral lesions, representing, at that time, 1977, economic losses of $4.40 per head and $14.00 per head, respectively. The relative number of calves affected varied from year to year and within geographical areas but, over the five year period studied, 33% of the calves were affected, 10% of them bilaterally. However, in this study, calves were evaluated at weaning on the basis of corneal scarring from healed or healing lesions. Thus, calves with affected but completely healed eyes would be allocated to the non-affected group.

Results of a British study (213) revealed that at 480 days affected animals had gained 4.6 kg less than non-affected animals with a 1.6% to 2.9% increase in time taken to reach slaughter weight depending upon the breed. It was also noted that the main effect of the disease was during the mid-growth period.

Surveys have been used to calculate the prevalence and occasionally the economic losses due to IBK in Australia, USA and New Zealand. From the results of two postal surveys of New Zealand beef farms (87), it was noted that between 15.8% and 27.9% of farms had been affected by IBK over a period of five years and that the incidence was increasing (87); unfortunately, economic and production losses were not estimated. A similar postal survey in Australia revealed the national incidence of IBK in calves and adult cattle to be 10% and 4.5%, respectively (198). Furthermore, it was calculated that annual losses in production at that time, 1982, totalled A$22M with additional labour costs involved from handling and treating affected animals of just over A$1.5M (199); these figures do not include the cost of medication. The major production loss was attributed to poor weight gain or even weight loss in affected calves. Loss of milk production was reported by less than 6% of respondents which probably reflected the lower prevalence of IBK in older cattle.

In the USA the prevalence in calves and feedlot cattle were estimated to be 20% and 10%, respectively. Nationally, production losses at that time, 1976, in beef

animals alone were estimated to be $120M per annum and when treatment costs were added, the total cost to the U.S. beef industry was estimated to be greater than $150M (222).

Clinical signs

There are marked discrepancies between authors in their descriptions of the clinical signs of IBK especially in respect of the order of development of conjunctivitis and keratitis, with some authors stating that conjunctivitis precedes keratitis (17,21,57,62,82,111) others that keratitis precedes conjunctivitis (58), and some even claiming that keratitis occurs alone (19,58,112). This variation may be due to the lack of sequential examination of developing cases by some authors or inadequate description of the lesions observed. However, the tendency to group all ocular infections under the umbrella heading of IBK has been noted (18). In cases where Moraxella bovis has been isolated or suspected from ocular smears the descriptions tend to show a greater degree of consistency.

Usually, the first signs that have been noted have been those of ocular irritation with the simultaneous development of epiphora, blepharospasm, iridospasm and conjunctivitis (17,21,62). Epiphora has been noted to be initially serous in all cases although on occasions a marked purulent discharge has developed laterally (21). The conjunctivae became reddened and oedematous with injection of superficial scleral and conjunctival blood

vessels, this latter feature being particularly marked at the corneoscleral junction, forming a distinct ring (17, 21,62). Within 24 to 48 hours a keratitis develops which appears initially as a slight cloudiness, usually on the anterior pole of the cornea and which develops rapidly to form an ulcer or a vesicle, while the cloudy area expands centripetally. It has been noted that, in many cases, expansion and excavation of the ulcer occurs, leading to hypopyon, keratoconus, descemetocoele formation, while in very severe cases, corneal rupture supervenes with the development of panophthalmitis. Death has been recorded in very rare cases as a sequel to infection tracking along the optic nerve (109).

Healing of severe ulcers is accompanied by vascularisation of the cornea and ulcer with the development of projecting granulation tissue over the ulcer floor (17,62). Active lesions are usually present for a period of 2-4 weeks (17,21,67) with complete healing in some cases by 4-6 weeks (180). In severe cases, permanent corneal scarring with the development of anterior synechiae have been recorded.

Pathology

Few detailed descriptions of the pathology of IBK have been published (18,180,230).

Histological studies of bovine corneas obtained 48 hours to seven days after experimental infection and from two field cases have shown the development of shallow corneal ulcers with loss of surface epithelium and

Bowman's membrane over the floor of the ulcer and degenerative changes in epithelial cells at the ulcer edge (41). Although there may be little damage to the stroma under the ulcer floor there may be local neutrophil infiltration extending under the ulcer edge. Large numbers of bacteria, similar in morphology to M.bovis, may be found on the surface of the ulcer and under and between epithelial cells at the ulcer edge: ultrastructural studies revealed the bacteria aligned with the lamellae of collagen fibrils.

Changes associated with healing of the ulcer were noted in eyes examined between one and three weeks post infection. Granulation tissue, consisting of a matrix containing neutrophils, lymphocytes, fibroblasts and capillaries, is often present covered with stratified epithelium. Capillaries have been noted extending from the corneoscleral junction and bacteria resembling M.bovis were seen in necrotic granulation tissue at the ulcer base and subepithelially at the ulcer edge.

Similar histopathological results were obtained during examinations of mouse corneas experimentally infected with M.bovis (44). In addition, it has been noted that the bacteria were predominately arranged perpendicular to the corneal tissue surface although it could not be determined whether this was due to preferential attachment sites on the bacteria or competition for space.

Additional, *in vitro*, scanning electron microscope (SEM) studies of the bovine corneal surface by the same workers (40) have been used to elucidate the early pathogenesis of corneal lesions in IBK. Normal bovine corneas were noted to be similar to other mammalian corneas and were shown to have a modified, squamous cell epithelium consisting of flat, polygonal cells with well-defined cell junctions. These cells were either light, due to the presence of many highly-stained microplicae, or dark, with a less well-stained surface; this difference in staining being attributed to the relative ages of the cell types. The surfaces of cells in the lower layers were revealed in some locations due to the presence, in the superficial layers, of holes with thickened edges. Fifteen to 30 minutes after *in vitro* exposure of corneas to suspensions of pathogenic strains of *M.bovis*, SEM revealed the bacteria mainly associated with the surface of dark cells, some being found in pit-like depressions and at cell junctions. Transmission electron micrographs (TEM) confirmed the association of bacteria with pit-like depressions in the most superficial layer of cells and it was suggested that these possibly were caused by the actions of proteolytic enzymes produced by the bacteria (42). Non-pathogenic strains tested did not adhere to the corneal epithelium or produce pits (42).

Micro-organisms implicated in the aetiology of IBK

While the bacterium *M.bovis* is now widely accepted as being the primary aetiological agent in IBK, a number of other agents have been implicated, either separately or

in conjunction with M.bovis (18,230).

- Isolation of M.bovis and experimental transmission

A Gram-negative, rod like bacterium was seen in smears of conjunctival scrapings in 1888 (24) although no attempts were made to isolate the organism. Later, in 1915 (137) and also 1919 (4) bacteria were isolated that were morphologically and culturally similar to the "bacillus of Morax and Axenfield", a known cause of keratoconjunctivitis in humans. In 1922 (112), a characteristic Gram-negative diplobacillus was consistently isolated from acute cases of IBK which was later classified as Haemophilus bovis (88), and subsequently reclassified as M.bovis (129). Haemophilus bovis or M.bovis have since been isolated from field cases of IBK by many workers (2, 16,19,33,57,58,62,63,91,96,98,149,165,224,233) and was first isolated and identified in Britain in 1950 (224).

Although occasionally the disease has been transmitted to susceptible cattle by the direct transfer of tears (4,57,113,184), attempts to produce lesions using instillation of pure cultures of M.bovis have frequently been unsuccessful (4,57,82,86,91,224). Prolonged laboratory passage was implicated as a possible reason for strains being non-pathogenic (224) and this may account for the lack of success of some other workers (57). Lesions have been produced following intracorneal and subconjunctival injection of M.bovis (142), although the relevance of such an approach to the field situation must be in doubt. On the other hand, many workers have been

successful in producing the disease using instillation of pure cultures into the conjunctival sac in gnotobiotic (45) and conventional calves (67,109,113,158,233) although the lesions they have produced have often been less severe than those seen in natural cases of IBK.

- The role of other bacteria

Moraxella (Branhamella) ovis, originally classified as Neisseria ovis (128), has been strongly implicated in the production of keratoconjunctivitis in sheep (56,128,204,205,206) although it has also been isolated from cases of keratoconjunctivitis in cattle (13, 18,56,203), deer (122) and goats (36). However, the role of this bacterium in the production of keratoconjunctivitis in cattle is obscure. In particular, some doubts can be expressed upon the accuracy of identification as it has frequently proven difficult to differentiate between field isolates of M.(B).ovis and M.bovis (63). Attempts to reproduce ocular disease in cattle by instillation of M.(B).ovis have failed to produce colonisation or disease (56). It is relevant to note that a similar challenge in sheep produced colonisation without the development of clinical signs (56), although corneal lesions have been produced in sheep by intracorneal inoculation (205). Again the relevance of this mode of infection must be doubtful.

Cases of keratoconjunctivitis in both sheep and cattle have occasionally been attributed to infection with Listeria monocytogenes (123,139). However, the syndrome

studied was considered to differ from classical IBK on epidemiological and clinical grounds (123,139). Other authors have noted that ocular infections with L.monocytogenes may be associated with encephalitis (26).

- The role of mycoplasmas

A number of mycoplasma species have been isolated from field cases diagnosed as IBK, but usually in conjunction with M.bovis. Acholeplasma laidlawii, Mycoplasma bovirhinis and unidentified large colony and T-strain mycoplasmas have been isolated from eyes affected by keratoconjunctivitis, in 1968 (85). In addition, a new species later identified as Myco.bovoculi (126), was isolated in 1972 (125), and has since been isolated by other workers (64,115,144,186).

Mycoplasma bovis has been isolated from cases diagnosed as IBK (108) in the absence of M.bovis, although the limited description of the lesions would not appear to justify such a diagnosis. Similarly, Myco.verecundum has been isolated in the absence of M.bovis from cases presenting with conjunctivitis alone (84). Mycoplasma bovoculi and A.oculi have been isolated from cases diagnosed as IBK in Scotland, although it was noted that M.bovis was the most frequently isolated organism (115). Acholeplasma oculi has also been isolated from cases of keratoconjunctivitis in sheep and goats (9,115).

In one series of transmission experiments (142), the instillation of Myco.bovoculi into the conjunctival sac did not produce clinical changes despite concurrent

exposure to ultraviolet (UV) irradiation, although severe keratoconjunctivitis was produced by intracorneal injection. In contrast, other workers (185), have reported that, following instillation into the lower conjunctival sac, Myco.bovoculi and Ureaplasma spp. both produced conjunctivitis in the absence of gross corneal changes despite the fact that these mycoplasmas had been shown to be toxic, in vitro, to corneal cells.

Combined instillation of an unidentified mycoplasma and M.bovis originally isolated from a field outbreak, failed to produce prolonged colonisation or lesions (125), although severe lesions were produced in five cattle infected by natural transmission (125) and the authors suggested that combined infection accentuated the severity of IBK. Unfortunately, control animals were not included in this study. Other studies have shown that neither pre-infection with Myco.conjunctivae nor A.laidlawii resulted in increased severity following subsequent M.bovis challenge (170). In contrast, it has been demonstrated that pre-infection with Myco.bovoculi enhanced the pathogenicity of M.bovis (64,185).

- The role of viruses

Adenoviruses have been isolated from conjunctival swabs taken from clinically normal bovine eyes (52,53,138). These viruses have also been incriminated in outbreaks of keratoconjunctivitis in Australia (231,234) which were alleged to have been clinically indistinguishable from outbreaks attributed to M.bovis. However, in these

outbreaks there was also a low level of M.bovis isolations. No ocular transmission experiments have been documented, although adenoviruses have been isolated from ocular swabs following respiratory challenge (53).

Bovine herpes virus 1 (BHV1), the aetiologic agent of infectious bovine rhinotracheitis (IBR), can cause a combination of symptoms including ocular lesions, respiratory disease, genital lesions (infectious pustular vaginitis (IPV) and balanoposthitis), abortion, fatal systemic disease in neonates and conjunctivitis (73). Although the ocular form of IBR is occasionally diagnosed as IBK (97,138,187), on most occasions the two diseases can be differentiated clinically. The ocular form of IBR usually presents as a severe conjunctivitis, with the presence of white diphtheritic plaques on the conjunctival mucosa (114,182,215). On those occasions when corneal changes have been noted there was usually the development of a diffuse corneal opacity (54,97,182). Corneal ulceration was a rare occurrence although the M.bovis status was not recorded (97,182). Indeed, some workers are of the opinion that corneal changes do not occur in uncomplicated cases of IBR (236). The ocular form of IBR can occur without the development of other signs of IBR (210), however, most reports indicate at least some level of respiratory or urogenital involvement (54,97,114,182, 187,236). Bovine herpes virus 1 could, however, play a role in the pathogenesis of IBK since it has been demonstrated experimentally that combined BHV1 and M.bovis infection produced more severe lesions than M.bovis

infection alone (168). Again, the situation in the field remains unclear.

- The role of Thelazia spp.

"Verminous ophthalmia" is a rarely diagnosed condition which is said occasionally to present with similar clinical signs to infectious bovine keratoconjunctivitis (7,59,60,145,201). Thelazia gulosa, T.skrjabini and T.rhodesi are the only species which have been reported in Britain (7,8,59,60,220). The former two have also been isolated in Australia (201) and were the only parasites found in two post mortem surveys in North America (68,124). Attempts to produce ocular lesions using these species have failed and this was attributed to an inability of the parasite to colonise the eye (10).

The worms are difficult to detect. They have rarely been found in the live animal, mainly being present behind the third eyelid, in the lacrimal ducts and in the ducts of the nictitating glands (8). Recent post mortem surveys involving detailed dissection of the external adnexa have reported isolation rates of between 12.2% and 41.9% (8,68,124) although it is important to note that the infections in these surveys were considered to have been almost entirely subclinical. Lesions were noted in only two out of 99 (2.0%) (68) and in 14 out of 327 (4.3%) infected animals (8) although it was by no means certain that the lesions described were due to the presence of the parasite.

Microbiological characteristics of *M.bovis*

Moraxella bovis is classically described as a Gram-negative diplobacillus and, when examined by light microscopy, appears as pairs of short plump rods with rounded ends. In fresh isolates the bacterium often appears coccoid and can be confused with bacteria of the *Neisseria*/*Branhamella* group (63); in older cultures a degree of pleomorphism develops.

The majority of strains of *M.bovis* are haemolytic, producing a clear zone of β-haemolysis on sheep or horse blood agar plates (BAP). Non-haemolytic strains have been isolated, particularly associated with periods of low disease incidence (13,49,98) or have arisen during subculture of haemolytic colonies (63). For initial isolation, Mattison and Cox (135) have recommended the combined use of both BAPs and 1% Tween 80 agar plates, on which *M.bovis* produces a characteristic zone of hydrolysis, thus allowing the proportion of haemolytic and non-haemolytic colonies to be estimated.

The cultural characteristics of *M.bovis* have been described (20,27,30,63,165,235) and may be summarised as Gram-negative, non-motile, oxidase positive, catalase variable, liquifies coagulated serum, produces peptonisation in litmus milk and liquifies gelatin. It does not produce acid from sugars, reduce nitrates to nitrites, utilise citrates or grow on MacConkey agar. Although *M.bovis* is non-motile when examined by conventional means a "twitching" motility has been seen in

cultures grown on solid medium (90).

Two main colony types can be differentiated in cultures of M.bovis grown on solid medium. However, the nomenclature used to describe them is extremely confused. In primary isolation the colonies produced are flat, umbonate, firm, dry, and produce pitting of the medium when scraped off. This type of colony has been described as both rough (149,153,190) and smooth (109,156,165,224, 232,235). After frequent passage, the second colony type can arise which is smooth, convex and mucoid and which does not corrode agar, although, again, there is confusion over whether this colony should be named rough or smooth. In order to avoid such confusion a third system has been developed in which the former colony type is described as an SC (spreading, corroding) type colony (28,66,153) and the latter called the N (non-spreading, non-corroding) type colony. Bacteria from the two colony types cannot be morphologically differentiated by light microscopy or by routine biochemical tests. However, the SC type auto-agglutinates, when suspended in distilled water (64,78,190), haemagglutinates chicken erythrocytes (76,78) and produces a surface pellicle when grown in liquid media (28). The infrequent occurrence of a third colony type, intermediate between SC and N types, designated NSC (non-spreading, corroding) has also been described (28).

Transmission EM studies have demonstrated that bacteria from SC type colonies are strongly fimbriated, the fimbriae (pili) consist of long, straight, unbranched appendages, 75-80 A° in diameter, with a peritrichous

distribution (29,39,190,195). In contrast, N type colonies lack fimbriae, while preparations from NSC type colonies display an intermediate degree of fimbriation (29).

Transmission experiments have demonstrated the importance of fimbriae in the colonisation of the conjunctiva and cornea and the production of ocular lesions (39,45,76,153,156) while in vitro experiments have demonstrated their importance in corneal attachment (40,42). The presence of fimbriae has also been implicated in the transfer of drug resistance through the ability to incorporate free DNA (30). It has also been noted that the twitching motility on solid agar has been found only with SC type colonies (90).

The presence of at least two cell-associated toxins have been described (93), a stable dermonecrotic toxin, considered to be responsible local necrosis when inoculated intradermally and a thermolabile haemolysin which, when injected intraperitoneally into mice, produced haemolysis and death within six hours when given in high doses. In addition, a cell-associated exotoxin has been described (169), which, when injected intravenously (i.v.), produced pulmonary emphysema and the development of a frothy material in the trachea and bronchi with widespread petechiation. A cell wall-associated endotoxin was also described, which produced dermonecrosis at the injection site and, in some mice, a severe anaphylactoid type reaction. The presence of a third, "oculospecific", toxin was also proposed (169).

The *in vitro* cytotoxic effect of some *M.bovis* isolates and cell wall preparations on phagocytes have been described (191). Pathogenic strains were found to contain three plasmids and killed phagocytes to a greater extent than non-pathogenic strains which contained five plasmids. Neither type of strain was found, *in vitro*, to have a cytotoxic effect on corneal cells.

Non-haemolytic colonies have been obtained from passage of haemolytic colonies (155), although it is interesting to note that further passage of non-haemolytic strains resulted in both haemolytic and non-haemolytic progeny. It is not clear whether the apparent loss of haemolytic ability was due to changes within the bacterium or suppression due to laboratory conditions. When instilled into eyes, the non-haemolytic isolates of *M.bovis* did not produce IBK. However, in some of the eyes that were infected, there appeared to be spontaneous development of haemolytic strains accompanied by the subsequent development of conjunctivitis or IBK. Although the possibility of contamination by other strains of *M.bovis* was minimised by prolonged isolation, it cannot be excluded as a source of the haemolytic strains isolated. The importance of haemolysin in the production of ocular lesions is further supported by field studies in which non-haemolytic strains appear to predominate during periods of low disease incidence and haemolytic strains predominate during periods of high incidence (33,49,98). However, sub-optimal growth by some strains of *Moraxellae* during biochemical tests (27) may result in erroneous

identification of other, non-haemolytic species as M.bovis.

Moraxella bovis haemolysin has been described as a labile protein, filterable with reduced activity through 0.22 μm membrane filters but not Seitz EK pads (189). Maximum production of haemolysin was found during the log phage of growth (140,147,189). Other workers (147) concluded, from the results of centrifugation tests and PAGE analysis, that the haemolysin is probably membrane-associated.

Use of laboratory models

The high costs incurred by the requirement to use cattle in experimental infections with M.bovis has led to several laboratory animals being tested as the basis of an experimental model for the disease.

Unsuccessful attempts to produce lesions by conjunctival instillation of M.bovis have been made in rabbits (67,109,166), guinea pigs (43,67,166), rats (166), hamsters, gerbils and voles (43). In sheep, while lesions were not produced, successful reisolation of the bacterium was reported (166). Even the intracorneal injection of M.bovis has failed to result in lesions in rats (92), although intraocular injection has produced conjunctivitis and keratitis in rabbits (92). On the other hand, subcutaneous (s.c.) injection has induced local dermonecrosis in rabbits while intraperitoneal injection has resulted in the death of mice (92).

Successful laboratory models have been developed using mice. Ten out of 18 mice developed lesions similar to those of IBK when suspensions of a strain of M.bovis, previously shown to be pathogenic in cattle, were flooded onto the open eye (166). These mice were, however, also exposed to high doses of UV radiation for 30 minutes prior to challenge, and for 30 minute periods daily thereafter. Other authors (71) were also able to produce lesions, similar to those of IBK, in mice using UV irradiation as a predisposing factor and found that the administration of an allergen in the form of rag weed extract produced more severe disease and prolonged the course of infection. This was presumed to be due to the local release of inflammatory by-products damaging the integrity of the corneal epithelium.

The C57 strain of mouse has been found to be particularly susceptible (43) and, in this strain, simple instillation of M.bovis has resulted in the production of disease. However, more dramatic lesions were consistently produced in mice to which high doses of corticosteroid had been administered s.c. prior to infection (43). While this system has proved to be useful in studying ocular pathology (44), it clearly has limitations for studying immune events due to the immuno-suppressive effect of corticosteroids.

Epidemiology of IBK

In temperate climates, IBK is a mainly seasonal disease with a higher incidence during the late summer and early autumn months (13,18,33,55,87,98,198,203,228),

however occasional winter epizootics have been reported (96,110,159).

The disease may arise in all ages of cattle (55, 87,198,230) although, presumably in endemic areas, the highest incidence of disease and most severe lesions are usually to be found in cattle under two years of age (58, 98,198,228). In localised areas, a similar incidence in both cows and calves has been reported (55), possibly suggesting the local occurrence of susceptible populations of all ages or alternatively the importation of a new strain of M.bovis.

Marked differences in susceptibility to IBK have been noted. These have been particularly marked when European breeds (Bos taurus) have been compared with the more resistant tropical breeds (Bos indicus) (55,65,82, 198) with cross-bred progeny being of intermediate susceptibility. Similar differences have also been alleged between European breeds, the Hereford and the Channel Island breeds being highly susceptible, Aberdeen Angus and their crosses being the most resistant (109,198, 228). It has been postulated that the lower susceptibility of Bos indicus cattle may be due to either higher immunological resistance, the protection afforded by the relatively more hooded orbit, or else a combination of the two (55). This second factor might also account for the relative susceptibility of Channel Island cattle. Other workers have related resistance to IBK to the degree of periocular pigmentation (38), cattle with pigmented eyelids being less susceptible than those with non-

pigmented lids. This view has, however, been challenged (47). In this context it is interesting to note that a lower incidence of chlamydial keratoconjunctivitis has been reported in breeds of sheep with non-pigmented eyes (221). In other studies it has been suggested that "resistance" in horned sheep may simply be due to the horns preventing transmission of infection via direct eye-to-eye contact (223).

- The role of predisposing factors

The seasonal incidence reported by many workers for outbreaks of IBK suggests that environmental factors may be involved in the transmission of infection and the development of the disease. Surveys carried out in Australia and New Zealand, canvassing the opinions of farmers, have associated a high prevalence of IBK with increased temperature, low rainfall, dusty conditions, flies, yarding and mustering, long grass and the introduction of new cattle (87,198). It has also been noted that most of these conditions are prevalent during the months of peak solar UV radiation (198). However, only two of these factors have received significant experimental investigation.

- Ultraviolet radiation

As previously stated, the seasonal incidence of IBK indicates a possible association between increased solar radiation, increased infection and disease (18,230), although obviously it has been difficult to prove this association in the field due to the presence of other

widely accepted potential predisposing factors such as flies, dust and raised temperature. Possibly in support of this theory, some winter epizootics in cattle which have been positive for M.bovis, have been associated with high solar radiation as a result of reflection from fresh snow when other predisposing factors have been absent (96, 159). In 1968, Hughes and his colleagues (105) demonstrated the potentiating effect of UV irradiation in calves experimentally exposed to M.bovis and, since then, this system has frequently been used to produce lesions in cattle (99,100,101,103,106,107,142,153,155,156,168,161,162, 170,171,173,176) and mice (71,166).

Although originally it was stated that the optimum wavelength of radiation used was 2880 A° (288nm) (106), comparable to summer solar radiation levels in Iowa, more recent work (119) has shown that the UV light source that was used was also emitting at shorter wavelengths, down to 270 nm, whereas solar radiation below 285 nm is known to be filtered out by the atmosphere. Whether or not calves were exposed to filtered UV light (wavelengths >285 nm) prior to M.bovis challenge had no significant effect upon infection rates, or prevalence and severity of disease (119).

- Fly infestation

Correlation has been noted by several authors between fly infestation and the prevalence of IBK (46,72, 87,112,198,207,209) and, in particular, infestation with the face fly, Musca autumnalis, which commonly feeds on

lachrymal secretions. Musca autumnalis has the ability to induce ocular irritation and can produce corneal lesions during its normal feeding activity (22,32,194) and this may predispose the eye to subsequent disease when exposed to pathogenic M.bovis. The fly may also have a role in the transmission of M.bovis within and between herds. Under laboratory conditions M.bovis has been recovered from artificially contaminated M.autumnalis for periods of up to 12 hours (11) and for periods of up to three days when contaminated using infected lachrymal secretions (207). Furthermore, M.bovis was recovered from up to 83% of face flies closely associated with cattle suffering from active lesions of IBK (79) although less than 1% of flies caught in the field during outbreaks of IBK were contaminated (22). Transmission was originally thought to be purely mechanical via external contamination of the legs and body of the fly as M.bovis could not be recovered from the gastrointestinal tract (207). In contrast, a more recent study has demonstrated the ability of M.bovis to survive for up to 48 hours in the crop of face flies fed on pure cultures (81). Furthermore, it has been demonstrated that crop contents are regurgitated onto the surface of the eye during feeding (80). The ability of M.autumnalis to transmit M.bovis infection to cattle has been demonstrated (12,32,80) with subsequent development of IBK recorded where strains previously shown to be pathogenic were used (12).

Musca autumnalis was not found during a survey carried out in the south west of Scotland where the sheep

headfly, Hydrotaea irritans, and Morrelia simplex were the species most frequently present (212). Both of these flies feed mainly on lachrymal secretions on the faces of cattle and to a lesser extent on nasal secretions and open wounds (212,217). However, M.bovis was never isolated from the above species of flies caught in the proximity of cattle suffering from outbreaks of IBK (237) and therefore their role in the transmission of M.bovis has yet to be elucidated.

Immune events

- Resistance to infection

It has frequently been noted that IBK, in endemic areas, predominantly affects young animals (58,98,198,228) thereby inferring an age related immunity. In contrast, outbreaks have also been reported in older, presumably non-exposed, animals (55,87,198,230). In addition, increased, but incomplete, resistance to disease, following experimental infection, has been demonstrated in calves which had previously been infected with either homologous or heterologous strains of M.bovis (107) although it was noted that prior infection with homologous strains gave a greater degree of protection. Serological results were not reported.

By comparing the effect of a unilateral challenge to the eyes of 12 calves followed by a secondary challenge to the contralateral eye 21 days later, other workers (120) concluded that resistance to infection was mediated systemically although systemic antibodies were found in

the sera from only three calves. Clearly though, the possibility that a low grade infection had spread to the non-challenged eye, which itself would stimulate local immunity, cannot be discounted.

- Local ocular immunity

During the early post-colostral phase in neonatal calves, the dominant lachrymal antibody is IgG_1 of maternal origin. In contrast, in lachrymal secretions collected from healthy adult bovine eyes the major immunoglobulin present is secretory IgA (SIgA) (131,150), with IgG_1 and IgM being present in lower levels (150); IgG_2 is frequently undetectable. Although IgA is present in bovine serum it is found in low levels which do not correlate with the levels of SIgA in the tears, suggesting that SIgA is synthesised locally. Comparison of lachrymal and serum concentrations of IgM suggests local secretion of plasma-derived IgM (150), while IgG_1 is not synthesised locally, it is selectively transferred across the ocular mucous membranes while transfer of IgG_2 is selectively inhibited (151).

The situation in eyes affected with IBK differs from that in healthy eyes, with the ratio of immunoglobulins to albumin approaching that found in serum, probably due to leakage from damaged epithelial surfaces, while total protein concentration is lower due to increased glandular secretion of tears (151).

The role of IgA in protection against IBK is poorly understood. By comparing the results of PAGE and

RID tests, levels of SIgA have been shown to increase when convalescent tear samples are compared to pre-infection samples, although only in those cases with severe lesions were there increased concentrations in specific SIgA (143). However this study did not attempt to quantify lachrymal IgG and IgM or, for comparison, the development of serum antibodies. Other authors (118), have analysed tears from "naturally" infected calves using an indirect fluorescent antibody test (FAT) and reported that the highest specific antibody titre was in the IgG class, and that rises in titre of specific IgA only occurred much later. Furthermore, the presence of local antibody did not prevent the development of clinical IBK.

Antigenic relationships

The failure of vaccination or earlier infection to provide complete protection following heterologous challenge indicates the presence of differing serotypes (107,171). Comparison of 12 strains of M.bovis using DID indicated that they could each be placed in one of six distinct antigenic groups (77). Other workers (167), using GDPT compared the number of precipitin lines produced when antigens of 12 strains of M.bovis were tested against both homologous and heterologous antisera and found cross reactivity between all 12 tested but with variations in the number of precipitin lines formed. Results of immunodiffusion and immunoelectrophoresis tests (188) reported two antigens common to three strains tested which, it was concluded, were somatic antigens as they were present on both fimbriated and non-fimbriated colony

types. Fimbriated strains contained two additional antigens which differed between different strains. Using a plate epi-immunofluorescence test, it was found that conjugated antisera produced from three strains of M.bovis reacted with all 167 haemolytic and all five non-haemolytic strains of M.bovis that were tested (130). A weak reaction was found with one unidentified strain of Moraxella but not with M.liquefaciens and M.nonliquefaciens. Similar results have been reported from immuno-peroxidase studies (219). The latter two tests have also been used for the rapid identification of M.bovis in ocular smears (141,164,219). Agglutination tests have revealed shared antigens between M.bovis and the closely-related organisms M.nonliquefaciens and M.lacunata with weak reactions with M.phenylpyruvica (89). Apparent contradictions in some of the above findings may simply reflect the wide variety of techniques employed.

Prevention of IBK

- Vaccination

Intramuscular (i.m.) inoculation of viable M.bovis conferred incomplete protection against the development of ocular lesions when calves were subsequently challenged with the homologous strain (99). Twenty-six of 39 vaccinated calves (66.7%) developed precipitating antibodies against M.bovis after two doses. In a separate group, all ten calves developed antibodies after four doses. However, in some calves, the first dose of the vaccine produced a severe anaphalactoid reaction, which was attributed to the presence of toxins (99). In a later

paper, the same workers (100), stated that vaccines prepared from formalin killed or viable cultures conferred equal protection against experimentally-induced IBK, although the former induced a poorer serological response; heat killed vaccines gave a lower degree of protection. It has also been demonstrated that vaccines confer a higher degree of resistance to homologous rather than heterologous challenge (169), although some degree of cross protection is given. Studies using formalin killed vaccines have shown that at least 14 days were required between vaccinations for maximum efficacy (101). Moreover, vaccines prepared from formalin killed, non-haemolytic strains of M.bovis were reported to be as efficacious against heterologous challenge as those prepared from haemolytic strains (176).

Vaccines prepared from purified pili have had similar efficacy to formalin killed, whole cell vaccines. In contrast, vaccines prepared from disrupted whole cells have not been shown to confer significant protection (162). Vaccines prepared from purified ribosomes of M.bovis, while successful in stimulating the production of antibodies against whole cell and pili antigens, failed to confer protection against the development of lesions when challenged with a homologous strain (179).

Numerous attempts have been made to increase the efficacy of parenteral vaccines through the use of adjuvants. Serological responses and results following homologous challenge in calves vaccinated with either a pili preparation in oil injected s.c. or a pili

preparation in water injected i.m. for two strains of M.bovis have been compared (171). The oil adjuvanted vaccine produced measurable antibody titres in a greater number of calves than the water based vaccine. Furthermore, the former vaccine conferred higher resistance to disease following challenge, with shorter duration of lesions in those affected, when compared to the latter. In a similar experiment using formalin killed whole cells (103) the use of Freund's incomplete adjuvant did not enhance the efficacy of vaccination as compared to formalin killed whole cells suspended in distilled water. Diphtheria-tetanus toxoid and pertussis vaccine (DPT) as an adjuvant in vaccines prepared from M.bovis pili has also been shown to produce an increase in immunity (173). On the other hand, when Mycobacterium paratuberculosis bacterin was used to adjuvant a pili vaccine there was no increase in efficacy (178).

Site of vaccination may also influence the efficacy of vaccines although contradictory results have been obtained (174,227). Subcutaneous inoculation, either in the neck or periorbitally, of an oil adjuvanted, formalin killed, whole cell vaccine, conferred greater protection against homologous challenge than an identical vaccine administered sub-conjunctivally (227). In addition, a severe granulomatous reaction developed following sub-conjunctival vaccination and it is possible that this interfered with stimulation of the immune response. These results contrast with those of a later study, which used a DPT adjuvanted, purified-pili

preparation, where sub-conjunctival inoculation afforded greater protection than s.c. vaccination (174). The apparent superiority of the sub-conjunctival site, probably reflects more upon the poor, and unexplained serological response following s.c. vaccination. There appears to be no references in the literature to the use of live, attenuated vaccines.

Increased resistance to experimental infection, at two months of age, has been demonstrated in calves from vaccinated cows *via* the colostrum (177). Although, in a later field study, using a similar system, vaccination of the dams did not reduce the prevalence or severity of disease in spring born calves during their first grazing season (172). This failure may have been due to the natural decline in maternal antibodies prior to the period of peak challenge.

When autogenous vaccines have been tested under field conditions, little difference has been shown between vaccinated and non-vaccinated calves in infection rates and the development of ocular lesions (13,104). However, it was noted in one trial (104) that the prevalence of IBK in non-vaccinated cows was less than a quarter of that in either their vaccinated or non-vaccinated calves. This was presumed to be due to the development of resistance following previous exposure to *M.bovis*. Cases of IBK have allegedly been treated successfully using an autogenous vaccine (208) but control animals were not used.

Treatment of IBK

Prior to the introduction of antibiotics, treatment of IBK was limited to the topical application of astringent salt solutions, for example zinc sulphate, copper sulphate, silver nitrate, cyanide of mercury, or acids, such as hydrochloric and boric acid (180). These treatments have now been almost completely replaced by the use of antibiotics, although one Australian survey has revealed that some farmers still use topical kerosene as their treatment of choice (199).

The sensitivity of strains of M.bovis to a wide range of antibiotics has been extensively reviewed by Punch (180). All strains tested so far have been shown to be sensitive to penicillin, amoxycillin, tetracyclines and chloramphenicol with only a proportion of strains showing resistance to streptomycin (149,175,225,226,232).

Several routes of administration of antibiotics have been reported. Topical applications, either in the form of powders, aerosols, drops or ointments, are easily administered although repeated application at relatively short intervals are required to maintain adequate inhibitory concentrations at the surface of the eye. Retention of antibiotic may be prolonged by the use of oil based ointments which are immiscible with the tears and which have been shown to maintain MICs for 60 to 86 hours in healthy eyes (1,37).

Subconjunctival injection of 1-2 ml of either short-acting or depot formulations of antibiotics have been recommended (23,31,50,51,61,74,110,146). Pharmacokinetic studies on local antibiotic levels in lachrymal secretions from normal cattle following injection into the bulbar subconjunctiva suggest that therapeutic levels were attained (1,37). Unfortunately, there was a decline to non-therapeutic levels within 32 hours of administration of 100 mg oxytetracycline and 24 hours with 50 mg amoxycillin (37). Therapeutic levels of oxytetracycline could be maintained for up to 72 hours using long-acting formulations (37) but resulted in severe local reactions. In a similar study in which antibiotics were injected into the palpebral subconjunctiva, therapeutic concentrations of procaine penicillin against M.bovis were maintained in healthy eyes for 40 hours. However, when injected per cutaneously, therapeutic concentrations were maintained for 68 hours (1). Unfortunately, it would appear that studies of this nature have not been carried out in animals affected by IBK. Despite its apparent therapeutic advantages, this form of treatment demands good animal handling facilities, skill in applications and can be relatively time-consuming when large numbers of cattle have to be treated.

Systemic administration of antibiotics, either by the i.m. or i.v. routes, has been shown to produce therapeutic concentrations of some antibiotics in lacrymal secretions (69,70,152,172,175,202). Basic drugs such as trimethoprim were found in higher concentrations in tears

than in serum whereas, acidic drugs such as sulphonamides or benzyl penicillin were found in lower concentrations (152). Attempts have been made to eradicate infection in experimental animals by systemic treatment. Intra-muscular injection of oxytetracycline (7 mg/kg) reduced infection rates by two thirds (175) while an increased dose (11 mg/kg) halved the number of remaining infected animals. Two i.m. doses of a long acting formulation of oxytetracycline, 20 mg/kg, reduced isolation rates by 90% (202) and prevented the development of new lesions. Similarly, bacterial shedding has been reduced by prophylactic treatment with i.m. oxytetracycline, 20 mg/kg, prior to challenge (69) while infection was eradicated in calves treated, 20 mg/kg, 37 days following exposure. In healthy eyes this route of administration has been shown to produce lower concentrations of oxytetracycline in lachrymal secretions than in serum (70) although selective distribution to conjunctival epithelium and lachrymal gland ductiles has been noted. By comparison, i.m. injection of tylosin tartarate (175) at 5 mg/kg, followed by a dose of 7 mg/kg given to those still infected, was shown to be unable to eliminate infection in 12 experimental calves.

The use of antibiotic impregnated ocular inserts has been investigated (102,181,196,200). However, this approach has suffered from a number of problems including poor retention, local irritation, corneal anoxia and rapidly declining levels of antibiotic and therefore has not yet been developed commercially.

Contrary to the situation in similar ocular diseases, in other animals, the use of locally administered corticosteroids has been recommended in conjunction with antibiotic therapy in the treatment of IBK, including cases with ulceration. It has been alleged that this produces a more rapid improvement in the appearance of infected eyes than when antibiotics were used alone (23,192). Although results from controlled trials have not been reported it is possible that such improvements are the result of the anti-inflammatory effect of the corticosteroid acting to reduce irritation of the eye. In considering the above claims it should be noted that in experimental mice, the administration of corticosteroids has been shown to reduce the resistance of the cornea to experimental M.bovis infection (43).

Other treatments reported include the use of solcoseryl, which promotes corneal healing in conjunction with chloramphenicol (146) and the topical application of ethidium bromide (199). In cases which are severely ulcerated or perforated surgical intervention has been recommended and several techniques of suturing the third eyelid across the cornea or suturing the eyelids closed (tarsorrhaphy) have been described (5,23,31,51).

The fact that IBK is essentially a self-limiting disease, in which signs naturally abate after a period of two to three weeks, makes the interpretation of many of the above claims difficult, particularly since very few large scale clinical trials have been carried out with adequate controls. Furthermore, the lack of a

consistently successful system for reproducing the disease has hampered progress in evaluating preventative and therapeutic measures.

CHAPTER 2

GENERAL MATERIALS AND METHODS

CHAPTER 2

GENERAL MATERIALS AND METHODS

SECTION A

Experimental calves

The experimental infections described in this thesis were carried out in conventional dairy-cross calves acquired at an age of 2-3 months from commercial dairy units in the south west of Scotland which were free from IBK. The calves had a history of freedom from clinical ocular and respiratory disease from birth.

Following purchase, the calves were housed in groups of five in north-facing loose boxes using deep straw litter with natural ventilation and no direct communication of air-space with adjacent boxes. The calves were fed hay ad libitum from overhead hay racks and were given a compound feed (Calf rearing pencils, BOCM, Renfrew, Scotland) ration of 2 kg per calf per day; water was available ad libitum at all times from floor level troughs.

SECTION B

Virology

- Media

Standard virological media were employed for the growth and maintenance of cells. All samples were tested for virus using either bovine embryonic kidney cells or embryonic calf lung cells (Flow Laboratories, Richmansworth, Herts, UK).

- Culture methods

Nasal, nasopharyngeal and ocular swabs were suspended in conventional virus transport medium and stored at -70°C prior to use. The methods used for the isolation of viruses have been previously described (34).

- Identification methods

The methods used in the identification of viruses isolated have been previously described (34).

SECTION C

Mycoplasmology

- Media

Liquid and solid media were prepared for the isolation and identification of mycoplasmas as previously described (3).

- Culture methods

Nasal, nasopharyngeal and ocular swabs were inoculated into 1.8 ml bottles of glucose serum (GS) and U3 broth samples. Tenfold serial dilutions to 10^{-3} of the original were made in the appropriate media. Incubation of broth solutions and subculture onto solid media were as described by Allan (3).

- Identification methods

The methods used for identifying mycoplasmas were as described by Allan (3).

SECTION D

Bacteriology

Bacteriological tests were employed for the isolation and identification of Gram-negative aerobic bacteria.

- Media

Standard bacteriological liquid and solid media were utilised (50). Tween 80 agar was prepared using the methods that have been described previously (135). Forty grams of blood agar base (Blood Agar Base No.2, Oxoid Ltd., Basingstoke, Hampshire, UK), 10 ml of Tween 80 and 1 g of calcium chloride were mixed and dissolved in 1 litre of distilled water. This was sterilised by autoclaving at 121°C for 15 minutes and allowed to cool to 50°C prior to being dispensed into sterile petri dishes. Horse blood agar was prepared by the addition of 75 ml sterile horse blood (Gibco BRL Ltd., Paisley, Scotland) to a litre of sterile agar base (Blood Agar Base No.2, Oxoid Ltd) prepared using the method recommended by the manufacturer. Horse BAP were prepared by dispensing 15 ml aliquots into sterile petri dishes.

- Culture methods

Nasal, nasopharyngeal and ocular swabs (Exogen Ltd., Clydebank, Scotland) were streaked onto sterile Tween 80 agar plates, and/or sterile BAP within 30 minutes of collection of the samples. All plates were examined for growth, colony type and Gram staining of colonies following aerobic incubation at 35°C for 24 hours.

- Identification methods

Gram-negative bacteria were identified from their reactions to standard biochemical tests using the methods of Cowan and Steele (48).

Moraxella bovis was identified by its biochemical reactions using the criteria described by Fraser and Gilmour (63) and by its reaction on Tween 80 agar (135).

SECTION E

Serology

- Production of antiserum

Antiserum was raised against whole cell M.bovis (GS) in rabbits. Six to eight week old, Dutch, cross rabbits were obtained from a commercial source and maintained in individual cages in a single room under controlled temperature and humidity conditions.

An antigen suspension containing whole cell M.bovis was prepared: the bacteria were grown overnight on BAP, scraped off using an inoculating loop and suspended in sterile normal saline at an approximate concentration of 10^8 colony forming units (CFU)/ml. This suspension was administered to the rabbits intravenously using the following regime: 0.5ml (day 0), 1ml (day 7), 1.5ml (day 14) and 2ml (day 21).

The rabbits were sacrificed and bled out on day 42. The serum was separated and then stored in bijoux bottles at -20°C prior to use.

- Indirect haemagglutination test

The indirect haemagglutination (IHA) test used to detect antibody response to M.bovis is a modification of that described (193).

Moraxella bovis strain GS was grown overnight on one BAP and the growth suspended in 10ml of sterile phosphate buffered saline (sPBS). The bacteria were

killed by heating at 56°C for 30 minutes, shaken vigorously to break up the colonies and centrifuged at low speed to remove larger aggregates of bacteria. Glutaraldehyde-fixed ox red blood cells are added at a concentration of 0.5%, incubated at 37°C for 30 minutes, washed three times in sPBS to remove excess antigen and made up to the original concentration.

Dilutions of test serum or lachrymal secretions were made in 0.025 ml volumes of microtitre plates with normal calf serum (reciprocal titre 0) and anti-M.bovis (GS) rabbit serum (reciprocal titre 128) as negative and positive controls. Equal volumes of the sensitised cells were added, incubated for two hours at room temperature and the test was then read.

SECTION F

Clinical examination and clinical scoring

Clinical examinations were carried out using a set procedure with the aid of a hand-held torch. Calves were initially examined from a short distance for general demeanour, the presence of blepharospasm, increased blinking and gross epiphora resulting in tear staining. Following this, a closer examination was made of the eye, firstly under natural light to check for the presence of gross conjunctivitis, corneal lesions and the presence of iridospasm. Finally, a thorough examination of the eye was carried out with the aid of the torch with particular attention to minor corneal changes.

Clinical scores were allocated independently to each eye according to the criteria set out in table 1. Other relevant information and clinical data not included in the scoring system were recorded separately.

CLINICAL SIGN	SEVERITY OF LESION	SCORE
Increased lachrymation		1-2
Conjunctivitis		1-2
Increased blinking		1
Blepharospasm		1-3
Iridospasm		1
Corneal opacity	Haze with oblique light only	1-2
	Marked opacity	3-4
	Iris not visible	5-6
Corneal vesicle/ ulcer	Up to 2mm	1-2
	2 to 4mm	2-3
	More than 4mm	3-4
	Ulcer perforated	5
Purulent discharge		1

TABLE 1. Clinical signs used in scoring system and allocation of scores

SECTION G

Electron microscopy

Overnight cultures of M.bovis were examined for the presence of pili by negative staining. The culture was suspended in PBS (pH 7.4) and agitated to break up the clumps. One drop was added to palladium-coated grids, immediately blotted and a drop of 1% phosphotungstic acid added; this was also blotted immediately. The samples were examined with an AEl 6B electron microscope.

CHAPTER 3

EXPERIMENTAL STUDIES OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

CHAPTER 3

EXPERIMENTAL STUDIES OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

INTRODUCTION

Although the bacterium M.bovis is now widely considered to be the primary aetiological agent in IBK, attempts to reproduce the disease with this organism alone have not been consistently successful and in several instances have failed completely (4,59,83,86,91,218). Consequently, it has been suggested that M.bovis can only produce a clinical reaction in the presence of predisposing factors such as UV light, intercurrent infection with either viruses, mycoplasmas, particularly Myco.bovoculi, or trauma from foreign bodies (18). It has been demonstrated by several groups of workers that variations in the relative pathogenicity of M.bovis occurs both between different strains and between different subcultures of a single strain (39,105,142,155,156). However, much of the above work has not taken account of possible variations in host resistance.

The poor environmental survival of M.bovis together with seasonal variations in incidence of both infection and prevalence of disease indicate that a carrier state must exist in which clinically normal animals harbour the bacterium in the nasopharynx and conjunctiva (160). Such a hypothesis is supported by the isolation, in low numbers, of M.bovis from these sites. Furthermore, staining of ocular smears by

immunofluorescence have demonstrated that up to 40% of samples negative for M.bovis by cultural examination contained the bacterium in low numbers (130). Thus, the above predisposing factors, in addition to their role in pathogenesis in primary infections, could also instigate recurrence of the disease. In addition, little is known about the rates of spread of infection and disease among susceptible calves, irrespective of whether the source of infection is from clinically normal carrier animals or cattle with active lesions.

While many regimes have been described for the treatment of field cases of IBK (1,23,31,37,50,51,69,110, 183,199,208), there has been little consideration of the effects of strain differences or the severity of the lesions treated and therefore, the efficacy of these regimes must remain in doubt. It is certain that the lack of an experimental model has hampered progress in this respect.

The following sections will describe the effects of pathogenic strains of M.bovis in carefully-selected conventional calves leading to the development of a consistently-reproducible experimental model. The studies then proceed to investigate the spread of infection in susceptible calves, the effectiveness of two commonly adopted therapeutic procedures, the effect of re-exposure to M.bovis and the effect of corticosteroid on recovered cases.

SECTION A

STUDIES OF THE EXPERIMENTAL PRODUCTION OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

INTRODUCTION

In this section will be described the reproduction of ocular lesions in conventional calves using M.bovis alone and without recourse to corneal damage from UV light or scarification. Differences in pathogenicity will be investigated, using three groups of five calves, between a known pathogenic strain, a reisolate of that strain and a low passage, strongly fimbriated field strain.

MATERIALS AND METHODS

Experiment 1

Experimental animals

Two groups of five animals each were bought from separate commercial dairy sources and maintained in isolation as separate groups. Group 1 consisted of five Friesian bull calves numbered 1 to 5 and group 2 consisted of five Ayrshire bull calves numbered 6 to 10. Group 2 acted as controls and were removed from this experiment on day 14. Housing and feeding were as described in Chapter 2, Section A.

Sampling of animals

- Virology

Ocular (left and right, L,R) and nasal swabs were collected from all calves on days -28, -7 and 7 and from the calves in group 1 on days 21 and 35. Samples were processed for the isolation and identification of viruses as described in Chapter 2, Section B.

- Mycoplasmology

Left and right ocular, nasal and nasopharyngeal swabs were collected at weekly intervals from all calves from admission until day 70. Samples were processed for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Prior to challenge on day 0, ocular (L,R) nasal and nasopharyngeal swabs were collected twice weekly for a period of eight weeks. Following challenge, ocular (L,R) nasal and nasopharyngeal swabs were collected three times weekly until day 45 and weekly thereafter with additional samples collected during the first four days. Weekly samples were processed for the isolation and identification of Gram-negative bacteria and all samples were processed for the isolation of M.bovis as described in Chapter 2, Section D.

- Sera and lachrymal secretions

Samples of lachrymal secretions were collected on day 0 from both left and right eyes of all animals using

soft tipped plastic pipettes and stored at -20°C in sterile plastic bijoux bottles. Serum samples were also collected on day 0. All samples were examined for the presence of anti-M.bovis antibodies using an IHA test as described in Chapter 2, Section E.

Clinical examination and scoring

Both eyes of each animal were examined daily for eight weeks prior to inoculation and found to be free from disease at all times. Following inoculation, both eyes were examined daily for a period of 28 days and three times a week thereafter. Examination procedure and clinical scoring system are as described in Chapter 2, Section F.

Inoculation procedures

The microorganism used for inoculation was a strain of M.bovis (GS) previously shown to be pathogenic in cattle (39). This strain was stored at -70°C on sealed BAP before being thawed and passaged twice on BAPs at 35°C for 24 hours. The growth of M.bovis was scraped off three BAPs and suspended in 10 ml of sPBS, pH 7.2, and the colonies were broken up by gentle agitation to give the inoculum suspension.

The calves in group 1 were challenged on day 0 by instillation of 0.5 ml of this suspension into the lower conjunctival sac of the left eye within 30 minutes of preparation. The eyelids were then held closed by gentle digital pressure for a period of 30 seconds. The left eyes of the calves in group 2 were inoculated with 0.5 ml of sPBS using an identical procedure.

The inoculum was serially diluted in sPBS and 0.5 ml of each tenfold dilution was inoculated onto BAP and incubated overnight at 35°C and the challenge dose was calculated from this as 10^{12} CFU/ml.

Experiment 2

Experimental animals

The calves used in this experiment were the control calves from experiment 1, designated group 2 and numbered 6 to 10. Housing and feeding were as described in Chapter 2, Section A.

Sampling of animals

- Virology

Ocular (L,R) and nasal swabs were collected on days -43, -22, -8, 6, 20 and 34. Samples were processed for the isolation and identification of viruses as described in Chapter 2, Section B.

- Mycoplasmology

Left and right ocular, nasal and nasopharyngeal swabs were collected at weekly intervals from all calves from admission until day 63. Samples were processed for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Prior to challenge on day 0 ocular (L,R) nasal and nasopharyngeal swabs were collected twice weekly for

a period of ten weeks. Following challenge, ocular, nasal and nasopharyngeal swabs two times per week until day 45 and weekly thereafter. Weekly samples were processed for the isolation and identification of Gram-negative bacteria and all samples were processed for the isolation of M.bovis as described in Chapter 2, Section D.

- Sera and lachrymal secretions

Samples of lachrymal secretions were collected on days -15 and 0 from both left and right eyes of all animals using soft-tipped plastic pipettes and stored at -20°C in sterile plastic bijoux bottles. Serum samples were also collected on days -15 and 0. All samples were examined for the presence of M.bovis antibodies using an IHA test as described in Chapter 2, Section E.

Clinical examination and scoring

Both eyes of each animal were examined daily for a period of ten weeks prior to inoculation and found to be free from clinical disease at all times. Following inoculation, both eyes were examined daily for a period of 28 days and three times a week thereafter. Examination procedure and the clinical scoring system are as described in Chapter 2, Section F.

Inoculation procedures

The microorganism used was a strain of M.bovis GS (RI) isolated from the left eye of calf 5 of experiment 1 on day 10 and passaged three times on BAPs prior to inoculum preparation. Inoculum preparation and exposure

procedures were described in experiment 1. The inoculum dose was found to be 10^{12} CFU/ml.

Experiment 3

Experimental animals

A single group of five conventional dairy-cross calves, approximately two months of age, were used. The calves were clinically healthy on admission with a history of freedom from clinical ocular and respiratory disease. Feeding and housing were as described in Chapter 2, Section A.

Sampling of animals

- Virology

Ocular (L,R) and nasopharyngeal swabs were collected at weekly intervals from day -21 to day 28 and processed for the isolation and identification of respiratory viruses as described in Chapter 2, Section B.

- Mycoplasmology

Ocular (L,R) and nasopharyngeal swabs were collected at weekly intervals from day -21 to day 28 and processed for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Prior to challenge on day 0, ocular (L,R) and nasopharyngeal swabs were collected twice weekly for a period of three weeks. Following challenge exposure,

ocular (L,R) and nasopharyngeal swabs were taken daily until day 18 and then three times a week until day 28. Weekly, ocular samples were processed for the isolation and identification of Gram-negative bacteria and all samples were processed for the isolation of M.bovis as described in Chapter 2, Section D.

- Sera and lachrymal secretions

Serum and lachrymal secretions were collected from all calves on days 0, 14, 28 and 56 and submitted for examination by an IHA test, as described in Chapter 2, Section E.

Clinical examination and scoring

Both eyes of each animal were examined daily for three weeks prior to challenge exposure and found to be free from disease at all times. Following inoculation both eyes were examined daily for a period of 28 days. Examination procedure and clinical scoring system are described in Chapter 2, Section F.

Inoculation procedures

The microorganism used for inoculation was a low passage, β haemolytic strain of M.bovis (GM) which was isolated from a severe winter outbreak of IBK affecting a large dairy unit in south-west Scotland and which was strongly fimbriated when examined by electron microscopy. Storage of the strain, preparation of the inoculum and inoculation procedure were as described previously for experiment 1.

Within 30 minutes of challenge exposure, the inoculum was serially diluted in sterile 10% magnesium chloride solution and 0.5 ml of each tenfold dilution was inoculated onto fresh BAP. From this, the inoculum dose was found to be approximately 10^{10} CFU/ml.

RESULTS

Experiment 1

Microbiology

- Virology

Bovine herpes virus 1, adenovirus and PI3 virus were not isolated from any of the ocular or nasopharyngeal swabs submitted for virological examination.

- Mycoplasmology

Isolations of mycoplasmas from ocular swabs are presented in table 2 and, from nasal and nasopharyngeal swabs, in table 3. In group 1, U.diversum was isolated from only five of 50 (10%) ocular swabs collected prior to infection and A.laidlawii was isolated from only two (4%). From nasal and nasopharyngeal swabs, collected prior to infection, U.diversum was isolated from six of 50 (12%) and Myco.bovirhinis from three (6%). Mycoplasmas were not isolated from any samples collected following infection.

In the control group, U.diversum was isolated from nine of 50 (18%) ocular swabs collected prior to infection. From nasal and nasopharyngeal samples, U.diversum was isolated from eight of 50 (16%), Myco.bovirhinis from

ANIMAL SAMPLED	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) AND SOURCE											
		-32		-25		-18		-11		-4		3-52	
		L	R	L	R	L	R	L	R	L	R	L	R
1	U.diversum	-	-	-	-	-	+	-	-	-	-	-	-
2	U.diversum	-	-	-	-	+	+	-	-	-	-	-	-
3	U.diversum	-	-	-	-	-	+	-	-	-	-	-	-
4	U.diversum	-	-	-	-	+	-	-	-	-	-	-	-
	A.laidlawii	+	+	-	-	-	-	-	-	-	-	-	-
5	-												

L - left eye
R - right eye

\+ Sample positive
\- Sample negative

TABLE 2. Isolations of mycoplasmas from ocular samples, experiment 1.

ANIMAL SAMPLED	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) AND SOURCE											
		-32		-25		-18		-11		-4		3-52	
		N	NP	N	NP	N	NP	N	NP	N	NP	N	NP
1	U.diversum	-	-	-	-	+	-	-	-	-	-	-	-
2	U.diversum	-	-	-	-	+	+	-	-	-	-	-	-
3	U.diversum	-	-	-	-	-	+	-	-	-	-	-	-
	Myco.bovirhinis	-	-	-	+	+	-	-	-	+	-	-	-
4	U.diversum	-	-	-	-	+	+	-	-	-	-	-	-
5	-												

N Nasal cavity
NP Nasopharynx

\+ Sample positive
\- Sample negative

TABLE 3. Isolations of mycoplasmas from nasal and nasopharyngeal samples, experiment 1.

three (6%), A.laidlawii from one (2%) and Myco.bovis from one (2%). Mycoplasmas were not isolated from any of the samples collected from this group on days 3 and 10.

- Bacteriology

The Gram-negative bacteria, other than M.bovis, isolated from ocular, nasal and nasopharyngeal samples collected during this experiment are listed (table 4). Moraxella bovis was not isolated from any of the ocular, nasal or nasopharyngeal samples collected prior to inoculation.

Following inoculation M.bovis was not isolated from any of the ocular, nasal or nasopharyngeal samples collected from the control group (group 2). In contrast, in group 1, M.bovis was isolated from 61 out of 75 (81.3%) swabs taken from left eyes up to day 42 although all samples taken subsequent to day 42 were negative (table 5). In the right eyes of this group, 17 (22.7%) isolations were made during the same period, 14 (18.7%) of these isolations being from two eyes only, calves 1, 2 and 3 giving only one positive isolation each.

Isolations were made from nasal and nasopharyngeal swabs on only a few occasions.

Immunological status

All calves were shown to have negative serum and lachrymal titres immediately prior to infection on day 0.

BACTERIA ISOLATED	SOURCE OF SAMPLE																			
	1				2				3				4				5			
	L	R	N	NP	L	R	N	NP	L	R	N	NP	L	R	N	NP	L	R	N	NP
A.anitratus	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A.lwoffi	+	-	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	+	-	-
M.catarrhalis	+	-	-	-	+	-	+	-	-	-	+	+	+	+	-	+	+	-	-	+
M.nonliquefaciens	-	+	-	-	-	+	-	-	+	+	-	-	-	-	+	-	-	+	-	-
E.coli	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+
P.haemolytica	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

L Left eye
R Right eye
N Nasal cavity
NP Nasopharynx

\+ Bacterium isolated
\- Bacterium not isolated

TABLE 4. Isolations of Gram-negative bacteria (excluding M.bovis) from ocular, nasal and nasopharyngeal samples, experiment 1.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)															
	1	2	3	4	7	9	11	14	16	18	25	29	32	37	42	46-70
1 L	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	-
1 R	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
1 N	ND	-	ND	-	-	+	-	ND	-	ND	-	-	-	-	-	-
1 NP	ND	-	ND	+	-	-	-	ND	-	ND	-	-	-	-	-	-
2 L	+	-	+	+	+	+	+	-	+	+	+	+	-	-	-	-
2 R	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
2 N	ND	-	ND	-	-	-	-	ND	-	ND	-	-	-	-	-	-
2 NP	ND	-	ND	-	-	+	-	ND	-	ND	-	-	-	-	-	-
3 L	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
3 R	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
3 N	ND	-	ND	+	-	-	-	ND	-	ND	-	-	-	-	-	-
3 NP	ND	-	ND	-	-	-	-	ND	-	ND	-	-	-	-	-	-
4 L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
4 R	-	-	-	+	-	+	+	-	+	+	-	+	+	-	-	-
4 N	ND	-	ND	-	-	-	-	ND	-	ND	-	-	-	-	-	-
4 NP	ND	-	ND	-	-	-	-	ND	-	ND	-	-	-	-	-	-
5 L	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-
5 R	-	-	-	-	+	+	+	+	+	-	-	-	+	+	-	-
5 N	ND	-	ND	+	-	+	-	ND	-	ND	-	-	-	-	-	-
5 NP	ND	-	ND	-	+	-	+	ND	-	ND	-	-	-	-	-	-

L Left eye
R Right eye
N Nasal cavity
NP Nasopharynx

\+ Sample positive
\- Sample negative
ND Sample not taken

TABLE 5. Isolations of M.bovis from ocular, nasal and nasopharyngeal samples, experiment 1.

Clinical features

The eyes of all five control calves remained clinically normal after instillation of sPBS into the left eyes. Overflow of the inoculum was noted at the medial canthus during instillation which resulted in wetting of the cheek. On examination the following day the cheeks were dry with no disruption of the hair below the eye.

Clinical signs of IBK developed in all of the artificially-infected left eyes and in one right eye. Abnormalities were first noted in the left eyes of calves 1, 2, 4 and 5 within 24 hours of inoculation, and on day 18 in calf 3. However, in the case of calf 4, only epiphora was present until other signs were noted on day 35. The right eye of calf 5 developed clinical signs on day 25 despite the fact that M.bovis had been isolated on days 7, 9, 11, 14 and 16.

Two of the six affected eyes developed only mild lesions (Appendix 1; calves 2,5), attaining maximum clinical scores of 8 and 12 respectively. The ulcers were less than 2 mm in diameter and healed rapidly without corneal vascularisation. Of the four remaining affected eyes, the maximum clinical scores ranged from 14 to 18 (Appendix 1; calves 1,3,4,5) the latter being in the left eye of calf 4 on days 37 and 38. The ulcers reached diameters of greater than 3 mm. Healing was much slower and was accompanied by corneal vascularisation.

The individual daily scores for the period from day 0 to 28 are presented in table 6 and the mean scores illustrated in figure 1. The mean score for all left eyes during this period was 5.3 and for those left eyes in which IBK was diagnosed during this period was 6.1. The mean clinical score for all right eyes, over the same period, was 0.2 and for those in which IBK was diagnosed was 0.8.

Experiment 2

Microbiology

- Virology

Bovine herpes virus 1, adenovirus and PI3 virus were not isolated from any of the ocular, nasal or nasopharyngeal swabs submitted from virological examination.

- Mycoplasmology

Isolations of mycoplasmas from ocular swabs are presented in table 7 and, from nasal and nasopharyngeal swabs in table 8. Prior to infection, U.diversum was isolated from only nine of 70 (12.9%) ocular swabs; from nasal and nasopharyngeal swabs, U.diversum was isolated from eight of 70 (11.4%), Myco.bovirhinis from two (2.8%), A.laidlawii from one (1.4%) and Myco.bovis from one (1.4%).

Following infection, U.diversum was isolated from one of 58 (1.7%) ocular swabs and A.laidlawii from one (1.7%). From nasal and nasopharyngeal swabs, Myco.bovirhinis was isolated from five of 58 (8.6%), U.diversum from two (3.4%) and A.laidlawii from one (1.7%).

	EXAMINATION TIMES (DAYS)																													
CALF	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1L	0	6	12	16	15	15	15	16	16	15	15	15	15	15	14	13	12	11	16	8	7	7	7	0	0	0	0	0	0
1R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2L	0	6	8	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	13	12	12	13	14	14	14	14	13
3R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4L	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
4R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5L	0	8	14	16	14	14	14	13	13	13	13	13	13	12	12	12	12	12	10	9	9	8	6	0	0	0	0	0	0
5R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	12	7	2

L Left eye
R Right eye

TABLE 6. Clinical scores from day 0 to day 28, experiment 1.

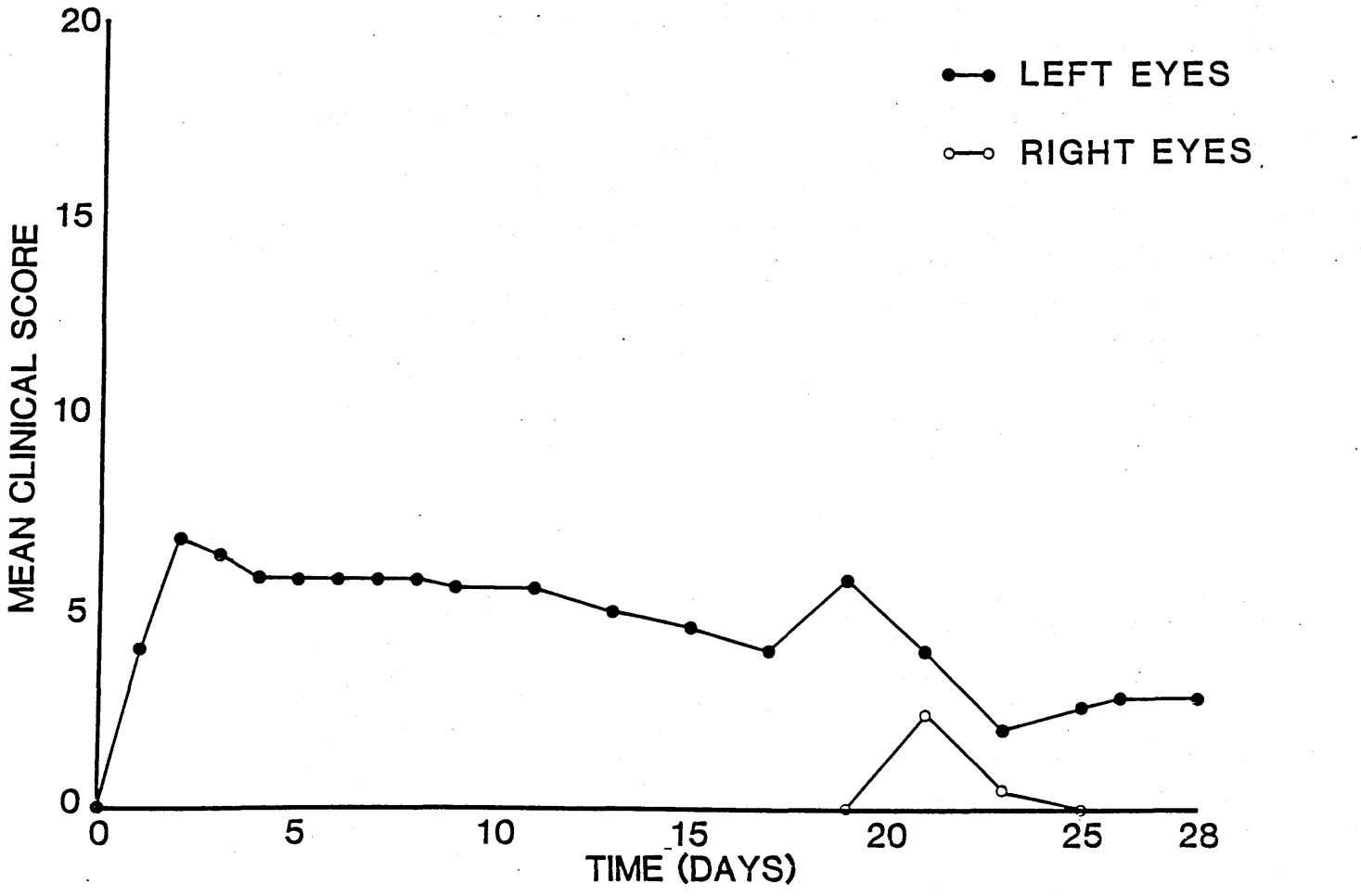


FIGURE 1. Experiment 1. Mean clinical scores following the instillation of M.bovis strain GS into each left conjunctival sac.

ANIMAL SAMPLED	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) AND SOURCE																					
		-47		-40		-33		-26		-19		-12		-5		3		10		17-38		45	
		L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
6	U.Diversum	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	U.diversum	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	U.diversum	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	A.laidlawii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
9*	-																						
10	U.diversum	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

L Left eye
R Right eye
* Calf 9 slaughtered day 3

\+ Sample positive
\- Sample negative

TABLE 7. Isolations of mycoplasmas from ocular samples, experiment 2.

ANIMAL SAMPLED	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) AND SOURCE																					
		-47		-40		-33		-26		-19		-12		-5		3		10		17-38		45	
		N	NP	N	NP	N	NP	N	NP	N	NP	N	NP	N	NP	N	NP	N	NP	N	NP	N	NP
6	U.diversum	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	Myco.bovirhinis	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	A.laidlawii	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	U.diversum	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Myco.bovirhinis	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
8	U.diversum	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	Myco.bovis	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	A.laidlawii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
9*	U.diversum	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	U.diversum	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-

N Nasal cavity
NP Nasopharynx
* Calf 9, slaughtered day 3

\+ Isolation positive
\- Isolation negative

TABLE 8. Isolations of mycoplasmas from nasal and nasopharyngeal samples, experiment 2.

- Bacteriology

Gram-negative bacteria, other than M.bovis, isolated from ocular, nasal and nasopharyngeal samples collected during this experiment are listed in table 9. Prior to inoculation, M.bovis was not isolated from any of the ocular, nasal or nasopharyngeal samples collected.

Moraxella bovis was isolated from 32 of 46 (69.6%) samples collected from the left eyes between days 0 and 40 (table 10) and from 28 of 46 (60.9%) right ocular swabs. Four right eyes were infected with M.bovis by day 3, and all five by day 10. Isolations of M.bovis were not made from any samples collected after day 40 in this group except for two isolations from the left eyes of calves 7, 8 on day 70.

Isolations of M.bovis were made from nasal and nasopharyngeal swabs sporadically.

Immunological status

All calves were shown to have negative serum and lachrymal titres prior to infection on day 0.

Clinical features

Signs of IBK developed in left eyes of calves 7,8, 9,10 and in right eyes of calves 6,7,8,10. Signs were first noted in left eyes on day 1 in calves 7,10, day 2 in calf 9 and day 4 in calf 8. In right eyes, signs were noted on day 11 in calf 10, day 17 in calf 7, day 18 in calf 8 and day 21 in calf 6.

BACTERIA ISOLATED	SOURCE OF SAMPLE																			
	6				7				8				9				10			
	L	R	N	NP	L	R	N	NP	L	R	N	NP	L	R	N	NP	L	R	N	NP
A.anitratus	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
A.lwoffi	+	+	-	+	-	-	-	+	+	-	-	+	-	+	+	-	-	-	+	-
M.catarrhalis	-	-	-	-	-	+	+	-	-	-	+	+	-	-	+	-	+	-	-	+
M.nonliquefaciens	-	-	-	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-
E.coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

L Left eye
R Right eye
N Nasal cavity
NP Nasopharynx

\+ Bacterium isolated
\- Bacterium not isolated

TABLE 9. Isolations of Gram-negative bacteria (excluding M.bovis) from ocular, nasal and nasopharyngeal samples, experiment 2.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)													
	1	3	7	10	14	17	22	27	31	33	37	40	43-67	70
6L	+	+	+	+	+	+	+	+	+	-	-	-	-	-
6R	-	-	-	+	+	+	+	+	+	-	-	-	-	-
6N	-	ND	-	-	+	-	-	-	-	-	-	-	-	-
6NP	-	ND	-	-	+	-	-	-	-	-	-	-	-	-
7L	+	+	-	+	+	-	+	-	+	-	-	+	-	+
7R	-	+	-	+	+	-	+	+	+	-	-	-	-	-
7N	-	ND	-	-	-	-	-	-	-	-	-	-	-	-
7NP	-	ND	-	-	-	-	-	-	-	-	-	-	-	-
8L	+	+	-	+	+	+	-	+	+	-	-	-	-	-
8R	+	-	-	-	+	+	+	+	+	+	-	+	-	-
8N	+	ND	-	-	-	-	-	-	-	-	-	-	-	-
8NP	-	ND	-	+	-	-	-	-	-	-	-	-	-	-
10L	+	+	-	+	+	+	+	+	-	+	-	-	-	-
10R	+	+	-	+	+	+	-	+	-	-	-	-	-	-
10N	+	ND	-	-	+	-	-	+	-	-	-	-	-	-
10NP	+	ND	-	+	-	-	-	-	-	-	-	-	-	-
9L*	+	+												
9R	+	+												
9N	-	-												
9NP	-	-												

L Left eye
R Right eye
N Nasal cavity
NP Nasopharynx
* Calf 9, slaughtered day 3

\+ Sample positive
\- Sample negative
ND Sample not taken

TABLE 10. Isolations of M.bovis from ocular, nasal and nasopharyngeal samples, experiment 2

Three eyes, 7L,R, 6R, were only mildly affected (Appendix I; calves 6,7) and healed without vascularisation with individual maximum clinical scores ranging from 10 to 14. However, two of these eyes, 6R,7R developed fresh mild lesions 14 and 9 days, respectively, after the original lesions had healed and 7R was affected for a third time seven days after the second lesion had resolved. Both eyes of calves 8,10 developed severe lesions with ulcers of greater than 3mm diameter and individual maximum scores of 13 to 19 (Appendix I; calves 8,10). These lesions took longer to resolve and healing was accompanied by corneal vascularisation.

Individual daily clinical scores from day 0 to day 28 are presented in table 11 and mean scores illustrated in figure 2. The mean score for all left eyes was 5.0 and for the left eyes, excluding calf 9 (slaughtered day 3), was 4.9. The mean score for the right eyes, again excluding calf 9, was 5.2

Experiment 3

Microbiology

- Virology

Neither BHV1, adenovirus nor PI3 virus were isolated from any of the ocular or nasopharyngeal swabs submitted for virological examination.

- Mycoplasmology

Isolations of mycoplasmas from ocular and naropharyngeal samples are presented in table 12.

	EXAMINATION TIMES (DAYS)																												
CALF	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
6L	0	0	4	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	8	10	9	9	5	0	0	0
7L	0	2	7	11	9	9	10	7	8	8	7	7	7	7	7	7	4	4	0	0	0	0	0	0	0	0	0	0	0
7R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	14	0	0	0	0	0	0	0	0	0	15	14
8L	0	0	0	0	13	13	13	9	8	12	12	12	12	12	11	10	10	11	10	9	6	0	0	0	0	0	0	0	0
8R	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	10	12	12	13	18	18	16	16	15	15	16	15
9L*	0	0	10	14																									
9R	0	0	0	0																									
10L	0	1	4	10	17	17	16	12	14	15	15	15	14	14	13	11	11	11	11	10	6	0	0	0	0	0	0	0	0
10R	0	0	0	0	0	0	0	0	0	0	3	13	15	16	16	17	18	19	18	17	16	16	18	17	16	15	15	15	16

L Left eye
R Right eye

* Calf 9, slaughtered day 3

TABLE 11. Clinical scores from day 0 to day 28, experiment 2.

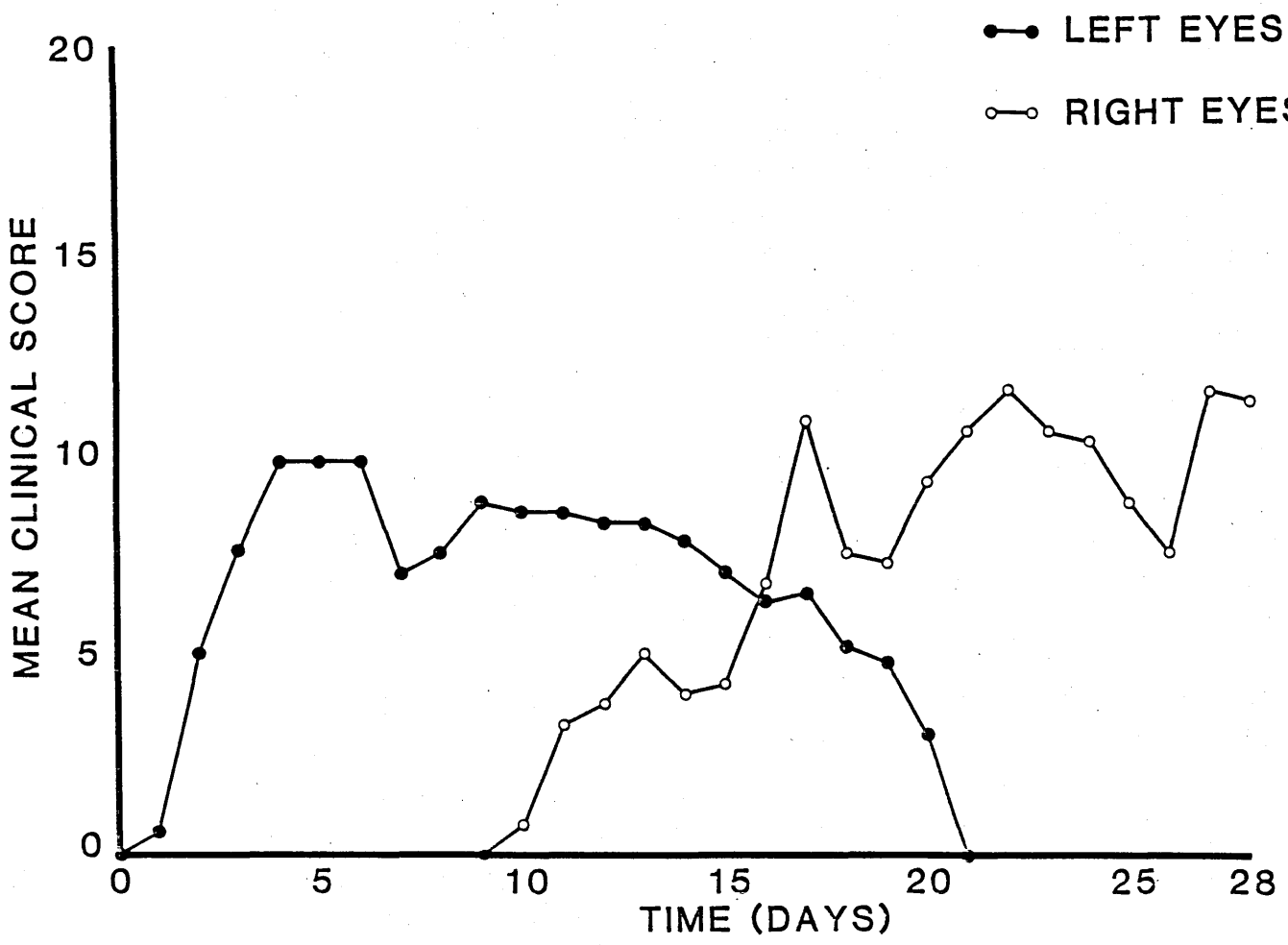


FIGURE 2. Experiment 2. Mean clinical scores following the instillation of M.bovis strain GS (RI) into each left conjunctival sac.

ANIMAL SAMPLED	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) AND SOURCE																	
		-11			-4			3			11			18			25		
		L	R	NP	L	R	NP	L	R	NP	L	R	NP	L	R	NP	L	R	NP
41	A.laidlawii	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	A.laidlawii	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-
43	A.laidlawii	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
	Myco.dispar	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
44	A.laidlawii	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	A.laidlawii	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	+
	Myco.dispar	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	Myco.bovis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

L Left eye
R Right eye
NP Nasopharynx

\+ Sample positive
\- Sample negative

TABLE 12. Isolations of mycoplasmas from ocular and nasopharyngeal samples, experiment 3.

Mycoplasmas were not isolated from any samples collected prior to infection. Following infection, A.laidlawii was isolated from four of 40 (10%) ocular swabs and Myco.bovirhinis from one (2.5%). From nasopharyngeal swabs, A.laidlawii was isolated from seven of 20 (35%), Myco.bovirhinis from three (15%), Myco.dispar from two (10%) and Myco.bovis from one (5%).

- Bacteriology

The Gram-negative bacteria, other than M.bovis, isolated from ocular samples collected during this experiment are listed (table 13).

Moraxella bovis was not isolated from any of the ocular or nasopharyngeal samples collected prior to inoculation on day 0. Isolations of M.bovis from samples collected following inoculation are given in table 14. From samples collected from the left eyes, M.bovis was isolated from 96 of 110 (87.3%) ocular swabs collected up to day 28. In the right eyes, M.bovis was isolated from 85 of 110 (77.3%) ocular swabs. Moraxella bovis was isolated from 42 out of 100 (42%) swabs collected from the nasopharynx.

Immunological response

None of the lachrymal or serum samples collected prior to challenge contained significant levels of antibody. The results of serological testing of lachrymal secretions and serum are given in tables 15 and 16, respectively. Significant levels of antibody were present in lachrymal secretions collected from calf 41L,R

BACTERIA ISOLATED	SOURCE OF SAMPLE									
	41		42		43		44		45	
	L	R	L	R	L	R	L	R	L	R
A.anitratus	+	+	-	-	-	-	-	-	-	-
A.lwoffi	+	+	-	-	+	+	-	+	+	-
M.caviae	-	+	-	-	-	-	-	+	-	-
M.catarrhalis	+	+	-	-	-	-	-	+	-	-
M.nonliquefaciens	+	+	+	+	+	+	+	+	+	+
E.coli	-	-	-	-	-	+	-	+	+	-

L Left eye
R Right eye

\+ Bacterium isolated
\- Bacterium not isolated

TABLE 13. Isolations of Gram-negative bacteria (excluding M.bovis) from ocular samples, experiment 3.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	18	20	22	25	27	28
41L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
41R	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
41NP	+	-	-	-	+	+	+	+	-	+	+	+	+	+	ND	ND	+	+	-	+	+	+
42L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42NP	-	-	+	-	-	+	+	-	-	-	+	-	-	+	ND	ND	+	-	+	-	+	-
43L	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
43R	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
43NP	-	-	-	-	-	+	-	-	-	-	-	-	+	-	ND	ND	-	+	-	-	-	-
44L	-	-	-	-	-	-	+	+	-	-	+	-	-	+	+	+	+	+	-	+	+	+
44R	-	+	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+
44NP	+	-	-	+	-	-	-	-	-	-	-	-	-	-	ND	ND	-	-	-	+	-	-
45L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
45R	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
45NP	+	-	-	-	+	+	+	+	-	+	+	+	-	+	ND	ND	-	+	+	+	-	+

L Left eye
R Right eye
NP Nasopharynx

\+ Sample positive
\- Sample negative
ND Sample not taken

TABLE 14. Isolations of M.bovis from ocular and nasopharyngeal samples, experiment 3.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS) -1	14	28
41L	-	-	16
41R	-	-	16
42L	-	-	-
42R	-	-	2
43L	-	-	-
43R	-	-	-
44L	-	-	-
44R	-	-	-
45L	-	-	-
45R	-	-	-

L Left eye
R Right eye

- No haemagglutination
N Haemagglutination in neat dilution only

TABLE 15. Reciprocal IHA titres against whole cell M.bovis in lachrymal secretions, experiment 3.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS) -1	14	28
41	-	-	2
42	-	-	2
43	2	-	4
44	-	-	2
45	-	-	2

- No haemagglutination
N Haemagglutination in neat dilutions only

TABLE 16. Reciprocal IHA titres against whole cell M.bovis in serum samples, experiment 3.

on day 28, with the highest titre present in the left eye.

Clinical features

Prior to challenge, the eyes of all five calves were clinically normal. In the left eyes, signs of ocular irritation were first noted on day 1 calves 42, 43 and 45, day 2 calf 41 and on day 20 calf 44. Corneal changes were first noted in calves 42, 43 and 45 on days 4, 7 and 1, respectively, while corneal lesions were never noted in calves 41 and 44. In the right eyes, signs of ocular irritation were first noted on day 4 in calf 41, day 7 in calf 43, day 8 in calf 42 and day 9 in calf 45. Corneal changes were first noted on days 8, 9, 10 and 16 in calves 41, 42, 45 and 43, respectively. The ulcers produced in all cases were less than 2 mm in diameter. All eyes healed without visible corneal vascularisation except the left eye of calf 45 in which the three pinpoint ulcers were sited close to the corneoscleral junction (Appendix I; calf 45). Mild lesions recurred in calves 41R, 43L, 45L,R.

Individual daily clinical scores for both left and right eyes over the 28 day examination period are presented in table 17 and the mean scores illustrated in figure 3. Mean score over this period for all left eyes was 3.6 and for those left eyes in which IBK was diagnosed was 4.5. In the right eyes, the mean score for all eyes was 3.0 and for those eyes in which IBK was diagnosed was 3.6.

	EXAMINATION TIMES (DAYS)																												
CALF	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
41L	0	0	8	0	0	0	0	0	4	0	0	2	5	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
41R	0	0	0	0	2	8	3	0	4	8	9	4	4	13	11	11	11	7	3	5	2	0	0	0	0	0	0	11	2
42L	0	6	2	2	8	8	4	11	9	2	3	3	4	2	0	11	10	9	7	0	11	9	9	6	6	6	2	2	11
42R	0	0	0	0	0	0	0	0	7	11	5	4	10	2	2	2	2	7	7	2	11	7	6	6	7	9	4	2	10
43L	0	7	0	0	0	0	4	5	0	0	0	0	0	0	0	11	2	4	6	0	0	4	9	3	3	3	2	9	5
43R	0	0	0	0	0	0	0	2	0	0	9	0	0	0	7	0	2	2	10	4	7	3	5	2	3	4	2	12	8
44L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
44R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45L	0	9	11	10	12	7	12	10	12	7	4	12	10	10	4	13	12	10	8	10	9	4	6	3	3	3	14	10	5
45R	0	0	0	0	0	0	0	0	0	5	5	4	4	0	0	0	0	0	0	5	2	3	12	10	9	8	5	5	3

L Left eye
R Right eye

TABLE 17. Clinical scores from day 0 to day 28, experiment 3.

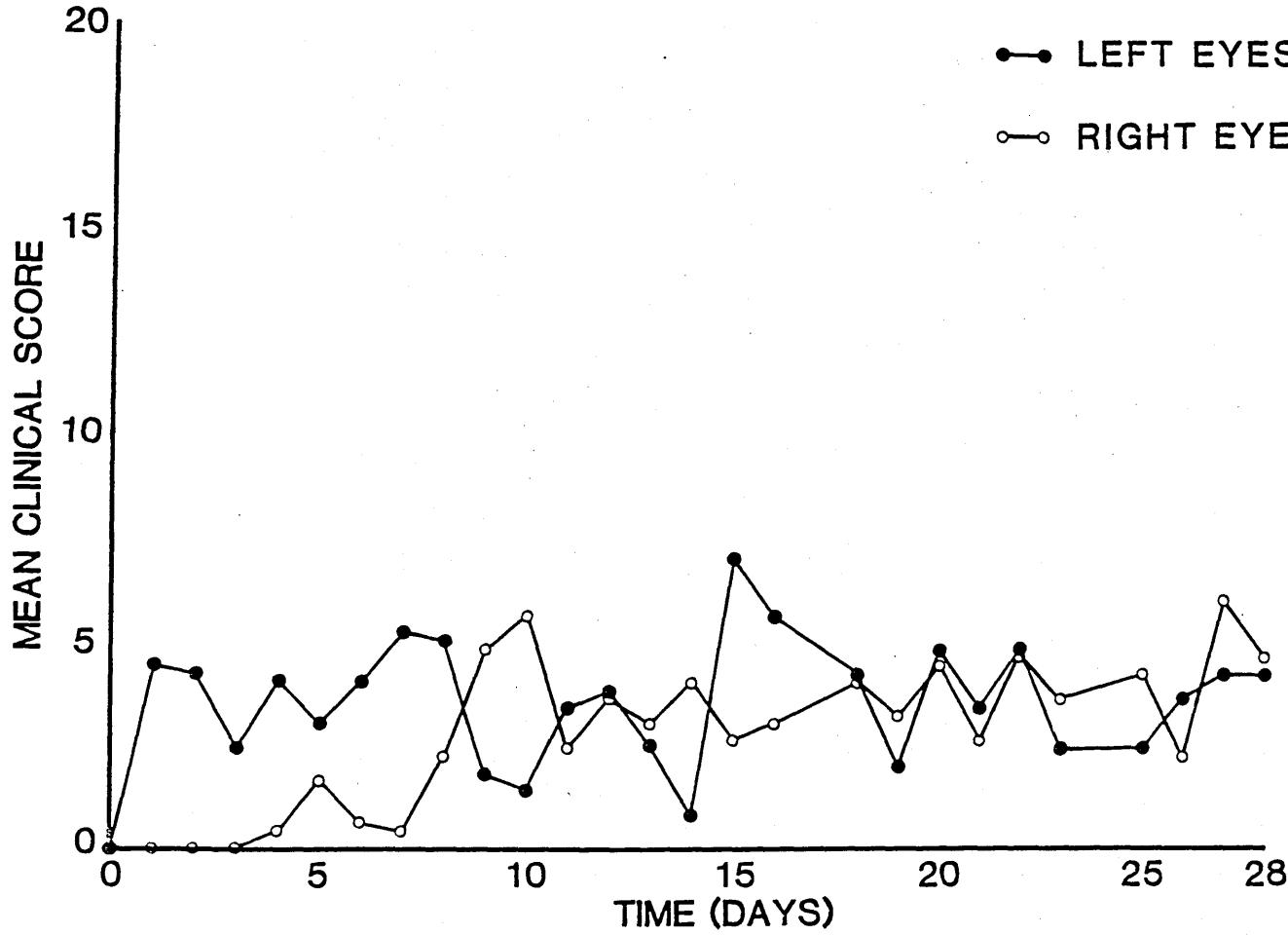


FIGURE 3. Experiment 3. Mean clinical scores following the instillation of M.bovis strain GM into each left conjunctival sac.

Sequential development of clinical signs and lesions

The results in this section are comprised of data from all three of the above experiments and are summarised in table 18.

In the healthy eye, (figure 4) the eyelids are normally held open with occasional blinking and there is no tear overflow. The conjunctivae are pale pink with small vessels present, the cornea is clear and shiny and the iris is normally dilated and responsive to light.

In calves challenged with either strain GS or the reisolate of that strain, epiphora was the first sign noted in all 14 eyes in which corneal lesions developed. Blepharospasm and conjunctivitis were first noted concurrently with epiphora in nine eyes, within 48 hours in four. However, in one eye, the left eye of calf 4, epiphora alone was noted for a period of 32 days before blepharospasm and conjunctivitis developed. Increased blinking and iridospasm were noted either simultaneously with blepharospasm or within 48 hours of its development. Conjunctivitis preceded corneal changes by 24 hours in eight of the eyes (figure 5), was noted concurrently in the remainder and epiphora preceded corneal changes in ten of the 14 affected eyes. A sporadic but mild purulent discharge was noted in all affected eyes. A head jerk movement consisting of a rearward jerk of the head immediately after blinking or during walking, which lasted 24 to 48 hours, was noted in three animals. This sign occurred within 48 hours subsequent to the development of corneal lesions.

Eye affected	Days to development of epiphora	Days from the appearance of epiphora to the development of: Conjunctivitis	Blepharospasm	Blinking	Iridospasm	Corneal ulceration	Corneal opacity
1L	1	L^+	0	0	1	2	1
1R	-						
2L	1	0	0	0	0	1	-
2R	-						
3L	18	0	0	0	1	0	0
3R	-						
4L	1	32	32	32	32	32	32
4R	-						
5L	1	0	0	0	0	1	1
5R	25	1	1	1	1	1	1
6L	-						
6R	21	0	0	0	0	1	1
7L	1	0	1	2	2	1	1
7R	16	0	0	0	0	1	1
8L	4	0	0	0	0	0	0
8R	17	0	0	0	1	0	0
9L*	2	0	0	0	1	0	0
9R	-						
10L	1	1	1	3	2	2	2
10R	10	0	1	1	1	1	1

TABLE 18.

Eye affected	Days to development of epiphora	Days from the appearance of epiphora to the development of: Conjunctivitis	Blepharospasm	Blinking	Iridospasm	Corneal ulceration	Corneal opacity
41L	2	0	0	0	0	-	-
41R	4	1	1	1	1	6	6
42L	4	-3	-3	-3	-3	1	1
42R	8	0	0	0	4	1	1
43L	1	0	0	0	14	14	14
43R	7	3	3	3	3	9	9
44L	20	0	0	0	-	-	-
44R	-						
45L	1	0	0	0	1	0	0
45R	9	1	0	0	13	1	1

L Left eye
R Right eye
* Calf 9, slaughtered day 3

\- Eye remained free of lesions
\+ 0 indicates that epiphora and other signs appeared simultaneously

TABLE 18. Time to development of clinical signs and order of appearance, experiments 1, 2 and 3. (contd.)

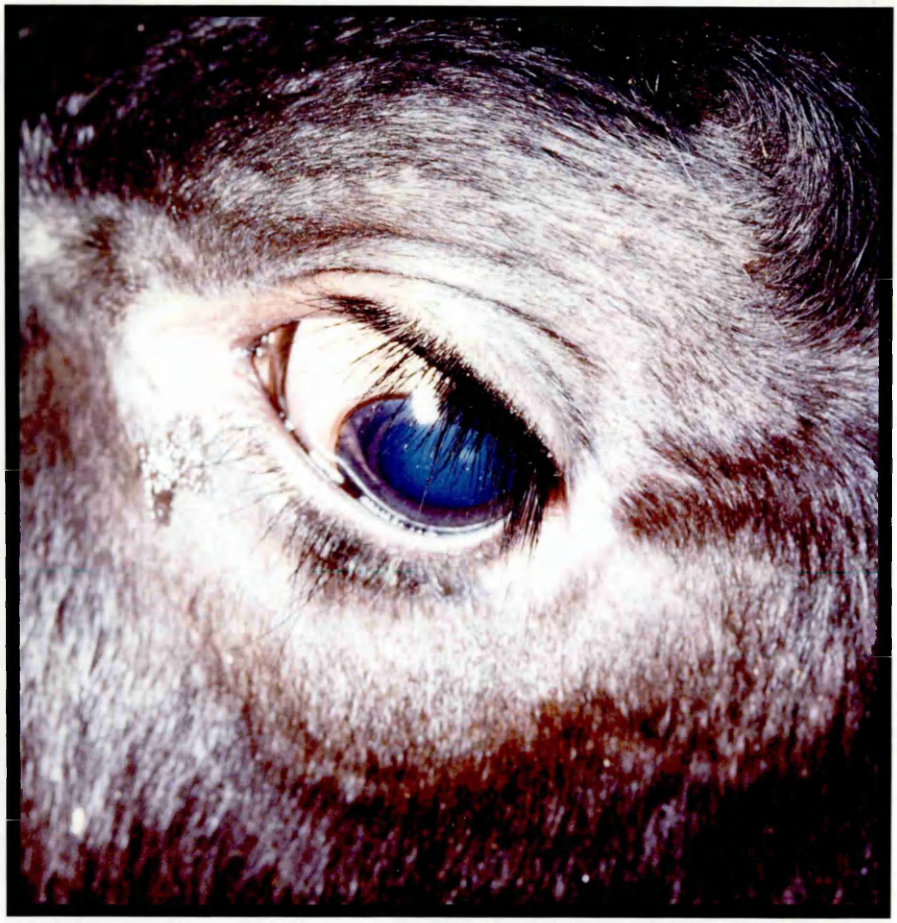


FIGURE 4. Calf 10L, day 0, normal bovine eye.

FIGURE 5. Calf 10L, day 2, two day old lesions. There are signs of ocular irritation, with marked epiphora present while the eyelids are held closed. The conjunctivae were inflamed at this stage although there were no corneal lesions.

In calves challenged with strain GM, epiphora was the first sign noted in six of the seven eyes which developed corneal lesions while in the remaining eye conjunctivitis, blepharospasm and blinking were present three days prior to the development of epiphora. Conjunctivitis, blepharospasm and increased blinking were noted concurrently in six eyes while blepharospasm and increased blinking preceded conjunctivitis by one day in one. Iridospasm was noted within 24 hours subsequent to the development of conjunctivitis in three instances.

Conjunctivitis preceded corneal changes in five eyes by a mean interval of 4.1 days and the two signs were noted concurrently in two. In three eyes, the delayed development of corneal lesions followed the initial resolution and recurrence of signs of irritation. A head jerk was noted in one animal on one occasion only and was seen to be associated with signs of acute irritation in the absence of corneal changes.

Course of disease

- Mild cases

Eleven of the eyes were classified as being mildly affected on the basis of low maximum clinical scores and healing of corneal lesions without vascularisation.

In nine of these, the ulcers did not increase in size after first being noted but in the other two they increased from 2 to 3 mm in diameter over a seven-day

period. Signs of ocular irritation (ie, epiphora, conjunctivitis and blepharospasm) abated one to 14 days after first being noted. Corneal opacities faded and ulcers resolved within three to 13 days, in some cases leaving a shallow depression.

A similar pattern was present during recurrent attacks with ulcers reaching maximum size on the first day noted. Signs of ocular irritation lasted two to 14 days and corneal changes were noted for one to 18 days.

- Severe cases

In the nine other affected eyes, corneal lesions were more severe with maximum clinical scores of 14 or above with lesions persisting over a longer period.

Healing of the ulcers was accompanied by vascularisation which started to appear three to 17 days after the development of initial signs. The ulcers in these cases grew most rapidly within 48 hours of first being noted, reaching a diameter of over 1 cm in the most severe cases. The surrounding corneal opacity increased over the same period while the floor of the ulcer gradually became totally opaque.

In cases with typical anterior polar ulcers, vascularisation was first noted as a dense red capillary bed, 1 mm wide, extending around the entire corneoscleral junction (figure 6). This capillary bed advanced centripetally in all areas at a rate of approximately 1 mm/day (figure 7).

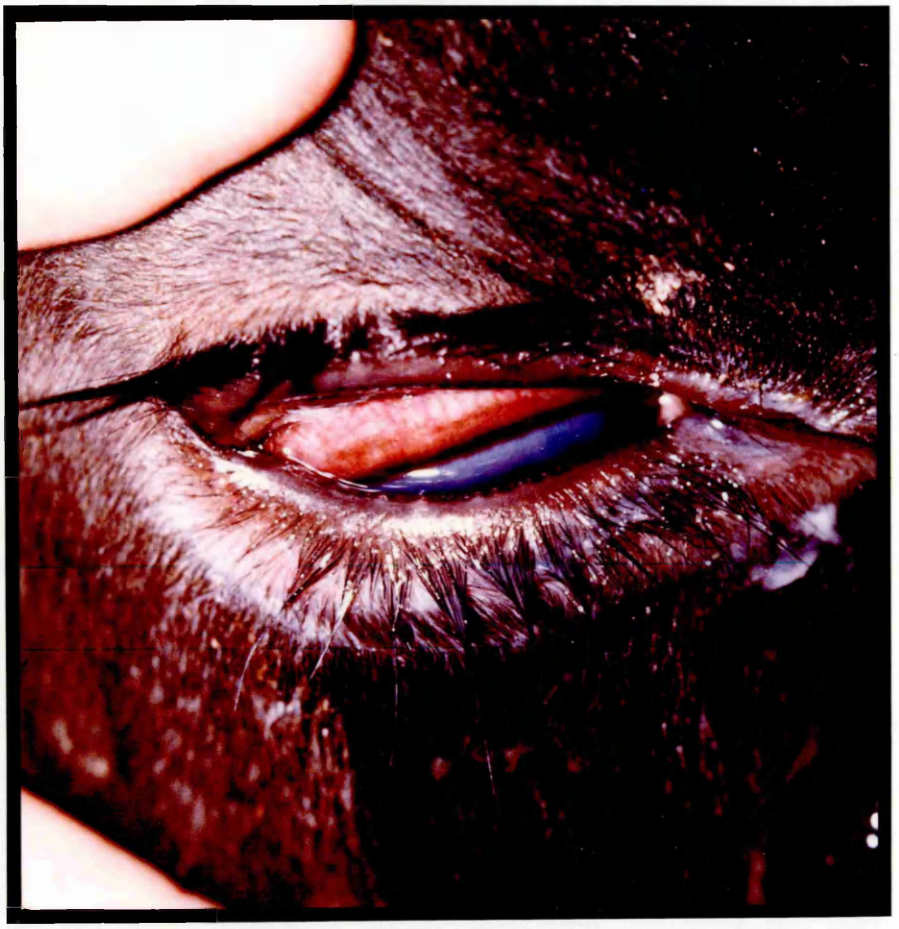


FIGURE 6. Calf 10R, day 13, three day old vesicle. There is severe irritation with epiphora, conjunctivitis, with injection of episcleral vessels, blepharospasm and iridospasm. A small amount of pus is present at the medial canthus and vascularisation can be seen at the corneoscleral junction.

FIGURE 7. Calf 10R, day 15, five day old vesicle. There is still severe irritation although blepharospasm has decreased in intensity and the iridospasm present is obscured by a generalised corneal opacity. There is no pus present and vascularisation has extended approximately 2mm from the corneoscleral junction.

When the capillary bed had extended 4 to 5 mm from the corneoscleral junction the blood supply to the leading edge became organised, with many vessels atrophying, leaving a small number of larger vessels to supply the capillary edge (figure 8). The ulcers at this point had developed a flat opaque white floor. After seven to ten days (figure 9) the capillary bed reached the ulcer edge and continued to advance over the ulcer floor again at a rate of about 1 mm/day. Granulation tissue formed over the advancing blood supply, resulting in dorsal and ventral ridges projecting 2 to 3 mm above the corneal surface (figure 10). These ridges coalesced as the underlying capillary beds met forming a single projecting mass of granulation tissue covering the whole of the ulcer floor (figure 11).

The area covered by the granulation tissue contracted over a period of five to six days until it appeared as a red mass, 2-3 mm in diameter and projecting by 2-3 mm (figure 12). Over the following 48 hours the granulation tissue flattened, leaving a dense scar at the site of the ulcer (figure 13), which although initially pink soon faded to become white (figure 14). The scar became progressively less opaque (figure 15) although in all eight severe cases it was still visible at the end of the experiments on days 90 and 76 for groups 1 and 2, respectively.

FIGURE 8. Calf 10R, day 20, ten day old ulcer. Vascularisation is present along the entire corneoscleral junction with most advanced development dorsal and ventral to the ulcer.

FIGURE 9. Calf 10R, day 22, 12 day old ulcer. Signs of irritation are much reduced in intensity. The capillary bed has reached the dorsal ulcer edge and the cornea is clearing near to the corneoscleral junction as capillaries become organised.

FIGURE 10. Calf 10R, day 27, 17 day old ulcer. Capillaries extend 3mm across the dorsal and ventral ulcer base with formation of projecting granulation tissue. Blood is supplied to the ulcer by several main corneal vessels.

FIGURE 11. Calf 10R, day 29, 19 day old ulcer. The ulcer is reduced in area to approximately 1.0 cm^2 and the floor is covered by granulation tissue projecting 2mm above the corneal surface.

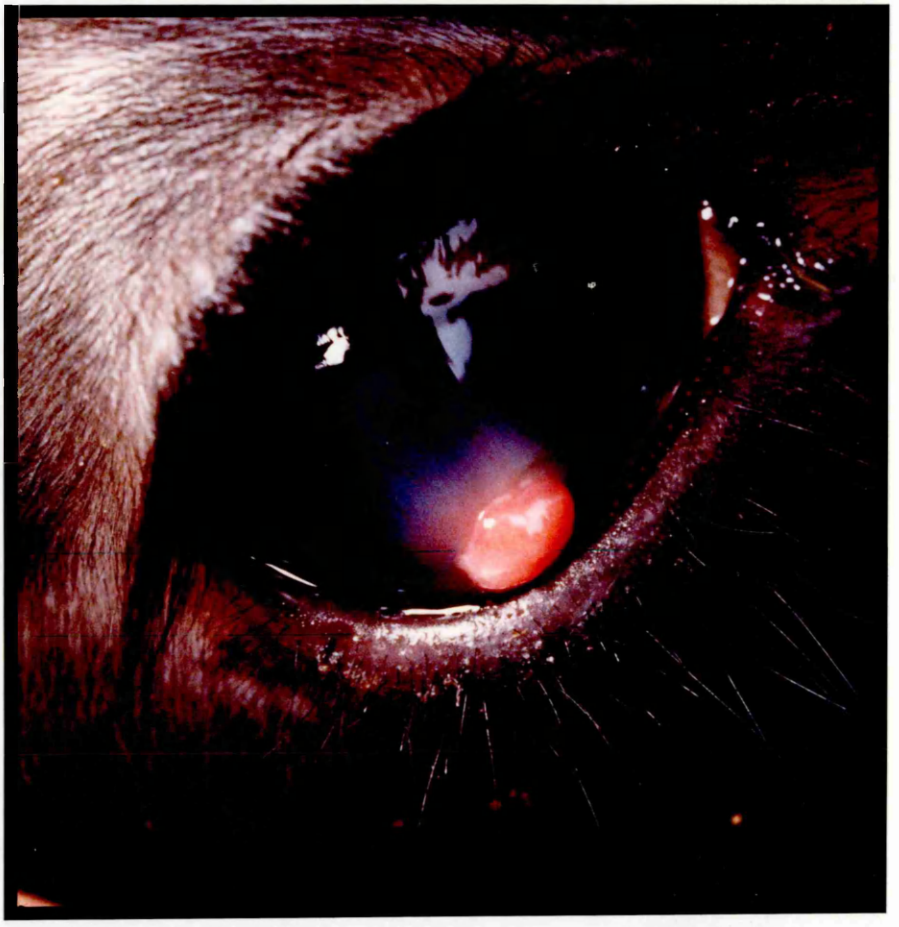


FIGURE 12. Calf 10R, day 31, 21 day old ulcer. The ulcer is reduced in area to approximately 0.6 cm^2, the granulation tissue is becoming less vascular but projecting 3mm. The cornea is clearing at the periphery with a slight haze surrounding main supply vessels.

FIGURE 13. Calf 10L, day 20, 18 day old lesion. Granulation tissue is confluent with the corneal surface, a vascular plexus is clearly visible at the ulcer site surrounded by a slight haze.

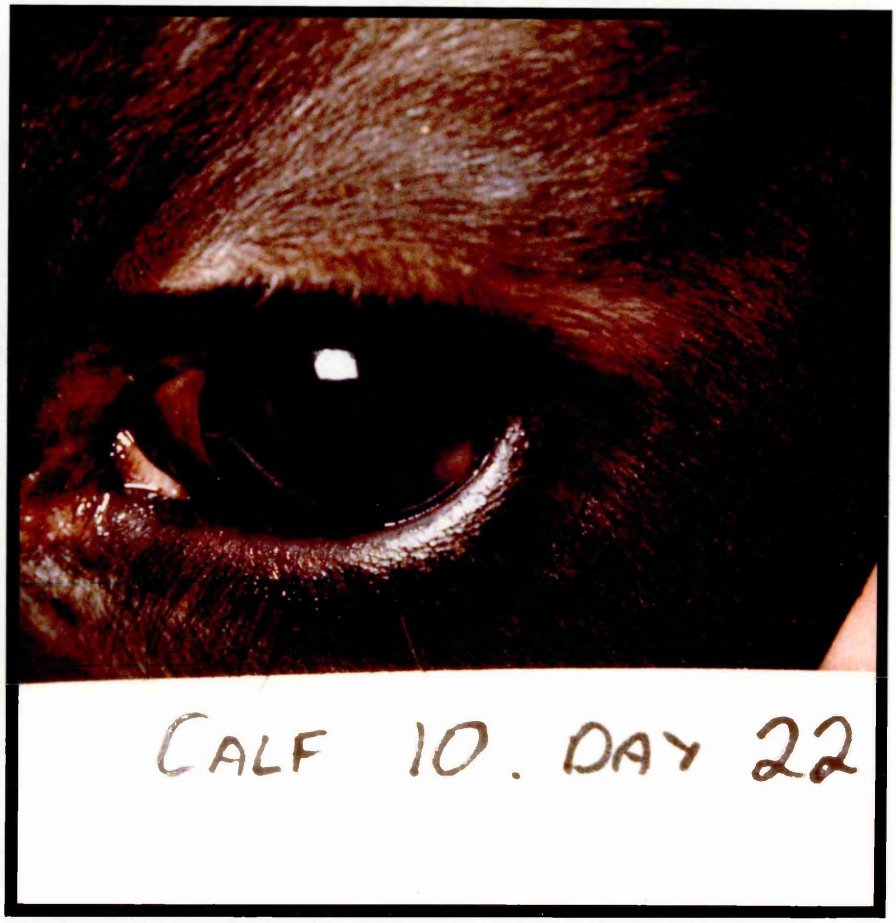


FIGURE 14. Calf 10L, day 22, 20 day old lesion. The cornea is transparent over most of its surface with a nebulous pink opacity at the ulcer site.

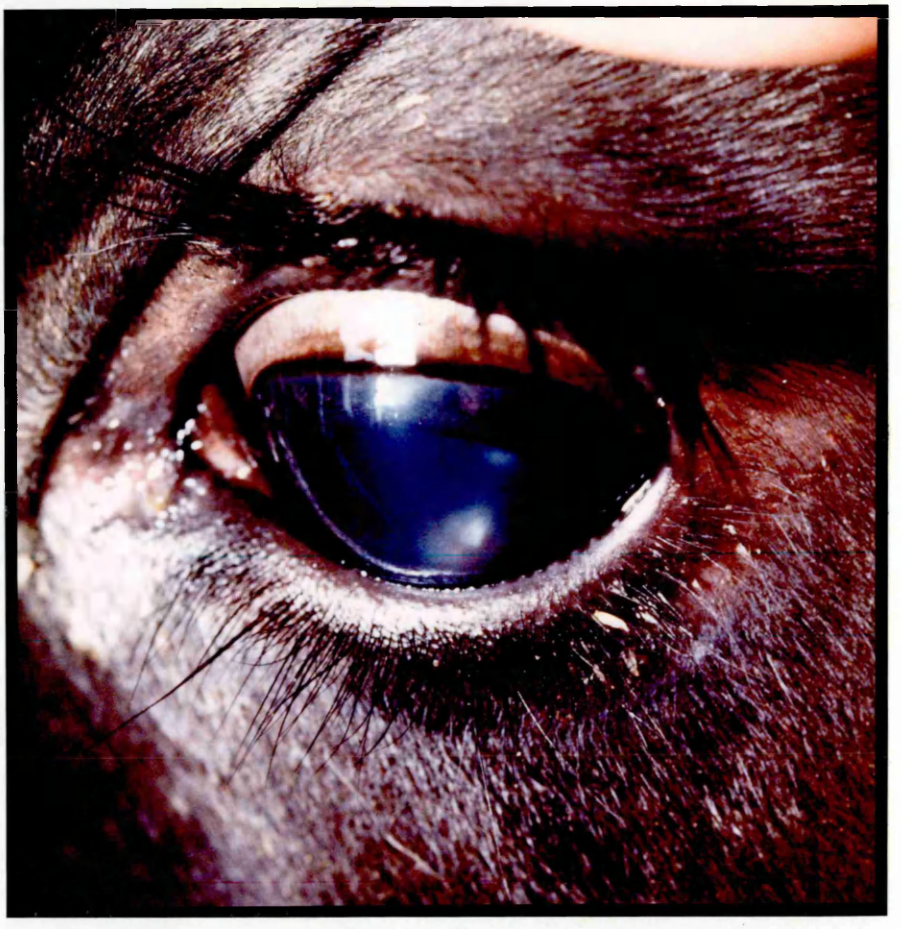


FIGURE 15. Calf 10L, day 31, 29 day old lesion. A tenuous white scar is present at the ulcer site. Some vascularisation is still present but supply vessels are much reduced in diameter and cannot be easily seen.

DISCUSSION

While M.bovis is now widely accepted as being the causal agent of IBK (18), its capability of producing severe ocular lesions, per se, has been questioned (105). Apparently, such doubts have often evolved as a consequence of, (i) the variable and often disappointing results that have followed attempts to reproduce the disease with field isolates which appeared to be highly pathogenic, (ii) interpretive problems when, in addition to M.bovis, other microbial agents have been isolated from field material and, (iii) the limited number of authoritative descriptions of the development and nature of the lesions seen in either the field or the experimentally-induced disease, with the result that other ocular disorders may occasionally have been included under an "umbrella diagnosis" of IBK (18).

Since the simple instillation into the conjunctival sac of M.bovis cultures has frequently failed to produce the disease (4,57,82,86,91,224) workers have occasionally resorted to such extreme measures as UV irradiation (105) or even more severe methods of traumatising the cornea (142). At least the former approach would appear to bear relevance to some outbreaks of IBK. Concurrent infection with viruses (138,168,228, 231), mycoplasmas (64,85,108,115,125,170,185) and other species of bacteria (56,203) have frequently been noted and sometimes been implicated in the pathogenesis of IBK.

The success achieved in the above study probably reflects the selection procedures aimed at identifying both susceptible calves and a highly pathogenic strain of M.bovis. In the first instance, calves were selected from known situations and had no history of any form of ocular disease before acquisition. In addition they were screened extensively for the presence of M.bovis and other potential ocular pathogens by both conjunctival and nasopharyngeal swabbing and were retrospectively found to be seronegative for M.bovis. Since strain variations in pathogenicity have been demonstrated (39,156) there was also a clear need to identify and use a pathogenic isolate of M.bovis. Therefore, it was decided to use a strain (GS) which was both strongly fimbriated and which had previously been shown to be highly pathogenic in mice and calves (39,43). Similarly, in the third study a strongly fimbriated haemolytic strain, isolated from a severe outbreak of IBK, was used.

M.bovis colonised each of the ten left eyes that were experimentally infected with GS and in seven epiphora had developed within 48 hours of inoculation. In a further two, despite the continued presence of M.bovis, clinical signs did not appear until four and 18 days after inoculation. The organism spread to the right eyes of all calves at some time during the experimental period although in three it was only recorded on single occasions. Its first appearance in the right eyes ranged from one to nine days and IBK was diagnosed in the right eyes of five calves. The time taken for clinical signs to arise in

the right eyes ranged from 10 to 18 days, which perhaps more closely reflects the natural situation than do the figures for the artificially infected left eyes. The variable and often lengthy incubation period may indicate the requirement for a build-up in numbers of organisms before any pathogenic effects may be visible. In another study (233), cross infections occurred from four to 12 days after artificial infection, with lesions becoming apparent one to nine days later. In this latter study lesions in the cross-infected eyes were less severe than those that had been artificially infected; however, in the latter instance each of the corneas had been extensively traumatised before infection.

Moraxella bovis was isolated frequently until days 40 and 42 in experiments 1 and 2, respectively, and, in the latter study, again on day 70 by which time many of the less severely affected eyes were normal or near normal. The persistence of the organism for such a lengthy period is in agreement with other studies in which M.bovis was isolated sporadically for up to 70 to 83 days following infection (158,229,233).

The timing and development of clinical signs seen in the present study correlate well with those recorded in both experimental (18) and natural outbreaks of IBK (98,111). Since there has been confusion over whether conjunctivitis precedes keratitis or vice versa (18) it is interesting to note that conjunctivitis (as judged clinically) preceded keratitis in more than half of the

affected eyes and was noted concurrently in the remainder.

Mildly affected eyes recovered quickly with corneal lesions disappearing without vascularisation. On the other hand, lesions persisted in the severely affected eyes for a far longer period and vascularisation preceded healing in every instance. In many, a corneal scar was still present at the end of the experimental period. Lesions recurred in two of the mildly affected eyes nine and 14 days after the original lesions had healed and in both instances a third attack occurred.

In the above studies, lesions were successfully produced without recourse to UV irradiation or trauma and in the absence of other significant infectious agents. Thus it would appear that M.bovis was playing a primary role in the production of IBK. All 15 eyes that were inoculated became colonised and ocular lesions of varying severity subsequently developed in all save one. The lesions were such that a confident diagnosis of IBK was made in 12 of the 15 eyes.

SECTION B

TRANSMISSION STUDIES IN SUSCEPTIBLE CALVES

INTRODUCTION

In the following section, the effects of natural challenge are studied in two groups of experimental calves. In each instance infection was introduced by the artificial inoculation of a single eye of a single calf by either strain GS or GM of M.bovis. The spread of infection and development of disease is studied in each instance and comparisons between strains made.

MATERIALS AND METHODS

Experiment 4

Animals and housing

Five Hereford cross calves, approximately two months of age, consisting of four male calves and one female were used. The calves were acquired as a group from a commercial dairy unit, had a history of freedom from clinical ocular disease and were free from lesions. On admission the calves were allocated ear tag numbers 11 to 15 at random. Feeding and housing were as described in Chapter 2, Section A.

Sampling of animals

- Virology

Ocular (L,R) and nasopharyngeal swabs were taken at weekly intervals from days -10 to 25 and submitted for

the isolation and identification of viruses as described in Chapter 2, Section B.

- Mycoplasmology

Ocular (L,R) and nasopharyngeal swabs were taken at weekly intervals from days -10 to 25 and submitted for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Prior to challenge, swabs were taken from both left and right eyes and from the nasopharynx three times weekly for three weeks. Following challenge ocular (L,R) and nasopharyngeal swabs were taken daily until the calves were treated on day 28. Weekly ocular samples were processed for the isolation and identification of Gram-negative bacteria and all samples were submitted for the isolation of M.bovis as described in Chapter 2, Section D.

- Sera and lachrymal secretions

Serum samples and bilateral tear samples were collected on days -1, 14, 21 and 28. The lachrymal secretions were collected using the method described in Chapter 3, Section A (Materials and Methods, experiment 1). Antibody levels were measured using an IHA test as described in Chapter 2, Section E.

Clinical examination and scoring

Calves were examined daily from three weeks prior to inoculation until the group was treated with antibiotics

on day 28. All examinations were carried out and clinical scores allocated according to the methods described in Chapter 2, Section F.

Inoculation procedures

The inoculum was prepared using the same strain of M.bovis (GS) as that previously described in Chapter 3, Section A (Materials and Methods, experiment 2). It had been passaged six times on BAP prior to storage on BAP at -70°C.

The stored M.bovis plates were thawed on day -1 and inoculated onto fresh BAP and incubated at 35°C for 24 hours. The growth from one BAP was scraped off using a sterile inoculating loop, and suspended in 5ml of sPBS which was then gently agitated to break up the colonies.

Within 30 minutes of preparation, 0.5 ml of this inoculum was administered using the methods described in Chapter 3, Section A (Materials and Methods, experiment 1) to the left eye of a single calf chosen at random (calf 12). The inoculum was serially diluted in sPBS, 0.5 ml of each tenfold dilution was inoculated onto BAP and the concentration of bacteria in the inoculum was found to be 10^{13} CFU/ml.

Experiment 5

Animals and housing

Five conventional dairy-cross calves approximately two months of age were used. The calves were acquired as a group from a commercial dairy unit in the south-west of

Scotland with a history of freedom from clinical ocular disease and were free from ocular lesions. On admission the calves were randomly allocated ear tag numbers from 69 to 73. Feeding and housing were as described in Chapter 2, Section A.

Sampling of animals

- Virology

Ocular (L,R) and nasopharyngeal swabs were collected at seven day intervals from days -10 to 25 and submitted for the isolation and identification of viruses as described in Chapter 2, Section B.

- Mycoplasmology

Ocular (L,R) and nasopharyngeal swabs were taken at seven day intervals from days -10 to 25 and submitted for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Prior to challenge, swabs were taken from both left and right eyes and from the nasopharynx three times weekly for three weeks. Following challenge ocular (L,R) and nasopharyngeal swabs were taken daily until the calves were treated on day 28. Weekly ocular samples were processed for the isolation and identification of Gram-negative bacteria and all samples were submitted for the isolation of M.bovis as described in Chapter 2, Section D.

- Sera and lachrymal secretions

Serum samples and bilateral lachrymal secretions were collected on day -1. The lachrymal secretions were collected using the method described in Chapter 3, Section A (Materials and Methods, experiment 1). Antibody levels were measured using an IHA test as described in Chapter 2, Section E.

Clinical examination and scoring

The calves were examined daily from three weeks prior to challenge exposure on day 0, until day 28. All examinations were carried out and clinical scores allocated according to the methods described in Chapter 2, Section F.

Inoculation procedures

The inoculum was prepared from low passage cultures of the strain of M.bovis (GM) used previously in Chapter 3, Section A, experiment 3 and stored at -70°C on BAPs.

The stored plates were thawed on day -1, inoculated onto fresh BAP and incubated at 35°C for 24 hours. The growth from one BAP was scraped off using a sterile inoculating loop and suspended in 5 ml of sPBS, which was then gently agitated to break up the colonies.

Within 30 minutes of preparation, 0.5 ml of the inoculum was administered using the procedures described in Chapter 3, Section A (Materials and Methods, experiment 1) to the left eye of a single calf which was chosen at

random (calf 69). The inoculum was serially diluted in sterile 10% magnesium chloride and 0.5 ml of each tenfold dilution inoculated onto fresh BAP, incubated overnight and the concentration of bacteria found to be 10^{10} CFU/ml.

RESULTS

Experiment 4

Microbiology

- Virology

Bovine herpes virus 1, adenovirus or PI3 virus were not isolated from any of the swabs submitted for virological examination.

- Mycoplasmology

Mycoplasmas were isolated from ocular swabs on only four occasions, Myco.bovirhinis from both eyes of calves 3 and 1 on days 21 and 28, respectively. The results of isolations from nasopharyngeal swabs are given in table 19.

- Bacteriology

Other than M.bovis, the Gram-negative bacteria isolated from ocular samples were M.nonliquefaciens, M.lacunata, A.lwoffi, A.anitratus, M.catarrhalis and E.coli.

Moraxella bovis was not isolated from any of the ocular or nasopharyngeal swabs taken prior to challenge exposure. The results for M.bovis isolations from ocular and nasopharyngeal swabs collected between day 0 and day

ANIMAL SAMPLED	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) -7	0	7	14	21	28
1	Myco.bovirhinis	-	-	-	-	-	+
2	Myco.bovirhinis	-	-	+	-	+	-
	Myco.bovis	-	-	+	-	-	-
3	Myco.bovirhinis	-	-	+	-	-	+
	Myco.bovis	-	-	-	+	-	-
	A.laidlawii	-	-	+	-	-	-
4	Myco.bovirhinis	-	-	+	-	-	+
	Myco.dispar	-	-	-	-	+	-
5	Myco.bovis	-	-	+	+	-	-
	Myco.dispar	-	-	-	-	+	-
	A.laidlawii	-	-	-	+	-	-

+ Sample positive
- Sample negative

TABLE 19. Isolations of mycoplasmas from nasopharyngeal samples, experiment 4.

28 are illustrated in table 20. Excluding calf 12L, M.bovis was isolated from 113 of 252 (44.8%) ocular swabs collected from in-contact eyes between days 1 and 28. Five of 63 (7.9%) samples were positive for days 1-7, 30 of 63 (47.6%) for days 8-14, 20 of 63 (31.7%) for days 15-21 and 58 of 63 (92.1%) for days 22-28.

Moraxella bovis was isolated from 18 out of 140 (12.8%) nasopharyngeal swabs collected from day 1 to day 28. Two isolations were made from calf 14, five from calf 11, five from calf 12 and six from calf 15.

Immunological response

Antibody titres from lachrymal secretions are illustrated in table 21 and from serum samples in table 22.

From lachrymal secretions collected on day 0, significant antibody titres (ie.≥8) were present only in 15R. On day 28, significant titres were present in 12L and 15L, although significant titres were also present in 12R on day 21.

From serum samples, significant titres were not found in any samples collected on day 0. Significant titres were present in calf 11 on day 14, calf 12 on days 14, 21 and 28, calf 14 on day 21 and calf 15 on day 28.

Clinical features

A diagnosis of IBK was made in seven out of the ten eyes. Two of these were mildly affected (Appendix II; calves 11,12) and four, severely (Appendix II; calves 11,

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
12L*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+
12R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+
12NP	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-
11L	-	-	-	-	-	-	+	-	+	+	+	+	+	+	-	-	+	-	+	-	+	+	+	+	+	+	+	+
11R	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	+	-	+	+	+	+	+
11NP	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-	+
13L	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+
13R	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+
13NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14L	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+
14R	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+
14NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
15L	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
15R	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+
15NP	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-

L Left eye
R Right eye
NP Nasopharynx
* Calf 12 inoculated with M.bovis on day 0

\+ Sample positive
\- Sample negative

TABLE 20. Isolations of M.bovis from ocular and nasopharyngeal samples, experiment 4.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS) 0	14	21	28
11L	-	-	-	N
11R	-	-	N	N
12L	-	8	32	64
12R	-	N	32	-
13L	-	-	-	-
13R	-	-	-	-
14L	-	-	-	-
14R	-	-	-	-
15L	4	4	16	32
15R	16	32	2	8

L Left eye
R Right eye

\- No haemagglutination
N Haemagglutination in neat dilutions only

TABLE 21. Reciprocal IHA titres against whole cell M.bovis in lachrymal secretions, experiment 4.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS) 0	14	21	28
11	-	16	4	4
12	-	32	32	16
13	-	N	N	-
14	-	N	8	N
15	2	2	-	8

- No haemagglutination
N Haemagglutination in heat dilutions only

TABLE 22. Reciprocal IHA titres against whole cell M.bovis in serum samples, experiment 4.

13, 14, 15). The infection was terminated before the severity of the lesions could be judged in the remaining affected eye (Appendix II; calf 13).

Individual daily clinical scores for both left and right eyes for the calves in this group are presented in table 23. Mean scores (figure 16) remained low from day 1 to day 10 reflecting only one mild case of clinical IBK arising during this period but four new cases arose between days 11 and 13 resulting in a rapid increase in the mean score, which reached a peak of 7.0 on day 14. Thereafter, the mean score fluctuated slightly, reaching a low point of 5.5 on day 23 and a new peak of 8.5 on day 28. Over the entire 28 day period, the mean clinical score of all eyes in this group was 4.4.

- Incubation period

The times from inoculation of M.bovis to the left eye of calf 12, the transmission and establishment of infection, the development of disease and diagnosis of IBK are presented in table 24. The mean time to first isolation of M.bovis from in-contact calves was 9.8 days and to establishment of persistent infection, ie. three positive isolations out of four consecutive samples, was 14.0 days. Ocular lesions were first noted in six of nine in-contact eyes 13.8 days following challenge and IBK diagnosed in all six after 16.2 days.

	EXAMINATION TIMES (DAYS)																												
CALF	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
12L*	0	2	2	2	1	6	9	7	5	10	6	13	11	9	5	5	3	2	9	8	6	8	7	9	12	10	11	11	7
12R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	13
11L	0	0	0	0	0	0	0	0	0	1	4	12	16	16	15	14	14	14	14	14	15	11	13	13	14	12	14	14	14
11R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	10	4	6	1	0	12	14	13	13
13L	0	0	0	0	0	0	2	1	2	2	2	0	15	17	15	15	15	15	13	12	12	11	12	13	11	9	11	10	11
13R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14L	0	0	0	0	0	0	0	0	0	0	0	10	16	17	18	18	18	18	18	18	17	17	16	15	17	18	18	16	17
14R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15R	0	0	0	0	0	0	0	0	0	0	0	0	5	8	17	14	14	16	14	9	5	5	5	4	5	4	12	7	10

* Calf 12L inoculated with M.bovis on day 0

L Left eye
R Right eye

TABLE 23. Clinical scores from day 0 to day 28, experiment 4.

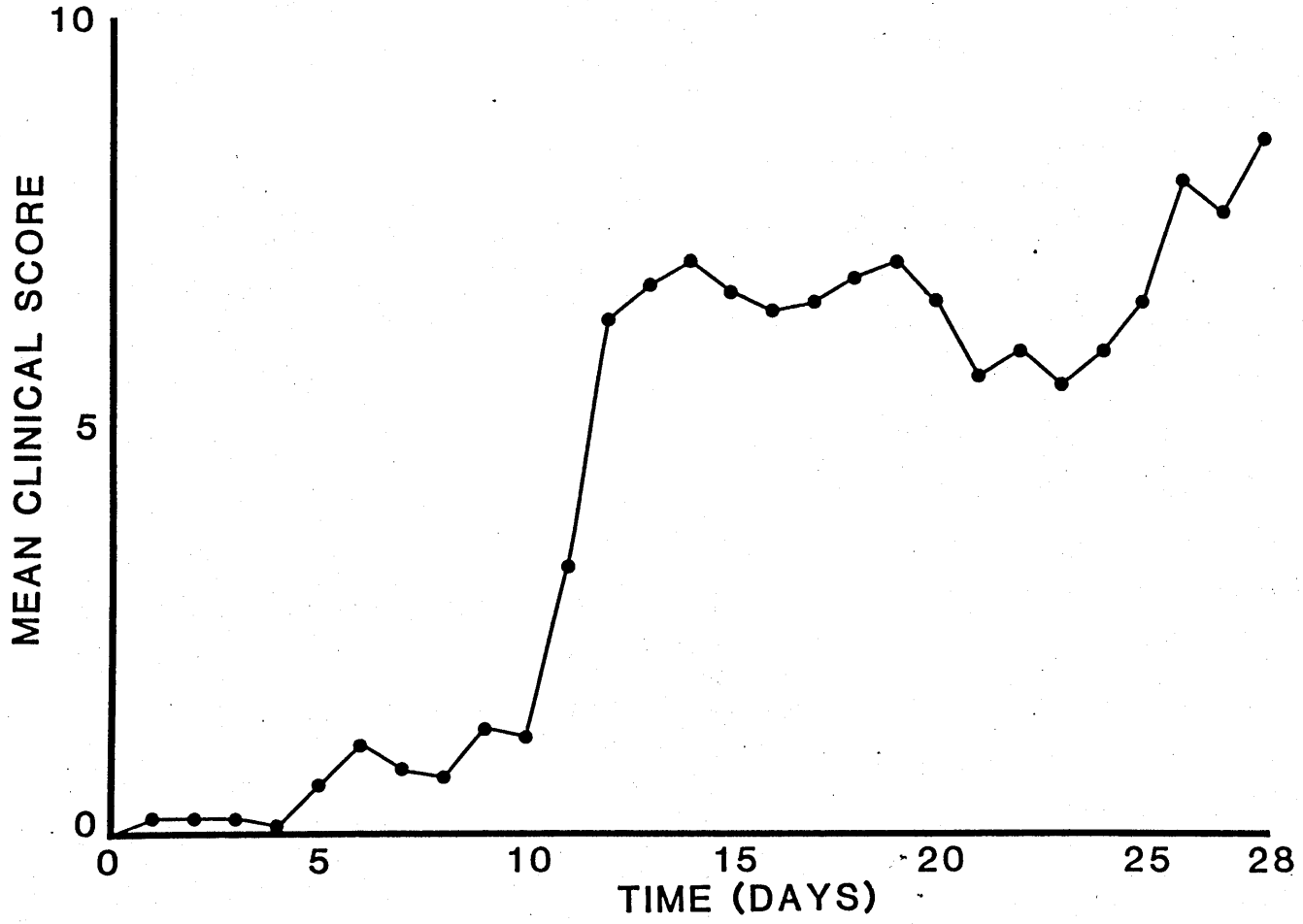


FIGURE 16. Experiment 4. Mean clinical scores following the instillation of M.bovis strain GS into the left eye of calf 12 on Day 0.

CALF IDENTIFICATION	12		11		13		14		15	
	L	R	L	R	L	R	L	R	L	R
Day of first isolation of M.bovis	1	20	7	14	2	9	9	10	8	9
Day persistent infection established	1	20	7	14	5	26	14	10	21	9
Day signs of disease first noted	1	27	8	19	6	-	11	-	-	12
Day IBK diagnosed	5	28	11	20	12	-	12	-	-	14

L Left eye
R Right eye

TABLE 24. Time (days post inoculation) to transmission and establishment of infection, development of clinical signs and diagnosis of IBK, experiment 4.

Experiment 5

Microbiology

- Virology

Bovine herpes virus 1, adenovirus or PI3 were not isolated from any of the ocular or nasopharyngeal swabs submitted for virological examination.

- Mycoplasmology

Mycoplasmas were isolated from four of the ocular swabs submitted for isolation and identification. On day -3 Myco.bovirhinis was isolated from the right eye of calf 69 and A.laidlawii was isolated from the left eyes of calves 69 and 71 and on day 25 Myco.bovirhinis was isolated from the right eye of calf 71. The results for isolation of mycoplasmas from nasopharyngeal swabs are illustrated in table 25.

- Bacteriology

Other than M.bovis, the Gram-negative bacteria isolated from ocular samples were M.nonliquefaciens, M.lacunata, A.lwoffi, A.anitratus, M.catarrhalis and E.coli.

Moraxella bovis was not isolated from ocular or nasopharyngeal swabs taken prior to challenge exposure. The results for M.bovis isolations from ocular and nasopharyngeal swabs collected between days 0 and 28 are illustrated in table 26. Moraxella bovis was isolated from 34 of 180 (18.9%) ocular swabs collected from in-contact eyes between days 1 and 28. One of 63 (1.6%)

ANIMAL SAMPLED	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) -3	4	11	18	25
69	Myco.bovis	-	-	-	+	+
70	A.laidlawii	-	-	+	-	-
	Myco.bovis	-	-	-	+	-
	Myco.bovirhinis	-	-	-	-	+
71	A.laidlawii	-	-	+	-	-
72	Myco.bovirhinis	-	-	-	+	-
73	Myco.bovirhinis	+	-	-	+	-

+ Sample positive
- Sample negative

TABLE 25. Isolations of mycoplasmas from nasopharyngeal samples, experiment 5.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)																				
	0	1	2	3	4	5	6	7	8	9	11	12	13	14	16	18	20	22	24	26	28
69L*	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
69R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
69NP	-	-	-	-	-	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	+
70L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
70R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
70NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
71L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
71R	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
71NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
72L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-
72R	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
72NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
73L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
73R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
73NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

L Left eye
R Right eye
NP Nasopharynx
* Calf 69L inoculated with M.bovis on day 0

\+ Sample positive
\- Sample negative

TABLE 26. Isolations of M.bovis from ocular and nasopharyngeal samples, experiment 5.

samples were positive for days 1-7, one of 54 (1.9%) for days 8-14, eight of 27 (29.6%) for days 15-21 and 24 of 36 (66.7%) for days 22-28.

Moraxella bovis was isolated from ten of 100 (10%) nasopharyngeal swabs collected from day 1 to 28, six positive isolations were made from calf 69, on days 5, 7, 11, 13, 14 and 28, two from calf 70 on days 26 and 28, one from calf 71 on day 28 and one from calf 73 on day 20.

Immunological status

All calves were shown to have negative serum and lachrymal titres immediately prior to infection on day 0.

Clinical features

In this group of calves, IBK was diagnosed in five of ten eyes and only one was severely affected (Appendix II; calf 73). Three were mildly affected (Appendix II; calves 69, 70, 72) and in one eye (Appendix II; calf 72) the infection was terminated before severity of the lesions could be judged. Four of the remaining eyes (Appendix II; calves 69, 70, 71) demonstrated periods of mild ocular irritation although these were frequently during periods when M.bovis was not isolated.

Individual daily clinical scores for both left and right eyes for the calves in this group are given in table 27. Mean scores (figure 17) remained low until day 15 before rising to a peak of 2.0 on day 19. Mean scores thereafter fluctuated between 1.2 and 2.0 between day 19

	EXAMINATION TIMES (DAYS)																												
CALF	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
69L*	0	0	0	0	0	0	0	7	0	2	0	0	0	0	0	0	10	8	5	4	3	3	1	0	0	0	0	0	2
69R	0	0	0	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	2	2	1	0	0	0	0	1	0
70L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	0	0	0	11	6	5	2	0
70R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0
71L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	4	0	0	2	0	0	0	0
71R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	5	8	8	4	0	0	4
72L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	3	2	1	1	0	4	0	0	0	0	8	10
72R	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	2	5	7	12	11	10	9	8	7	4	4	1	3
73L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
73R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	13	13	14	14	14	14	12	14	13	13

L Left eye
R Right eye
* Calf 69L inoculated with M.bovis on day 0

TABLE 27. Clinical scores from day 0 to day 28, experiment 5.

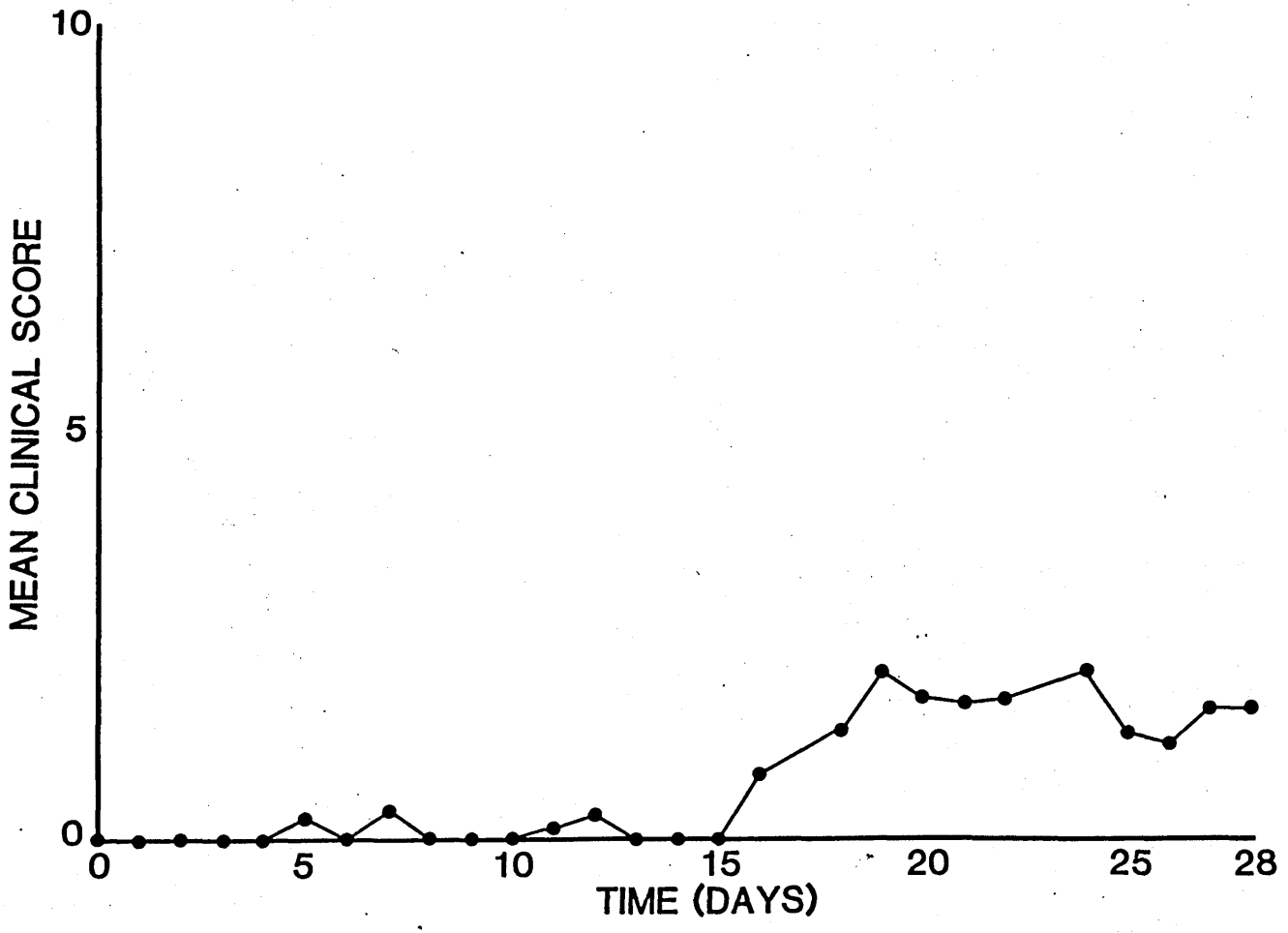


FIGURE 17. Experiment 5. Mean clinical scores following the instillation of M.bovis strain GM into the left conjunctival sac of calf 69 on Day 0.

and 28. Over the entire 28 day period, the mean score of all nine in-contact eyes in this group was 0.7.

- Incubation period

The times from inoculation of M.bovis to the left eye of calf 69, the transmission and establishment of infection, the establishment of disease and diagnosis of IBK are tabulated in table 28. Mean time to first isolation of M.bovis from in-contact eyes was 17.6 days and to establishment of persistent infection, 20.6 days. Moraxella bovis was never isolated from one eye and infection did not become established in another two. In the five affected eyes, mean time to development of ocular lesions following initial infection was 21.8 and a confident diagnosis of IBK was possible after 22.0 days.

DISCUSSION

The above investigations have clearly demonstrated the successful transmission of M.bovis from an experimentally infected calf to susceptible in-contact animals. The morbidity of the disease produced was similar to that reported in previous experimental infections which have relied upon the use of exceptionally high challenge doses of bacteria and predisposing factors such as UV irradiation (105,106,119). Furthermore the severity of disease produced was comparable to that reported in previous experiments using these strains.

Despite the use of large numbers of organisms to infect the two inoculated eyes, corneal lesions did not

CALF IDENTIFICATION	69		70		71		72		73	
	L	R	L	R	L	R	L	R	L	R
Day of first isolation of M.bovis	1	-	20	24	20	14	18	5	20	20
Day persistent infection established	1	-	20	24	-	14	22	24	20	20
Day signs of disease first noted	9	-	24	-	-	-	27	18	-	18
Day IBK first diagnosed	16	-	24	-	-	-	28	18	-	18

L Left eye
R Right eye

TABLE 28. Time (days post inoculation) to transmission and establishment of infection, development of clinical signs and diagnosis of IBK.

develop immediately. Strain GS produced mild conjunctivitis and epiphora only for a four day period following challenge while corneal changes were first noted on day 5. The other strain used (GM) did not produce signs for 11 days following challenge and corneal lesions were not noted until day 20. In both instances, this was despite early establishment and persistence of infection. Such a situation might suggest that preformed toxins had little pathogenic effect and that disease in both cases was produced following a progressive build up of infection.

Similar patterns of infection were displayed by both groups of calves although differences were noted in the timing of events. Transmission of infection was initially slow in both groups. Infection did not become established in any in-contact eyes until day 5 (strain GS) or day 14 (strain GM) following which only one or two ocular samples were positive for M.bovis from each set of samples collected. However, in both groups there was a sudden increase in the proportion of samples positive for M.bovis, on days 9 and 16 in experiments 4 and 5, respectively. This coincided with a general rise in mean clinical score and in the number of eyes affected with epiphora which may reflect the importance of the latter as a source of infection to in-contact animals. In addition, this provides a possible explanation for the difference in transmission times displayed by the two groups.

In the present studies, transmission of M.bovis between calves during handling was minimised by examining the artificially infected calves last. Moraxella bovis was occasionally isolated from the nasopharynx, leading to the possibility of transmission being accomplished due to droplet infection following coughing. However, transmission of infection was found to occur only after the development of marked epiphora despite earlier isolations from the nasopharynx.

Spread of infection from the upper respiratory tract to the eye via the nasopharyngeal duct is a theoretical possibility. However, this is unlikely as M.bovis is regarded as being non-motile by conventional tests (48) and would thus probably be unable to ascend the nasolachrymal duct against the normal flow of tears. This view is supported by the results of isolations from calves 12 and 69 in which M.bovis was not isolated from the right eyes until after other calves had been infected and despite positive isolations from the nasopharynx.

The role of flies, particularly the face fly, M.autumnalis, has been extensively studied and these flies have been implicated in transmission and pathogenesis of disease (11,12,32,46,72,207). In fact, while small numbers of unidentified flies were present in the animal accommodation it is unlikely that they were involved in transmission of infection as they were seen feeding only on dung and were not seen around or on the faces of the calves at any time.

The incubation period of the disease was found to be relatively short, with an average of 4.6 days between first isolation of M.bovis and the development of signs of ocular irritation. This is shorter than that recorded by Wilcox (233). Corneal changes were noted concurrently or up to seven days after initial development of irritation. This, combined with the rapid transmission of infection, produced a sudden outbreak of IBK within both groups with a morbidity similar to that seen in field incidents.

SECTION C

RESULTS OBTAINED FOLLOWING TREATMENT AND REINFECTION WITH M.BOVIS

INTRODUCTION

In this section will be described the effects of two commonly adopted therapeutic procedures, subconjunctival administration of oxytetracycline hydrochloride and the topical application of cloxacillin benzathine formulated in an ophthalmic base. Furthermore, the effect of subsequent inoculation with a pathogenic strain of M.bovis (GS) to animals previously exposed to homologous and heterologous strains will be described.

MATERIALS AND METHODS

Experiment 6

Experimental animals

The five calves used in this experiment were those described previously in Chapter 3, Section B (Materials and Methods, experiment 4). Their previous history, up to day 28, of ocular disease and M.bovis (GS) isolations is as described in Chapter 3, Section B (Results, experiment 4).

Sampling of animals

- Virology

Prior to treatment, nasopharyngeal and ocular swabs were collected as described in Chapter 3, Section B (Materials and Methods, experiment 4). Ocular and

nasopharyngeal swabs were further collected 7, 14 and 21 days following initial treatment. All samples were processed for the isolation and identification of viruses as described in Chapter 2, Section B.

- Mycoplasmology

Prior to treatment, nasopharyngeal and ocular swabs were collected as described in Chapter 3, Section B (Materials and Methods, experiment 4). Ocular and nasopharyngeal swabs were further collected on days 35, 42 and 49. All samples were processed for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Prior to treatment, nasopharyngeal and ocular swabs were collected at the times described in Chapter 3, Section B (Materials and Methods, experiment 4). Bilateral ocular and nasopharyngeal swabs were collected daily from days 29 to 36, every two days from days 36 to 63, daily from days 63 to 72 and every two days until day 80. All samples were processed for the isolation and identification of Gram-negative bacteria and of M.bovis as described in Chapter 2, Section E.

- Sera and lachrymal secretions

Bilateral tear and serum samples were collected from all animals on days 0, 14, 28 and 65 using the methods described in Chapter 3, Section A (Materials and Methods, experiment 1). Samples were stored at -20°C and antibody

response was measured using an IHA test as described in Chapter 2, Section F.

Clinical examination and scoring

Prior to treatment, examination was carried out at the periods described in Chapter 3, Section B (Materials and Methods, experiment 4). Following treatment, the eyes were examined daily for a period of 14 days and from days 63 to 80. All examinations were carried out and clinical scores allocated as described in Chapter 2, Section F.

Treatment

All ten eyes in this group were treated on four occasions. Initial treatment was administered on day 28 by subconjunctival injection into the upper eyelid of 0.5 ml of a 5% solution of oxytetracycline hydrochloride (Engemycin 5%; Gist-Brocades Animal Health, Braintree, Essex, UK). This treatment was repeated three days later. On days 35 and 37, all eyes were further treated using a proprietary solution of 16.7% W/W cloxacillin benzathine, suspended in an ophthalmic base containing 3% aluminium stearate (Orbenin ophthalmic ointment; Beecham Animal Health, Brentford, Middlesex, UK). Approximately half of a tube (1.5ml) was administered evenly into the dorsal and ventral conjunctival sacs.

Antibiotic sensitivities

Three isolates of M.bovis, one collected prior to initial treatment, the second following initial treatment and the third following the second treatment dose, were

tested for sensitivity to the oxytetracycline hydrochloride suspension used in treatment.

BAPs were made up containing the following dilutions of the oxytetracycline hydrochloride suspension that was used.

Plate 0	-	No antibiotic
Plate 1	-	10^{-8}
Plate 2	-	$5x10^{-9}$
Plate 3	-	10^{-9}
Plate 4	-	$5x10^{-10}$
Plate 5	-	10^{-10}

A single colony of each isolate tested was suspended in 5 ml of sPBS and 0.5 ml of each suspension was placed in triplicate on each dilution of antibiotic impregnated BAP, incubated aerobically at 35°C for 24 hours and examined for the growth of M.bovis.

Reinfection with homologous strain M.bovis (GS)

The five calves in this group were challenged on day 63 using strain GS of M.bovis which was a subculture of the isolate used for initial challenge in this group as described in Chapter 3, Section B (Materials and Methods, experiment 4). This low passage strain had been stored on BAP at -70°C prior to use.

The inoculum was prepared and administered to the left eyes of all the calves in this group and the dose of M.bovis inoculated using the methods described in Chapter 3, Section A (Materials and Methods, experiment 1). The inoculum was calculated to contain 10^6 CFU/ml.

Experiment 7

Experimental animals

The five calves used in this experiment were those described previously in Chapter 3, Section A (Materials and Methods, experiment 3). Their history of ocular disease and M.bovis (GM) isolations up to day 28 are as described in Chapter 3, Section A (Results, experiment 3).

Sampling of animals

- Virology

Prior to treatment, nasopharyngeal and ocular swabs were collected as described in Chapter 3, Section A (Materials and Methods, experiment 3). Ocular and nasopharyngeal swabs were further collected on days 32, 39 and 46. All samples were processed from the isolation and identification of viruses as described in Chapter 2, Section B.

- Mycoplasmology

Prior to treatment, nasopharyngeal and ocular swabs were collected as described in Chapter 3, Section A (Materials and Methods, experiment 3). Ocular and nasopharyngeal swabs were collected on days 32, 39 and 46. All samples were processed for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Prior to treatment, nasopharyngeal and ocular swabs were collected as described in Chapter 3, Section A

(Materials and Methods, experiment 3). Bilateral ocular and nasopharyngeal swabs were further collected daily for 12 days following initial treatment. All samples were processed for the isolation and identification of Gram-negative bacteria and M.bovis as described in Chapter 2, Section D.

- Sera and lachrymal secretions

Bilateral tear and serum samples were collected from all animals on days 0, 14, 28 and 56 using the methods described in Chapter 3, Section A (Materials and Methods, experiment 1). Samples were stored at -20° and antibody response was measured using an IHA test as described in Chapter 2, Section E.

Clinical examination and scoring

Prior to treatment examination was carried out at the periods described in Chapter 3, Section A (Materials and Methods, experiment 3). Following treatment the eyes were examined daily for a period of 12 days. All examinations were carried out and clinical scores allocated as described in Chapter 2, Section F.

Treatment

All ten eyes in this group were treated on days 28 and 31. Treatment was by instillation into the dorsal and ventral conjunctival sac of a proprietary ophthalmic ointment containing 16.7% W/W cloxacillin benzathine suspended in an ophthalmic base containing 3% aluminium stearate (Orbenin Ophthalmic ointment, Beecham Animal

Health, Brentford, Middlesex, UK). Approximately half of a tube (1.5ml) was administered to each eye.

Reinfection with heterologous strain M.bovis (GS)

The calves in this group were challenged on day 40 using strain GS of M.bovis which was a subculture of the isolate used for reinfection in experiment 6. This low passage strain had been stored on BAP at -70°C prior to use. The inoculum was prepared and administered to the left eyes of all the calves in this group and the dose of M.bovis calculated using the methods described in Chapter 3, Section A (Materials and Methods, experiment 3). The inoculum was found to contain 10^9 CFU/ml.

RESULTS

Experiment 6

Microbiology

- Virology

Viruses were not isolated from any of the ocular or nasopharyngeal swabs submitted for virological examination.

- Mycoplasmology

The results of mycoplasma isolations from ocular and nasopharyngeal swabs taken prior to initial treatment on day 28 are given in Chapter 3, Section B (Results, experiment 4). Isolations of mycoplasmas from ocular and nasopharyngeal swabs collected on days 35, 42 and 49 are illustrated in table 29. Mycoplasmas were isolated from

ANIMAL SAMPLED	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) AND SOURCE								
		35			42			49		
		L	R	NP	L	R	NP	L	R	NP
11	Myco.bovirhinis	+	+	+	-	-	+	-	-	+
	A.laidlawii	-	+	-	+	+	+	+	+	-
12	Myco.bovirhinis	-	-	+	-	-	+	-	-	+
	Myco.bovis	-	-	-	+	-	+	-	-	-
	A.laidlawii	-	+	+	-	+	-	+	+	-
13	Myco.bovirhinis	-	-	-	-	-	+	-	-	-
	A.laidlawii	-	+	+	-	+	+	-	+	+
14	Myco.bovirhinis	-	-	+	-	-	+	+	-	+
	A.laidlawii	+	+	-	+	+	-	-	-	-
15	Myco.bovirhinis	-	-	+	-	-	+	-	-	-
	Myco.bovis	-	-	-	-	-	-	-	-	+
	A.laidlawii	+	+	+	+	+	-	-	-	-

L Left eye
R Right eye
NP Nasopharynx

\+ Sample positive
\- Sample negative

TABLE 29. Isolations of mycoplasmas from ocular and nasopharyngeal samples, experiment 6.

four out of 30 (13.3%) ocular swabs collected on days 14, 21 and 28 and from 23 out of 30 (76.7%) collected on days 35, 42 and 49. Acholeplasma laidlawii was isolated on 20 occasions following treatment, Myco.bovirhinis on three and Myco.bovis on one.

- Bacteriology

Significant Gram-negative bacteria, other than M.bovis were not isolated from any of the samples examined.

The results from samples submitted for examination for M.bovis prior to treatment are given in Chapter 3, Section A (Results, experiment 3). The results of M.bovis isolations for samples collected between day 28 and day 42 are given in table 30. All samples between days 42 and 60 were negative for M.bovis.

Immunological response

The results from examination of lachrymal secretions are illustrated in table 31 and those from serum are illustrated in table 32. On day 28, significant titres of antibody were present in lachrymal secretions from 12L and 15L,R. On day 70, significant titres were present in a further four eyes. Significant titres were present in two of five serum samples collected on day 28 and all animals had seroconverted by day 70.

Clinical features

On day 28, seven of the ten eyes were affected by corneal lesions and signs of ocular irritation. Following initial treatment, signs of ocular irritation decreased in

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)													
	28[o]	29	30	31[o]	32	33	34	35[c]	36	37[c]	38	39	41	43-60
11L	+	-	-	-	-	-	-	-	-	ND		-	-	-
11R	+	+	+	+	+	-	-	-	-	ND	-	-	-	-
11NP	+	-	-	-	-	-	-	-	-	ND	-	-	-	-
12L	+	-	-	+	-	-	-	-	-	ND	-	-	-	-
12R	+	-	+	+	+	+	+	-	-	ND	-	-	+	-
12NP	-	-	-	-	-	-	-	-	-		-	-	-	-
13L	+	-	+	+	-	-	+	+	-	ND	-	-	-	-
13R	+	-	-	+	-	+	-	+	-	ND	-	-	-	-
13NP	-	-	-	+	-	-	-	-	-	ND	-	-	-	-
14L	+	-	+	+	-	+	-	-	-	ND	-	-	-	-
14R	+	+	-	-	-	-	-	-	-	ND	-	-	-	-
14NP	-	-	-	-	-	-	-	-	-	ND	-	-	-	-
15L	+	+	+	+	+	+	+	-	-	ND	-	-	-	-
15R	+	+	+	-	+	+	+	-	-	ND	-	-	-	-
15NP	-	-	-	-	-	-	-	-	-	ND	-	-	-	-

L Left eye
R Right eye
NP Nasopharynx
o All eyes treated with oxytetracycline hydrochloride
c All eyes treated with cloxacillin benzathine

\+ Sample positive
\- Sample negative
ND Not sampled

TABLE 30. Isolations of M.bovis from ocular and nasopharyngeal samples, experiment 6.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS) 0	14	21	28	70
11L	-	-	-	N	16
11R	-	-	N	N	64
12L	-	8	32	64	8
12R	-	N	32	-	32
13L	-	-	-	-	32
13R	-	-	-	-	32
14L	-	-	-	-	2
14R	-	-	-	-	N
15L	4	4	16	32	8
15R	16	32	2	8	64

L Left eye
R Right eye

\- No haemagglutination
N Haemagglutination in neat dilutions only

TABLE 31. Reciprocal IHA titres against whole cell M.bovis in lachrymal secretions, experiment 6.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)				
	0	14	21	28	70
11	-	16	4	4	16
12	-	32	32	16	16
13	-	N	N	-	16
14	-	N	8	N	8
15	2	2	-	8	8

- No haemagglutination
N Haemagglutination in neat dilutions only

TABLE 32. Reciprocal IHA titres against whole cell M.bovis in serum samples, experiment 6.

intensity and number and had resolved in four of the seven eyes (Appendix III; calves 11, 12, 15) by day 31 although corneal lesions persisted. In two of the remaining affected eyes (Appendix III; calves 13, 14), signs of irritation had resolved within three days of the second treatment on day 31. There was no clinical response to treatment in one of the affected eyes (Appendix III; calf 12R) and in two healthy eyes (Appendix III; calf 13) lesions developed subsequent to treatment, on days 31 and 34.

Individual daily mean clinical scores for all ten eyes in this group for the period from five days prior to treatment (day 23) to 14 days following initial treatment (day 42) are illustrated in table 33.

In the five days prior to treatment, the mean clinical score for all eyes (figure 18) rose from a level of 5.5 on day 23 to a level of 8.5 on day 28. Following treatment with oxytetracycline hydrochloride the mean clinical score fell to 3.3 on day 30, rising to 6.5 prior to second treatment on day 31. The score declined following second treatment, over a period of three days, to a mean of 3.1 on day 34 and remained at about that level until day 42.

Antibiotic sensitivities

No differences in antibiotic sensitivity to oxytetracycline hydrochloride were noted between pre-treatment, post-initial treatment and post-second treatment samples of M.bovis, all of which were highly

	EXAMINATION TIMES (DAYS)																					
CALF	23	24	25	26	27	28[o]	29	30	31[o]	32	33	34	35[c]	36	37[c]	38	39	40	41	42	43	44
11L	13	14	12	14	14	14	9	5	4	4	4	4	4	3	2	2	2	2	2	2	2	2
11R	1	o	12	14	13	13	8	4	2	2	3	3	2	1	1	1	1	1	1	1	1	1
12L	9	12	10	11	11	7	5	4	8	5	2	4	2	4	5	3	2	2	1	1	1	2
12R	1	o	o	1	5	13	13	15	14	14	16	14	15	15	16	16	15	15	14	15	15	15
13L	13	11	9	11	10	11	10	7	11	8	6	5	3	3	2	2	2	1	1	3	4	1
13R	o	o	o	o	2	2	o	o	9	10	10	7	4	1	1	2	o	o	o	4	2	o
14L	15	17	10	18	16	17	14	11	10	9	8	6	8	6	6	5	5	6	6	5	5	5
14R	o	o	o	2	2	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	1
15L	o	o	o	o	o	1	o	o	o	o	o	10	13	12	13	13	12	11	10	12	6	4
15R	4	5	4	12	7	10	4	4	7	5	5	3	2	2	2	1	1	1	1	1	1	1

L Left eye
R Right eye
o All eyes treated with oxytetracycline hydrochloride
c All eyes treated with cloxacillin benzathine

TABLE 33. Clinical scores for days 23 to day 44, experiment 6.

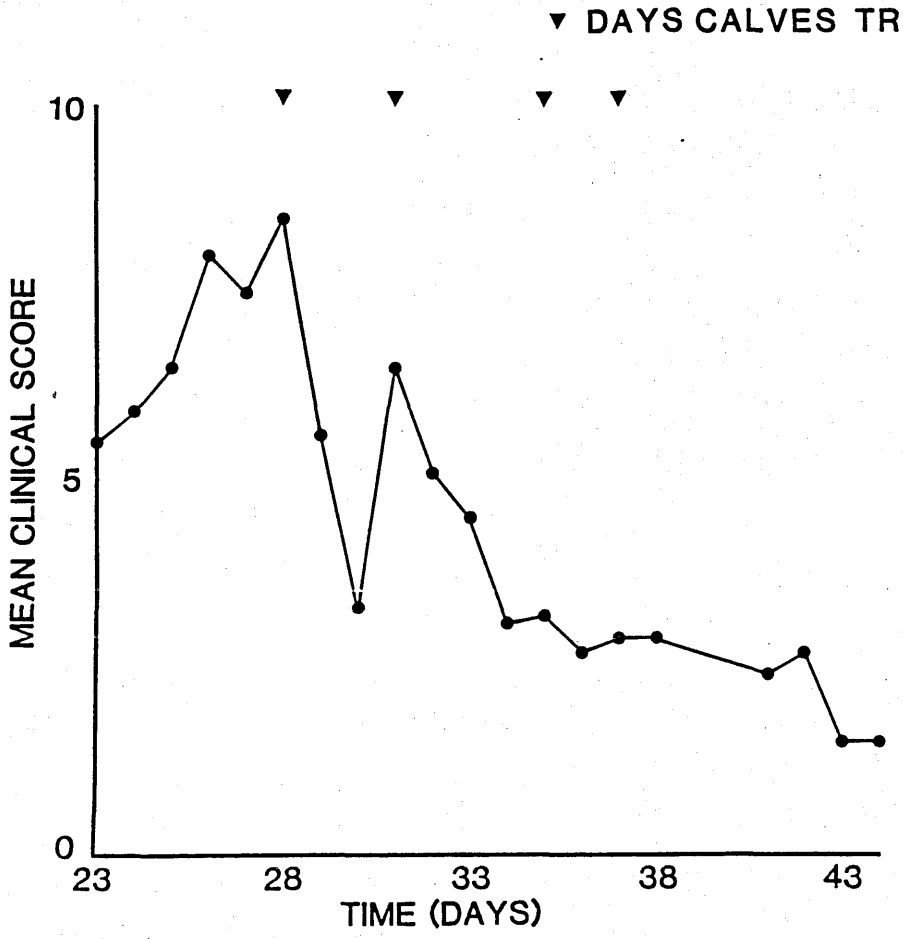


FIGURE 18. Experiment 6. The effect of treatment with subconjunctival oxytetracycline hydrochloride on days 28 and 31 and topical cloxacillin benzathine on days 35 and 37 on the mean clinical scores of calves affected by IBK.

sensitive to this antibiotic. In all three instances Growth inhibition was noted at identical dilutions of $5x10^{-9}$ of the original solution

Results following reinfection

- Moraxella bovis isolations

The results of M.bovis isolations from ocular and nasopharyngeal samples collected following reinfection on day 63 are illustrated in table 34.

Moraxella bovis was isolated from 20 of 65 (30.8%) samples collected from the left eyes up to day 80 and one of 65 (1.5%) from the right eyes. Although the bacterium was isolated from all left eyes, infection became established in only 11L and 13L. Moraxella bovis was not isolated from any of the nasopharyngeal samples collected.

- Clinical features

Prior to reinfection, all ten eyes were clinically normal apart from the presence of milky white corneal opacities at the sites of healed corneal ulcers in the eyes which had been most severely affected. Vascularisation of the cornea was not visible in any of the eyes.

All eyes remained clinically healthy following reinfection from day 63 to day 70 and thereafter only one eye developed lesions. On day 71, epiphora, conjunctivitis, blepharospasm, increased blinking and iridospasm were noted in the left eye of calf 11 and on day 72, a 2mm vesicle had developed on the lateral aspect of the cornea.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)												
	63*	64	65	66	67	69	70	71	72	75	76	78	80
11L	+	-	+	+	+	-	+	+	+	+	+	+	+
11R	-	-	-	-	-	-	-	-	-	-	-	-	-
11NP	-	-	-	-	-	-	-	-	-	-	-	-	-
12L	+	-	-	-	-	-	-	-	-	-	-	-	-
12R	-	-	-	-	-	-	-	-	-	-	-	-	-
12NP	-	-	-	-	-	-	-	-	-	-	-	-	-
13L	+	-	-	-	-	-	+	+	+	+	+	-	-
13R	-	-	-	-	-	-	-	-	-	-	-	-	-
13NP	-	-	-	-	-	-	-	-	-	-	-	-	-
14L	+	-	-	-	-	-	-	-	-	-	-	-	-
14R	+	-	-	-	-	-	-	-	-	-	-	-	-
14NP	-	-	-	-	-	-	-	-	-	-	-	-	-
15L	+	-	-	-	-	-	-	-	-	-	-	-	-
15R	-	-	-	-	-	-	-	-	-	-	-	-	-
15NP	-	-	-	-	-	-	-	-	-	-	-	-	-

L Left eye
R Right eye
NP Nasopharynx

\+ Sample positive
\- Sample negative
* Samples collected 2 hours post-infection

TABLE 34. Isolations of M.bovis from ocular and nasopharyngeal samples following homologous reinfection, experiment 6.

By day 73, extensive vascularisation of the medial and anterior aspects of the cornea was visible with a narrow rim of neovascularisation present at the lateral corneoscleral junction. The capillary beds encroached upon the non-vascularised areas of the cornea at a rate of about 1mm per day, reached the ulcer edge by day 78 and had infiltrated 2mm under the base of the ulcer by day 80 at which time there were still signs of moderate irritation.

The clinical scores following reinfection on day 63 are given in table 35. In the left eyes individual daily score remained low, reaching a peak of 3.0 on day 76. In the right eyes, the individual daily mean score was zero at all times.

Experiment 7

Microbiology

- Virology

No viruses were isolated from any of the ocular or nasopharyngeal samples collected and submitted for virological examination.

- Mycoplasmology

The results of mycoplasma isolations from ocular and nasopharyngeal swabs taken prior to initial treatment on day 28 are given in Chapter 3, Section A (Results, experiment 3). No mycoplasmas were isolated from any of the ocular or nasopharyngeal swabs collected following day 28.

	EXAMINATION TIMES (DAYS)																	
CALF	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
11L	0	0	0	0	0	0	0	0	8	10	11	11	12	15	14	14	14	14
11R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

L Left eye
R Right eye

TABLE 35. Clinical scores following homologous reinfection, experiment 6.

- Bacteriology

Ocular pathogens, other than M.bovis, were not isolated from any of the samples examined.

The results for samples submitted for examination for M.bovis prior to treatment are given in Chapter 3, Section A (Results, experiment 3). On day 28, M.bovis was isolated from all ocular swabs collected prior to treatment but was not isolated from any of the ocular and nasopharyngeal samples collected following treatment until reinfection on day 40.

Immunological response

The results from examination of lachrymal secretions are illustrated in table 36 and those from serum are illustrated in table 37.

On day 28, significant antibody titres were present in lachrymal secretions from one calf, 41L,R, and by day 54, increased titres were also present in another, 42L,R.

Significant serum titres were not present in any calves on day 28 although by day 54, a total of four calves, 41, 42, 44 and 45, had seroconverted.

Clinical features

On day 28, five eyes were affected by mild corneal ulceration accompanied by mild signs of ocular irritation with clinical scores ranging from five to 11 in the affected eyes. All eyes were found to be completely free from signs of ocular irritation by day 29 and in all cases,

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS) -1	14	28	54
41L	-	-	16	128
41R	-	-	16	32
42L	-	-	-	16
42R	-	-	2	8
43L	-	-	-	-
43R	-	-	-	-
44L	-	-	-	-
44R	-	-	-	-
45L	-	-	-	-
45R	-	-	-	-

L Left eye
R Right eye

\- No haemagglutination
N Haemagglutination in near dilution only

TABLE 36. Reciprocal IHA titres against whole cell M.bovis in lachrymal secretions, experiment 7.

SOURCE OF SAMPLE	-1	14	28	54
41	-	-	2	16
42	-	-	2	64
43	2	-	4	2
44	-	-	2	8
45	-	-	2	8

- No haemagglutination
N Haemagglutination in neat dilutions only

TABLE 37. Reciprocal IHA titres against whole cell M.bovis in serum samples, experiment 7.

corneal lesions resolved rapidly leaving small facets in the corneal surface at the sites of the corneal ulcer by day 30. By day 40 the eyes appeared completely healthy and facets were not visible.

Individual daily clinical scores for this group prior to treatment are given in table 17, Chapter 3, Section A (Results, experiment 3). The mean clinical score increased from 2.6 on day 23 to 4.4 on day 28 and, following treatment, fell to zero by day 29.

Results following re-infection

- Moraxella bovis isolations

The results of M.bovis isolations from ocular and nasopharyngeal swabs following reinfection on day 40 are given in table 38.

Moraxella bovis became established in all five left eyes and the right eye of calf 44 and was still present in those eyes on day 54. The bacterium was isolated from 31 out of 35 (88.6%) samples collected from the left eyes, from 7 (20%) samples collected from right eyes and from 6 (17.1%) samples collected from the nasopharynx.

- Clinical features

All ten eyes were clinically normal immediately prior to reinfection on day 40. Following reinfection signs of irritation and corneal ulceration were noted in all five left eyes (Appendix III; calves 41, 42, 43, 44, 45). Irritation was first noted on day 41 in four and on

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)							
	40	41	43	45	47	49	51	54
41L	-	+	+	+	+	+	+	+
41R	-	-	-	-	-	-	-	-
41NP	-	-	-	-	-	-	-	+
42L	-	+	+	+	+	-	+	+
42R	-	-	-	-	-	-	-	-
42NP	-	-	-	+	-	-	-	+
43L	-	-	+	+	+	+	+	+
43R	-	-	-	-	-	-	-	-
43NP	-	-	-	+	-	-	-	-
44L	-	-	-	-	+	+	+	+
44R	-	-	-	+	+	+	+	+
44NP	-	-	-	-	-	+	-	-
45L	-	+	+	+	+	+	+	+
45R	-	-	-	+	-	-	-	-
45NP	-	-	-	-	-	-	-	+

L Left eye
R Right eye
NP Nasopharynx

\+ Sample positive
\- Sample negative

TABLE 38. Isolations of M.bovis from ocular and nasopharyngeal samples following heterologous reinfection, experiment 7.

day 46 in the remaining eye and corneal changes were first noted on days 41 in three eyes, 45 in one and 50 in one. All lesions were mild, resolving in under five days in all but one eye; recurrent lesions were noted in 45L on one occasion and 43L on two occasions. Widespread, tenuous vascularisation of half of the corneal area was noted in 45L on day 46 which was absent by day 50.

Small corneal opacities were noted in the right eyes of calves 41, 42, 43 and 44 at the sites of previous corneal ulceration and in the absence of irritation.

The clinical scores for this group from day 40 to day 54 are given in table 39. Daily mean scores for the left eyes rose from zero on day 40, reached a peak of 6.2 on day 46 and then declined to 2.0 by day 53. For the right eyes, the individual daily scores remained low, reaching a peak of 1.6 on day 42.

DISCUSSION

In both groups of animals, treatment with antibiotics appeared to have a beneficial effect, resulting in decreased isolations of M.bovis and decreased signs of ocular irritation, including conjunctivitis. However, as a consequence of intrinsic differences in the severity of infection due to differences in the pathogenicity of the two strains of M.bovis used, direct comparisons cannot be drawn between the relative values of the two treatments.

In the first trial, the administration of oxytetracycline hydrochloride initially reduced the rate

	EXAMINATION TIMES (DAYS)												
CALF	40	41	42	43	45	46	47	48	49	50	51	53	54
41L	0	4	0	0	13	13	11	10	6	3	3	2	2
41R	0	0	2	1	0	0	0	0	0	0	0	0	0
42L	0	2	2	2	2	4	3	4	11	14	12	4	4
42R	0	2	2	1	2	2	2	2	2	1	1	1	1
43L	0	11	6	2	0	0	0	3	1	4	4	0	13
43R	0	2	2	2	2	2	2	2	2	2	2	2	2
44L	0	6	2	4	0	0	0	0	0	0	0	0	0
44R	0	0	0	0	0	0	0	0	0	0	0	0	0
45L	0	5	3	2	12	14	12	10	8	5	4	4	4
45R	0	2	2	2	2	2	2	2	2	2	2	2	2

L Left eye
R Right eye

TABLE 39. Clinical scores following heterologous reinfection, experiment 7.

of infection by M.bovis, although isolation rates subsequently increased over a period of two days. The chosen dose of 250 mg of oxytetracycline hydrochloride per eye should have given initial lachrymal concentrations higher than the demonstrated sensitivity for this strain However, work using cattle and rabbits with healthy eyes has shown that this route of administration produces adequate local concentrations initially but that these decline to below therapeutic levels in less than 24 hours (37). This may have been responsible for the subsequent increase in isolation rates. Minimum inhibitory concentrations were identical for M.bovis isolates collected both prior to and following treatment which suggests that persistence of infection may be due to either inadequate maintenance of therapeutic levels or due to the presence of bacteria in areas of necrotic tissue, or even within the nasopharynx. Low numbers of M.bovis in eyes may not be detected by isolation techniques (130). It is therefore equally possible that the subsequent rise in isolation rates may have been due to either reinfection from individual animals, which had remained infected, or else persistent infection within the individuals.

Although treatment with oxytetracycline hydrochloride appeared to result in a general improvement in the clinical condition of the eyes other factors must be considered. The number of animals involved was such that this group was not large enough to divide into treated and untreated controls. Moreover, the advanced age of the majority of the lesions was such that spontaneous

resolution would be expected at about the time that treatment was instituted (18,199,229,230). On the other hand two of the eyes were treated at an early stage of clinical disease. This resulted in a successful cure in one despite continued isolations of M.bovis, while in the other, treatment on the day lesions were first noted failed to prevent the development of severe lesions or eliminate infection. Much more significantly, perhaps, two eyes which had been free from disease developed lesions within three days of treatment. Both eyes were infected with M.bovis prior to, and continued to be infected following, treatment.

The subsequent use of the cloxacillin benzathine ointment on days 35 and 37 appeared to eliminate the infection from the majority of eyes. However, the single isolation of M.bovis on day 41 might suggest that infection persisted at a level below which M.bovis can be detected by conventional isolation techniques. Persistent infection could also explain the apparent lack of response to treatment in this calf which was still severely affected five days after treatment. On the other hand, the use of cloxacillin benzathine appears to have been justified in the above and in one other, field trial (37), despite the fact that strains of M.bovis tested for in vitro sensitivity to this drug have been reported to be between 52% (206) and 100% (225) resistant. The apparent success of the present treatment regime may reflect the particular properties of the base used in this formulation which has been found to produce prolonged

retention of antibiotic with resistance to leaching by excess tear production (37). In the second trial, treatment, with cloxacillin benzathine ointment, of animals infected by a less pathogenic strain of M.bovis, was apparently effective in eliminating both infection and disease in all calves.

The prevalence and severity of the disease that arose following reinfection with M.bovis (GS) were less than might have been expected following initial challenge of fully susceptible calves. This was most obvious in animals which had previously been infected by the homologous strain. This situation is analogous to that found in vaccinated cattle subjected to homologous and heterologous challenge (171).

Analysis of serum samples collected following reinfection indicate that all five calves in the first study and four out of five in the second contained significant antibody levels against strain GS. Significant titres were also present in eight of ten and four of ten lachrymal samples collected during the first and second studies, respectively. This is suggestive that the initial infection with M.bovis (GM) had not stimulated as good an immune response as the GS strain. It must be noted, however, that the latter group had been previously infected for a greater period, due to the inability of antibiotic treatment to eradicate infection from this group, and serum and lachrymal secretions had been collected 14 days later than those from calves in the GM

infected group. In addition, in the initial infection, strain GS also produced more severe inflammation and tissue penetration which may have resulted in greater stimulation of both local and systemic immune systems.

Resistance to reinfection appears also to be affected by previous corneal vascularisation. In the initial infection, strain GS produced only severe lesions with vascularisation of eight of ten corneas. Although vascular tissue was not visible in any eyes on reinfection, pathological and histological examination of healed lesions from other cases cited below suggests that small capillaries would still be patent. This would allow a rapid cellular and immunological response to infection and may have contributed to the apparent higher resistance to homologous reinfection. In addition, in the one calf that did develop lesions, the ulcer was found in a previously non-vascularised area of the cornea and obvious injection developed in areas which had been previously vascularised.

In primary infection, M.bovis became established in all right eyes whereas only one right eye became infected following reinfection. It is not clear whether this was due to increased resistance in these eyes or due to decreased challenge from partially-immune infected left eyes. It is also important to note that, in the homologous challenge, the infective dose was much lower than that used in previous experiments; this may have contributed to the failure of M.bovis to establish itself in three of the five eyes infected.

In four out of five right eyes originally challenged with GM small corneal opacities developed at the site of previous corneal ulceration despite the failure to isolate M.bovis from these eyes. The pathogenesis of these lesions is unknown but may have had some immunological basis.

The relative prevalence of IBK is strongly associated with age, younger animals being more susceptible than older stock (67,98,228). Although a specific age-related immunity has been proposed (98) this is unlikely because when older non-exposed cattle are introduced to IBK endemic areas the disease incidence may be as high as that in young cattle (160). It has also been noted that some animals can occasionally suffer from IBK on more than one occasion during consecutive seasons (98,104) which could be due to naturally declining levels of circulating antibody (227) or could equally be due to infection by new strains of M.bovis. This series of experiments demonstrates that previous exposure to M.bovis does provide some degree of protection against subsequent reinfection and that greater protection was afforded against homologous challenge.

SECTION D

THE EFFECTS OF CORTICOSTEROID ADMINISTRATION ON HEALED CASES

INTRODUCTION

In this section will be described the clinical and bacteriological results following i.m. administration of a short acting corticosteroid to a group of four animals that had recovered from a previous M.bovis (GS) infection.

MATERIALS AND METHODS

Experimental animals

The four animals used were those previously described in Chapter 3, Section A (Materials and Methods, Experiment 2). The clinical and microbiological histories of this group, up to day 70, are as described in Chapter 3, Section A (Results, experiment 2).

Bacteriology

Bilateral ocular and nasopharyngeal samples were collected daily immediately prior to corticosteroid administration on days 70 to 84. All samples were processed from the isolation and identification of M.bovis as described in Chapter 2, Section D.

Clinical examination

Clinical examination of the group was carried out daily from days 70 to 84 using the procedure described in Chapter 2, Section F.

Administration of corticosteroid

The short acting corticosteroid, betamethasone (Betsolan injection, Glaxo Animal Health Limited, Uxbridge, Middlesex, UK) was administered to all animals by deep i.m. injection at the rate of 0.1 mg/kg, on days 70, 71 and 72.

RESULTS

Bacteriology

Moraxella bovis was not isolated from any of the ocular or naropharyngeal swabs collected from days 46 to 69. The results of M.bovis isolations from days 70 to 84 are illustrated in table 40.

Two of the eight ocular and one of four naropharyngeal samples collected immediately prior to the initial treatment on day 70 were positive for M.bovis. By day 71, seven ocular samples were positive, the number declining thereafter, such that M.bovis was not isolated from days 75 to 84.

Clinical features

On day 70, the eyes of all the animals were healthy, apart from slight anterior opacities in calves 8L,R and 10L,R, due to scarring from the previous infection. They remained healthy from days 70 to 84.

DISCUSSION

Daily administration of pharmacological doses of short acting corticosteroid over a three day period

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)														
	70*	71	72	73	74	75	76	77	78	79	80	81	82	83	84
6L	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
6R	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
6NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7L	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
7R	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
7NP	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8L	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
8R	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
8NP	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
10L	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
10R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10NP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

L Left eye
R Right eye
NP Nasopharynx
* Samples collected immediately prior to treatment

\+ Sample positive
\- Sample negative

TABLE 40. Isolations of M.bovis following administration of betamethasone.

coincided with a rise in the proportion of ocular and nasopharyngeal swabs positive for M.bovis. Unfortunately, although M.bovis was not isolated from any samples collected from this group from days 44 to 69, two ocular samples collected immediately prior to the administration of the first dose of corticosteroid on day 70 were found to contain the organism in low numbers. It was, therefore, impossible to tell whether the rise in infection rate was due to the corticosteroid reducing immunity to M.bovis and allowing re-emergence of infection, or due to a natural increase in excretion by a single calf resulting in transmission to, and transient re-infection of, other calves in the group.

It has been shown that betamethasone reduces the resistance of mice to ocular disease produced by M.bovis (43). However, in this experiment, ocular changes did not occur following corticosteroid administration despite the presence of M.bovis. This may be due to the short period over which steroids were administered not permitting sufficient time for incubation of the disease although infection rates appeared to decline despite continued corticosteroid treatment.

The re-isolation of M.bovis after an absence of 28 days in animals which had apparently recovered from IBK is strongly supportive of the presence of carrier animals (145). However, the possibility of infection at this time by a fresh strain cannot be excluded although the probability of this is extremely low because there were no

other cases of IBK present in adjacent loose boxes at this time and the bacteria isolated were culturally and morphologically identical to strain GS.

CHAPTER 4

STUDIES ON THE PREVENTION OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS BY VACCINATION

CHAPTER 4

STUDIES ON THE PREVENTION OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS BY VACCINATION

INTRODUCTION

Although IBK is rarely a fatal condition, herd infection results in significant economic losses (87,117, 199,213,214,222) due to a combination of severe ocular discomfort and temporary or permanent blindness interfering with grazing. Severe handling difficulties may also occur in affected animals (199). The disease usually responds well to treatment (180) although this generally requires good handling facilities allowing animals to be captured, examined regularly and treated where appropriate. It is thus expensive, in terms of labour and equipment, and is not economically viable in extensive grazing systems.

Considerable interest, therefore, has been aimed at developing preventative measures against the disease, such as the use of breeds which are naturally more resistant (47,55,65,198), long term fly control measures (49,72,216,217,238) or by vaccination (99,160,218,227).

It has already been demonstrated in UV irradiated experimental animals that vaccination produces protection against homologous challenge (99,100,171,227) but protection against heterologous challenge or challenge in the field is poor (13,104,171). The following experiment was designed to test a homologous type vaccine challenged by a highly pathogenic strain of *M.bovis* without the use

of artificial predisposing factors.

MATERIALS AND METHODS

Experimental animals

Fifteen, three to four month old conventional dairy-cross calves, acquired from three separate commercial dairy farms in groups of five, with a history of freedom from ocular disease, were used. Ocular samples (L,R) were taken on three occasions, to ensure freedom from M.bovis infection, before all 15 calves were introduced to a single large box on day -14. The calves were identified by plastic ear tags, the control calves were numbered 1 to 5 and the vaccinated group numbered 7 to 16.

Feeding and housing were as described in Chapter 2, Section A.

Sampling of animals

- Virology

Nasopharyngeal and unilateral ocular swabs were taken at weekly intervals from days -12 to 79 and processed for the isolation and identification of BHV1, adenoviruses and PI3 virus as described in Chapter 2, Section B.

- Mycoplasmology

Nasopharyngeal and unilateral ocular swabs were taken at weekly intervals from days -12 to 79 and processed for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Bilateral ocular swabs were collected three times a week, from days -14 to 42, daily from days 43 to 83, and twice weekly thereafter until day 115. Nasopharyngeal samples were collected at weekly intervals, from days -12 to 79. At weekly intervals from days -7 to 77, ocular (L,R) samples were processed for the isolation and identification of all Gram-negative bacteria and all samples were submitted for the isolation of M.bovis as described in Chapter 2, Section D.

- Sera and lachrymal secretions

Sera and lachrymal (L eye only) samples were collected on day 0 and sera and bilateral lachrymal samples were subsequently collected on day 14 and at weekly intervals from days 28 to 77. Lachrymal secretions were collected by inserting sponge strips, approximately 6 mm in diameter and 20 mm long, into the lower conjunctival sac and leaving them in place until they became saturated or for a maximum of 30 seconds. Lachrymal secretions were expressed from the sponge into sterile plastic bijoux bottles using a plastic 2 ml syringe. This procedure was repeated up to a maximum of three times or until approximately 0.5 ml of tears had been collected. Antibody response was measured using an IHA test as described in Chapter 2, Section E. Samples in which no antibody was detected were given a nominal reciprocal titre of one and the results converted to log reciprocal titres for statistical analysis.

Clinical examination and scoring

Calves were examined three times weekly from admission on day -21 until challenge exposure on day 43, and daily from days 43 to 83. All examinations were carried out and clinical scores allocated according to the methods described in Chapter 2, Section F.

Vaccine preparation and administration

The vaccine was prepared using a low passage strain of M.bovis (GS) which had been reisolated from a previous experimental infection and stored at -70°C on BAP. Prior to vaccine preparation, M.bovis was thawed out, passaged onto fresh BAP and a high level of fimbriation confirmed by colony morphology and by electron microscopy using the methods described in Chapter 2, Section G. Bacteria were scraped off the BAP, killed with formaldehyde to a final concentration of 0.2% and adjusted to a dry weight concentration of 10 mg/ml. Ten ml of aluminium hydroxide was added to 30 ml of this suspension to give a final concentration of 7.5 mg/ml. To ensure sterility prior to use, samples of the vaccine were inoculated onto BAP. Three ml aliquots were dispensed into sterile syringes and administered, by s.c. injection, on the right side of the neck, to calves 7 to 16 on day 0. A repeat dose was administered on day 28.

Inoculation procedures

The inoculum was prepared from a low passage subculture of the vaccine strain, stored at -70 on BAP prior to use. The plate was thawed out on day 41 and

passaged once on BAP. On day 42, five BAPs were inoculated with single SC type colonies and incubated aerobically for 16 hours at 35°C. On day 43, the growth from the five plates was suspended in 10 ml of sPBS and gently agitated to break up the colonies. Within 30 minutes of preparation, 0.5ml was instilled into the lower right conjunctival sacs of all animals and each eye held gently closed for 30 seconds.

Immediately following administration the inoculum was serially diluted in sterile 10% magnesium chloride and 0.5ml of each tenfold dilution inoculated onto BAP; the inoculum dose was found to be 10^8 CFU/ml.

Pathology

Calves 3, 5 (controls) and 8 (vaccinated) were destroyed on humane grounds on days 52, 53 and 57, respectively, and the eyes removed for pathological examination. The methods used and results are described in Chapter 6.

RESULTS

Microbiology

- Virology

Bovine herpes virus 1, adenovirus or PI3 virus were not isolated from any of the samples submitted for virological examination.

- Mycoplasmology

The results for the isolation and identification of mycoplasmas from ocular samples are presented in table 41.

In the control group Myco.bovirhinis was isolated from three out of 62 (4.8%) ocular samples and A.laidlawii on one occasion only (1.6%). In the vaccinated group, Myco.bovirhinis was isolated from four out of 136 (2.9%) samples, A.laidlawii from three (2.2%), U.diversum from two (1.5%) and Myco.dispar from one (0.7%).

The results from nasopharyngeal samples are presented in table 42. In the control group, Myco.bovirhinis was isolated from ten out of 62 (16.1%) samples, A.laidlawii from nine (14.5%), Myco.bovis from five (8.1%) and Myco.dispar from one (1.6%). In the vaccinated group, Myco.bovirhinis was isolated from 32 out of 136 (23.5%) samples, A.laidlawii from 27 (19.9%), Myco.bovis from four (2.9%) and U.diversum from one (0.7%).

- Bacteriology

The results for the isolation and identification of Gram-negative bacteria from ocular swabs collected from the right eyes are presented in table 43 and those for the left eyes in table 44. Moraxella(B.) ovis was isolated from 17 out of 208 (8.2%) samples collected prior to exposure and from three out of 130 (2.3%) post-exposure samples.

SOURCE OF SAMPLE	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) -12	-5	2	9	16	23	30	37	44	51	58	65	72	79
1	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	A.laidlawii	-	-	-	-	-	-	-	-	-	-	-	+	-	-
2	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	-	+	-	-
3A	-														
4	Myco.bovirhinis	-	-	-	-	-	-	-	+	-	-	-	-	-	-
5B	-														
7	Myco.bovirhinis	-	-	-	-	-	-	+	-	-	-	-	-	-	-
8C	A.laidlawii	-	-	-	-	-	-	-	+	-	-	-	-	-	-
9	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	+	-	-	-	-
10	-														
11	-														
12	Myco.bovirhinis	-	-	-	-	+	-	-	-	-	-	-	-	-	-
13	U.diversum	-	-	-	+	-	-	-	-	-	-	-	-	-	-
14	U.diversum	-	-	-	+	-	-	-	-	-	-	-	-	-	-
15	A.laidlawii	-	-	-	-	+	-	-	-	-	-	-	-	-	-
16	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Myco.dispar	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.laidlawii	-	-	-	-	-	-	+	-	-	-	-	-	-	-

\+ Mycoplasma isolated
\- Mycoplasma not isolated

A Calf 3 slaughtered day 52
B Calf 5 slaughtered day 53
C Calf 8 slaughtered day 67

TABLE 41. Isolations of mycoplasmas from ocular samples, vaccination experiment.

SOURCE OF SAMPLE	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS)													
		-12	-5	2	9	16	23	30	37	44	51	58	65	72	79
1	Myco.bovirhinis	-	-	-	+	-	+	-	-	-	-	-	+	-	-
	Myco.bovis	-	-	-	-	-	+	-	-	-	-	-	+	-	-
	A.laidlawii	-	-	-	-	+	-	+	-	-	-	-	-	-	+
2	Myco.bovis	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	Myco.dispar	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.laidlawii	-	-	-	+	-	-	-	-	-	-	-	+	-	-
3A	Myco.bovis	-	-	-	-	-	-	-	-	-	+	-			
	A.laidlawii	+	-	-	-	-	-	-	-	-	-	-			
4	Myco.bovirhinis	-	-	-	-	+	+	+	-	+	-	-	+	-	-
	Myco.bovis	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	A.laidlawii	-	-	-	-	+	-	-	-	-	-	-	+	-	-
5B	Myco.bovirhinis	-	-	-	-	-	-	+	-	+	-	-			
	A.laidlawii	-	-	-	-	-	+	-	-	-	-	-			
7	Myco.bovirhinis	-	-	-	-	+	+	-	-	-	-	-	+	-	-
	Myco.bovis	-	-	-	-	-	-	-	-	-	-	-	+	-	-
8C	A.laidlawii	-	-	-	-	-	+	+	-	-	-	-	-		
9	Myco.bovirhinis	-	+	-	-	+	+	+	-	-	-	-	-	-	+
	A.laidlawii	+	-	-	-	-	-	-	+	-	-	+	-	+	-
	U.diversum	-	-	-	-	+	-	-	-	-	-	-	-	-	-
10	Myco.bovirhinis	-	-	-	-	-	-	-	-	+	-	-	+	+	+
	A.laidlawii	-	-	-	-	-	+	+	+	-	+	+	-	-	-
11	Myco.bovirhinis	-	-	-	-	+	-	+	+	+	-	-	-	-	+
	Myco.bovis	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	A.laidlawii	-	+	-	-	+	-	-	-	-	-	-	-	-	-
12	Myco.bovirhinis	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	Myco.bovis	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	A.laidlawii	-	-	-	-	-	-	+	-	-	-	-	-	-	-

TABLE 42.

SOURCE OF SAMPLE	MYCOPLASMA ISOLATED	SAMPLING TIMES (DAYS) -12	-5	2	9	16	23	30	37	44	51	58	65	72	79
13	Myco.bovirhinis	+	-	-	+	-	+	-	+	-	-	-	-	-	-
	A.laidlawii	-	-	-	-	+	+	-	-	-	-	-	-	-	-
14	Myco.bovirhinis	-	-	-	-	+	+	-	+	-	-	-	-	-	-
	Myco.bovis	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.laidlawii	-	-	-	-	-	+	-	+	-	-	-	+	-	-
15	Myco.bovirhinis	+	-	-	-	+	+	-	-	-	-	+	-	-	-
	A.laidlawii	-	-	-	+	+	+	-	+	-	-	-	-	-	-
16	Myco.bovirhinis	-	+	-	-	-	-	-	+	-	-	-	-	+	-
	A.laidlawii	-	+	-	-	+	+	-	+	-	-	-	-	-	-

\+ Mycoplasma isolated
\- Mycoplasma not isolated

A Calf 3 slaughtered day 52
B Calf 5 slaughtered day 53
C Calf 8 slaughtered day 67

TABLE 42. (contd.) Isolations of mycoplasmas from nasopharyngeal samples, vaccination experiment.

ANIMAL SAMPLED	BACTERIUM ISOLATED	SAMPLING TIMES (DAYS) -7	0	7	14	21	28	35	42	49	56	63	70	77
1	A.anitratus	-	-	-	+	-	-	-	+	+	-	-	-	-
	A.lwoffi	+	-	-	-	+	-	-	-	-	-	-	-	+
	M.catarrhalis	-	-	-	+	+	-	-	-	+	-	+	-	-
	M. ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	+	-	-	-	-	+	-
2	A.anitratus	-	+	-	-	-	-	-	-	-	-	-	-	-
	Alcagines spp.	-	-	-	-	-	+	-	-	-	-	-	-	-
	M.catarrhalis	-	-	-	-	+	-	-	+	+	+	+	+	-
	M.nonliquefaciens	-	-	-	-	+	-	-	-	-	-	-	-	-
3A	A.anitratus	-	-	+	-	-	-	-	-	-				
	A.lwoffi	-	-	-	+	+	-	-	+	-				
	M.catarrhalis	-	-	-	+	+	-	-	-	+				
	K.oxytoca	+	-	-	-	-	-	-	-	-				
	M.nonliquefaciens	-	-	-	-	+	-	+	-	-				
4	A.lwoffi	-	-	-	-	+	-	-	+	+	-	-	-	+
	M.catarrhalis	-	-	-	+	+	-	+	-	-	+	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	-	-	-	-	-	-	-
	N.caviae	-	-	-	-	-	-	-	-	-	-	-	+	-
5B	A.anitratus	-	-	-	-	-	-	+	-	-				
	A.lwoffi	-	-	+	-	+	+	-	-	-				
	M.catarrhalis	-	-	-	-	+	-	-	-	-				
	M. ovis	-	-	-	+	-	-	-	-	+				
	M.nonliquefaciens	-	+	-	-	+	-	-	-	-				
7	A.lwoffi	-	-	-	+	+	-	-	-	-	-	-	-	+
	M.catarrhalis	-	-	+	+	+	+	+	+	-	+	-	-	-
	M. ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	+	+	-	-	-	-	-	-	-	-	+	-

TABLE 43.

ANIMAL SAMPLED	BACTERIUM ISOLATED	SAMPLING TIMES (DAYS) -7	0	7	14	21	28	35	42	49	56	63	70	77
8C	A.anitratus	-	-	-	-	-	+	-	-	-	-	-		
	M.catarrhalis	-	-	-	-	+	-	-	-	-	+	+		
	M. ovis	-	-	-	+	-	-	-	-	-	-	-		
	M.nonliquefacians	-	+	-	-	+	-	-	-	-	-	-		
9	A.lwoffi	-	-	-	-	+	-	-	-	-	-	-	-	-
	M.catarrhalis	-	-	+	-	+	-	-	-	+	+	-	+	-
	E.coli	-	-	+	-	-	-	-	-	-	-	-	-	-
	M. ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	N.pharyngis	-	-	-	-	-	-	+	-	-	-	-	-	-
10	A.anitratus	-	-	+	-	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	+	-	-	-	-	-	-	-	-	-	-	-
	M.catarrhalis	-	-	-	+	+	-	-	-	+	-	+	+	+
	M. ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	-	-	-	-	-	+	-
11	A.anitratus	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	+	-	-	-	-	-	-	-	-
	M.catarrhalis	-	-	-	-	+	-	-	-	+	+	-	+	-
	Flavobacterium spp.	-	+	-	-	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	+	-	+	-	+	-	-	-	+	-	-
12	A.anitratus	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	+	+	-	-	-	-	-	-	-
	M.catarrhalis	-	-	+	+	+	-	+	-	+	+	-	-	-
	M.nonliquefaciens	-	-	+	-	+	-	-	-	-	-	-	-	-
13	A.lwoffi	-	-	-	+	-	+	-	-	-	-	-	-	+
	M.catarrhalis	-	-	-	-	-	-	+	-	-	-	-	+	+
	E.coli	-	-	-	-	+	-	-	-	-	-	-	-	-
	K.oxytoca	-	+	-	-	-	-	-	-	-	-	-	-	-

TABLE 43. (contd.)

ANIMAL SAMPLED	BACTERIUM ISOLATED	SAMPLING TIMES (DAYS) -7	0	7	14	21	28	35	42	49	56	63	70	77
13	M. [illegible]ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	-	-	+	-	+	-	-
14	A.lwoffi	+	+	-	-	+	-	-	+	-	-	-	-	-
	M.catarrhalis	-	-	-	-	+	-	-	-	-	-	+	-	-
	E.coli	-	-	-	-	-	-	-	-	-	-	-	+	-
	M. [illegible]ovis	-	-	+	-	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	+	-	+	-	-	-	+	-	-	-	-
15	A.anitratus	-	-	-	+	-	-	+	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	+	+	-	+	-	-	-	-	-
	M.catarrhalis	-	-	-	+	+	-	-	+	+	+	+	+	-
	M. [illegible]ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	-	-	-	-	-	+	-
16	A.anitratus	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	-	-	-	-	-	-	-	+	-
	M.catarrhalis	-	-	-	-	+	-	-	+	-	-	+	-	-
	M.(B.)ovis	-	-	+	-	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	+	-	+	-	-	-	-	-	+	-	-
	S.marcescens	-	-	-	-	-	-	-	-	-	-	-	+	-

\+ Bacterium isolated
\- Bacterium not isolated

A Calf 3 slaughtered day 52
B Calf 5 slaughtered day 53
C Calf 8 slaughtered day 67

TABLE 43. (contd.) Isolations of Gram-negative bacteria (other than M.bovis) from right ocular samples, vaccination experiment.

ANIMAL SAMPLED	BACTERIUM ISOLATED	SAMPLING TIMES (DAYS) -7	0	7	14	21	28	35	42	49	56	63	70	77
1	A.anitratus	-	-	+	+	-	-	-	+	-	-	-	-	-
	A.lwoffi	-	-	-	-	+	-	-	-	-	-	-	-	+
	M.catarrhalis	-	-	-	-	+	-	-	+	+	+	-	+	-
	M.(B.)ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	+	-	-	-	-	-	-
2	A.anitratus	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	+	-	-	-	-	-	-	-	-
	Alcagines spp.	-	-	-	-	-	+	-	-	-	-	-	-	-
	M.catarrhalis	-	-	+	-	+	-	-	+	-	-	+	-	-
3A	A.anitratus	-	-	-	+	-	-	-	-	-				
	A.lwoffi	-	-	-	-	+	-	-	-	-				
	M.catarrhalis	-	-	-	-	-	-	-	-	+				
	E.coli	-	-	+	-	-	-	-	-	-				
	M.(B.)ovis	-	-	-	+	-	-	-	-	-				
	M.nonliquefaciens	-	-	-	-	+	-	+	-	-				
4	A.lwoffi	-	-	-	-	+	-	-	-	-	-	-	-	-
	M.catarrhalis	-	-	-	-	+	-	+	-	-	+	+	-	+
	Flavobacterium spp.	-	+	-	-	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	-	-	-	-	-	-	-
5B	A.anitratus	-	-	-	-	-	-	+	-	-				
	A.lwoffi	-	-	-	+	-	+	+	-	-				
	M.catarrhalis	-	-	-	+	+	-	-	+	-				
	M.(B.)ovis	-	-	-	-	-	-	-	-	+				
	M.nonliquefaciens	-	-	+	-	+	-	-	-	+				
7	A.lwoffi	-	-	-	+	+	-	-	+	-	-	-	-	+
	M.catarrhalis	-	-	-	+	+	+	+	+	+	+	-	+	-
	E.coli	-	-	+	-	-	-	-	-	-	-	-	-	-

TABLE 44.

ANIMAL SAMPLED	BACTERIUM ISOLATED	SAMPLING TIMES (DAYS) -7	0	7	14	21	28	35	42	49	56	63	70	77
7	M.nonliquefaciens	-	+	+	-	+	-	-	-	-	-	-	-	-
	N.pharyngis	-	-	-	-	-	-	+	-	-	-	-	-	-
8C	A.lwoffi	-	-	-	+	+	-	-	-	-	-	-		
	M.catarrhalis	-	-	-	-	+	-	-	+	+	-	+		
	M.(B.)ovis	-	-	-	+	-	-	-	-	-	-	-		
	M.nonliquefaciens	-	-	-	-	-	-	-	-	-	-	-		
9	A.lwoffi	-	+	-	-	+	-	-	-	-	-	-	-	+
	M.catarrhalis	-	-	-	+	+	-	-	-	+	-	+	-	-
	E.coli	-	-	+	-	-	-	-	-	-	-	-	-	-
	K.oxytoca	+	-	-	-	-	-	-	-	-	-	-	-	-
	M.(B.)ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	-	-	+	-	+	-	-	-	-
	N.pharyngis	-	-	-	-	-	-	+	-	-	-	-	-	-
10	A.lwoffi	-	+	-	-	+	-	-	-	-	-	-	-	-
	M.catarrhalis	-	-	-	-	+	-	-	-	+	-	-	-	+
	K.oxytoca	+	-	-	-	-	-	-	-	-	-	-	-	-
	M.(B.)ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	+	-	-	-	-	-	-
11	A.anitratus	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	-	-	-	-	-	-	-	+	-
	M.catarrhalis	-	-	-	+	+	-	-	-	+	+	-	-	-
	M.nonliquefaciens	-	-	+	-	+	-	+	-	-	-	-	-	-
12	A.lwoffi	-	-	-	+	+	+	-	+	-	-	+	-	-
	M.catarrhalis	-	-	-	-	+	-	-	+	+	+	+	+	-
	M.(B.)ovis	-	-	-	-	-	-	-	-	+	-	-	-	-
	M.nonliquefaciens	-	-	-	-	+	-	-	-	-	-	-	-	-

TABLE 44. (contd.)

ANIMAL SAMPLED	BACTERIUM ISOLATED	SAMPLING TIMES (DAYS) -7	0	7	14	21	28	35	42	49	56	63	70	77
13	A.anitratus	-	-	-	-	-	-	+	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	-	-	-	-	-	-	-	-	+
	M.catarrhalis	-	-	-	-	+	-	+	-	-	-	-	-	-
	E.coli	-	-	-	-	-	-	-	-	-	+	-	-	+
	M.(B.)ovis	-	-	-	+	-	-	-	-	-	+	-	-	-
	M.nonliquefaciens	-	-	-	-	-	-	-	-	+	-	+	+	-
14	A.anitratus	-	-	-	+	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	-	-	-	+	-	-	-	-	-
	M.catarrhalis	-	-	-	+	-	-	-	-	+	+	+	-	-
	M.nonliquefaciens	-	-	+	-	+	-	-	-	+	-	+	-	-
15	A.anitratus	-	-	+	+	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	-	-	-	+	+	-	-	-	-	-	-	+
	A.lignieresii	-	+	-	-	-	-	-	-	-	-	-	-	-
	M.catarrhalis	-	-	-	-	+	-	-	+	-	+	+	+	-
	M.(B.)ovis	-	-	-	+	-	-	-	-	-	-	-	-	-
	M.nonliquefaciens	-	-	-	-	-	-	-	-	-	-	-	+	-
16	A.anitratus	+	-	-	+	-	-	-	-	-	-	-	-	-
	A.lwoffi	-	+	-	-	+	-	-	+	-	-	-	-	+
	M.catarrhalis	-	-	-	+	-	-	-	-	-	-	+	-	-
	M.nonliquefaciens	-	+	+	-	+	-	-	-	-	-	-	-	-

\+ Bacterium isolated
\- Bacterium not isolated

A Calf 3 slaughtered day 52
B Calf 5 slaughtered day 53
C Calf 8 slaughtered day 67

TABLE 44. (contd.) Isolations of Gram-negative bacteria (other than M.bovis) from left ocular samples, vaccination experiment.

Moraxella bovis was not isolated from any of the ocular or nasopharyngeal swabs taken prior to exposure on day 43.

Isolations of M.bovis between days 43 and 83 are illustrated in figure 19. In the control group, infection was established (i.e. three positive isolations of M.bovis from four sequential samples), in all five right eyes 24 hours following challenge. Infection had been transmitted to, and established in, two left eyes by day 44 and in the remaining three eyes by days 49, 50 and 52, a mean of 5.0 days post challenge.

In the vaccinated group, M.bovis was isolated from seven right eyes on day 44 but did not become established in all ten eyes until day 47. In the left eyes of this group, M.bovis was first isolated from four animals on day 45, one on day 47, one on day 49, three on day 50 and one on day 59 resulting in a mean time of transmission of 5.5 days. However in six animals, isolations were sporadic and the infection did not become established until 12.6 days post challenge.

Isolations of M.bovis between days 87 and 115 are illustrated in table 45. In the right eyes of the control group infection persisted until day 81 in calf 1, 83 in calf 2 with one subsequent isolation on day 115. Calf 4 was still infected at the end of the experiment. Of the 27 samples collected between days 87 and 115, eight were positive for M.bovis (29.6%). In the left eyes, calves 1 and 2 were persistently infected up to day 115 and in

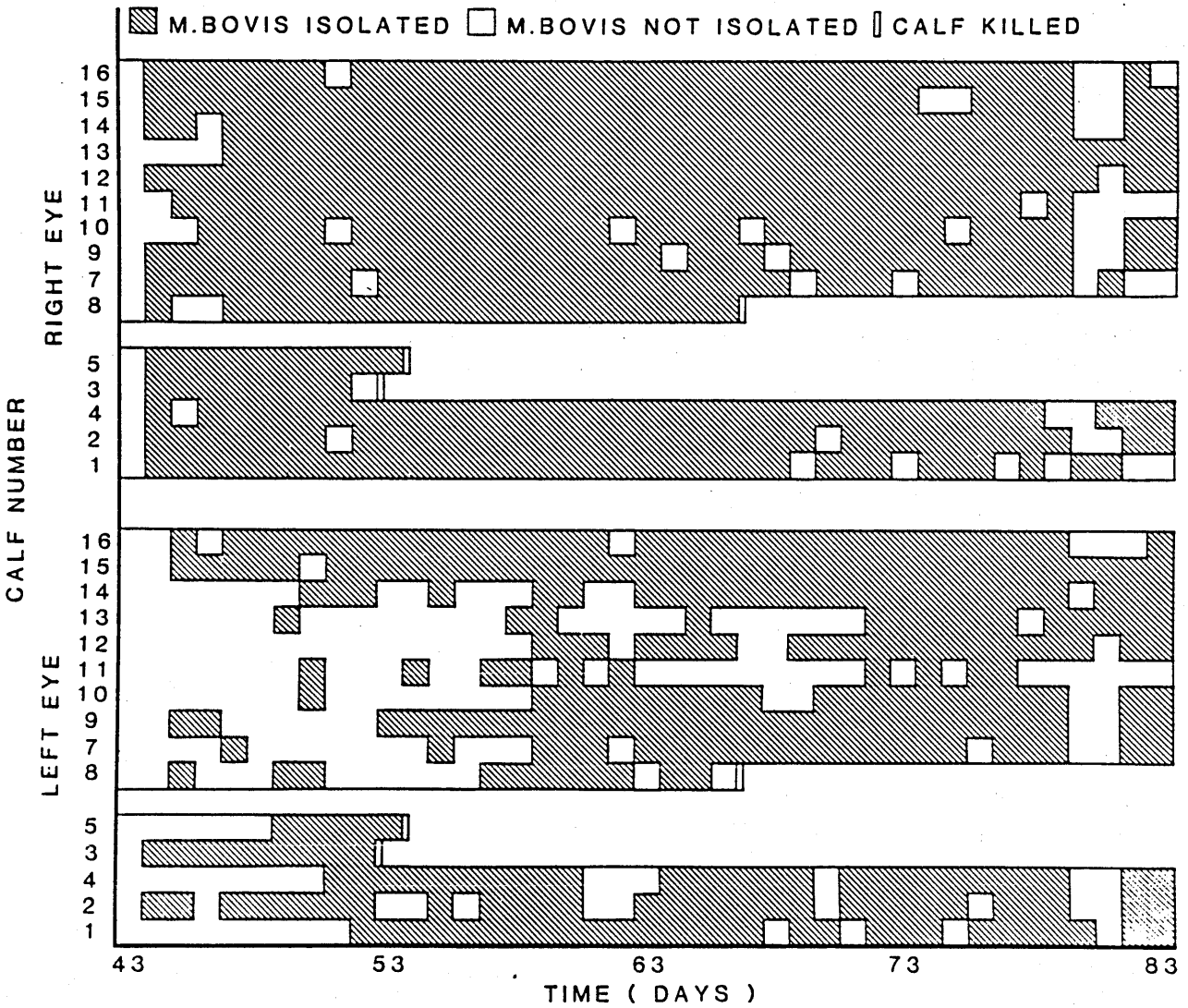


FIGURE 19. Vaccination experiment. Isolations of M.bovis from ocular swabs following instillation, into each right eye, on day 43.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)								
	87	91	95	98	101	105	108	113	115
1L	+	+	+	+	+	-	-	-	-
1R	-	-	-	-	-	-	-	-	-
2L	-	+	-	+	+	-	+	+	+
2R	-	-	-	-	-	-	-	-	+
4L	+	-	+	+	+	+	+	+	+
4R	+	+	+	+	-	-	+	+	+
7L	+	-	+	+	+	-	+	-	-
7R	-	-	-	-	-	-	-	-	-
9L	-	-	-	-	-	+	+	-	-
9R	+	-	-	+	-	+	+	-	-
10L	+	+	+	+	+	-	+	-	+
10R	+	+	+	+	+	+	+	+	+
11L	-	-	-	-	-	-	-	-	-
11R	-	-	-	-	-	-	-	-	-
12L	-	+	+	-	-	-	-	-	-
12R	+	-	-	-	-	+	-	-	-
13L	+	+	+	-	-	+	+	+	-
13R	+	-	+	-	-	+	-	+	+
14L	-	-	+	-	+	-	+	-	+
14R	+	-	+	+	-	+	+	-	-
15L	+	-	+	-	-	-	-	-	-
15R	+	-	+	+	-	+	+	+	-
16L	+	-	+	+	+	+	+	+	+
16R	-	+	+	+	+	+	+	-	+

L Left eye
R Right eye

\+ Sample positive
\- Sample negative

TABLE 45. Isolations of M.bovis from ocular samples collected from day 87 to day 115, vaccination experiment.

calf 4, the last sample positive for M.bovis was collected on day 101. Moraxella bovis was isolated from 19 of the 27 swabs taken between days 87 and 115 (70.4%).

In the right eyes of the vaccinated group, infection persisted until day 79 in calf 11, day 81 in calf 7, day 87 in calves 9 and 12, day 95 in calf 13, day 108 in calf 14, day 112 in calf 15 and calves 10 and 16 were still infected on day 115. In calves 9, 12 and 13, sporadic isolations were made subsequent to days 87 and 95, respectively. Eighty-one samples were collected from the right eyes of this group between days 87 and 115, 38 of which were positive for M.bovis (46.9%). In the left eyes, infection persisted until day 78 in calf 11, day 83 in calves 9 and 14, day 95 in calves 12, 13 and 15, day 108 in calf 7 and was still present in calves 10 and 15 on day 115. In calves 9, 14 and 13 sporadic isolations were made subsequent to days 83 and 95 respectively. Moraxella bovis was isolated from 36 samples collected between days 87 and 115 (44.4%).

Eighty nasopharyngeal swabs were collected between days 44 and 79. Moraxella bovis was isolated from three out of 22 (13.6%) and ten out of 58 (17.2%) samples collected from the control and vaccinated groups, respectively. In all thirteen positive samples only one or two colonies of M.bovis were found per plate.

Clinical features

In the control group, signs of IBK developed in all five of the artificially infected right eyes and in three left eyes. Abnormalities were first noted in the right eyes of all calves within 24 hours of inoculation and in the left eyes of calves 1, 3 and 4 on days 80, 50 and 57, respectively. The lesions in two of the eyes were mild (Appendix IV; calves 1,4) and healed without vascularisation with mild lesions recurring in one case (4L) on day 50, again resolving without vascularisation. In the remaining six eyes (Appendix IV; calves 1, 2, 3, 4, 5) the lesions were severe; healing was accompanied by vascularisation in calves 1R, 2R and 4L while two calves (3, 5) were slaughtered on humane grounds.

In the vaccinated group, signs of IBK developed in all ten of the artificially infected right eyes and in six left eyes. Abnormalities were first noted in the right eyes of calves 9, 11, 12 and 15 within 24 hours of inoculation and of calves 7, 8, 10, 13, 14 and 16 by days 65, 49, 67, 53, 45 and 61, respectively. In the left eyes, abnormalities were first noted in calves 9, 10, 12, 14, 15 and 16 on days 62, 74, 63, 66, 51 and 69, respectively. The lesions in five of the eyes affected were mild (Appendix IV; calves 7, 13, 14 and 16), resolved without vascularisation with mild lesions recurring once in calves 14L and 16R on days 79 and 76, respectively, and on three occasions in calf 13R on days 55, 64 and 73. In the remaining 11 eyes (Appendix IV; calves 8, 9, 10, 11, 12, 14 and 15) the lesions were more severe and healing was

accompanied by vascularisation. Mild lesions occurred in calves 12R and 15L,R on days 83, 76 and 72, respectively, and severe lesions recurred in calf 10R on day 79.

Individual daily clinical scores for the right eyes are given (table 46) and the mean scores are illustrated in figure 20.

In the control group, the mean clinical score rose rapidly to a peak of 14.2, three days following challenge exposure. Resolution of signs in calf 4 resulted in a temporary decrease on days 47 and 48 before rising to a new peak of 16.0 on day 52. The two most severely affected calves (3, 5) were slaughtered on days 52 and 53. The mean clinical score fell gradually, such that by day 67 no ocular discomfort was noted despite the continued presence of corneal ulceration and granulation tissue in the two severely affected eyes (calves 1,2). In this group, the mean clinical score over days 44 to 63 was 11.7 but from day 64 to 83 it was 7.9.

In the vaccinated group, the mean score rose most rapidly on days 44 and 45, and continued to rise more slowly thereafter to a peak of 10.0 on day 52. Between days 52 and 65 the scores reached a plateau, fluctuating between 10.0 and 7.6 before gradually falling to 0 on day 77. Increased mean scores were recorded on two further occasions due to the development of recurrent lesions in two calves (10 and 12). In this group, the mean clinical score over days 44 to 63 was 7.4 but from day 64 to 83 it was 5.6.

EXAMINATION TIMES (DAYS)

CALF	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
1	0	9	14	16	15	15	17	17	15	17	17	16	17	16	16	17	16	17	16	16	15	12	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	12	14	17	17	15	18	16	16	17	14	16	17	16	17	17	17	19	17	13	14	13	15	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3A	0	14	16	17	17	18	19	19	20	20																															
4	0	7	10	5	0	0	0	8	8	9	9	12	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5B	0	14	16	3	17	18	1	18	18	17	20																														
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8C	0	0	3	1	2	2	5	10	13	11	8	10	10	12	16	15	15	16	16	18	17	15	17	16	17																
9	0	12	14	14	15	13	16	16	16	17	16	16	14	14	16	15	14	15	16	13	11	10	8	6	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	4	10	12	13	13	12	8	0	0	0	0	0	0	11	11	8	0	0
11	0	10	14	13	16	17	16	15	17	18	18	18	18	17	16	16	14	14	9	11	8	8	9	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	10	11	14	14	14	14	14	15	15	16	15	15	16	17	16	11	8	11	12	13	11	11	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11
13	0	0	0	2	0	2	2	2	2	8	12	8	11	5	11	12	10	8	8	6	4	11	12	13	14	13	13	13	13	0	14	12	0	0	0	0	0	0	0	0	0
14	0	0	10	12	13	15	10	8	12	13	7	7	12	8	0	10	11	10	8	0	7	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	10	14	13	15	15	14	15	15	17	17	13	15	17	17	12	12	13	12	11	12	10	13	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	2	2	3	2	2	1	2	2	1	1	2	1	1	14	9	8	5	5	0	0	0	0	0	0	0	0	2	2	9	0	0	0	0	0	0	0

A Calf 3 slaughtered on day 52
B Calf 5 slaughtered on day 53
C Calf 8 slaughtered on day 67

TABLE 46. Clinical scores from right eyes, vaccination experiment.

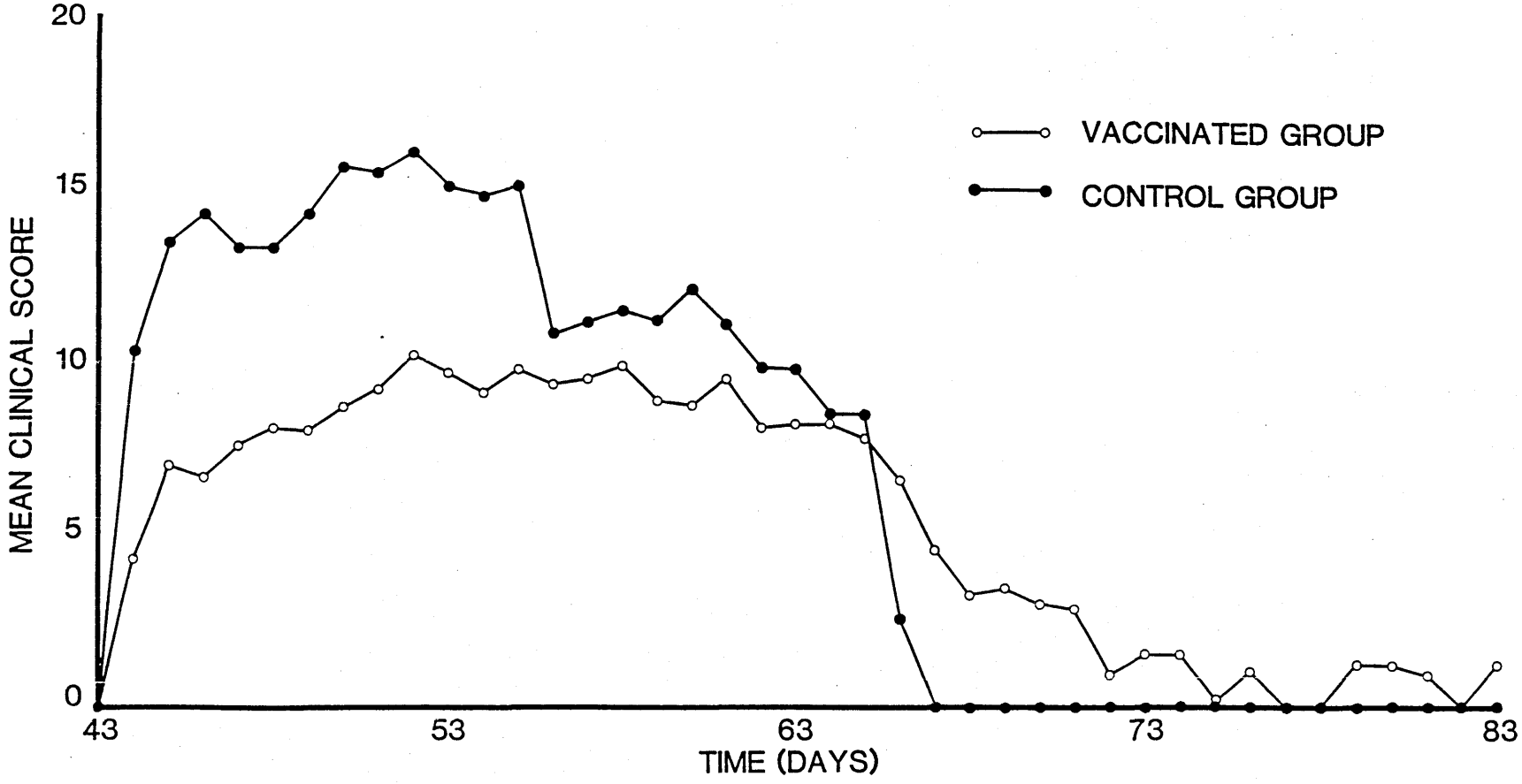


FIGURE 20. Vaccination experiment. Mean clinical scores in the right eyes of vaccinated and non-vaccinated calves following instillation of M.bovis (GS), into each right eye, on day 43.

Individual daily clinical scores for the left eyes are given in table 47 and the mean clinical scores are illustrated in figure 21.

In the control group the mean score was above zero on days 47 to 49 due to transient lesions in calf 3. The second peak was due to recurrence of lesions in the same animal which was slaughtered on day 52. The third rise in clinical score was due to the development of severe lesions in a single eye (calf 4) which persisted until day 80 at which time calf 1 developed lesions. The mean clinical score over the 40 day period was 3.2.

In the vaccinated group the left eye of calf 15 was affected on day 48 and the mean score did not increase further until day 61 when calf 8 developed lesions. A further rise was produced on day 65 due to lesions in calf 12. The score fluctuated thereafter due to healing of these lesions, the slaughter of calf 8 on day 67, and the development of new lesions in calves 10 and 16 on day 74 and calf 14 on day 78. The mean clinical score over the 40 day period was 2.0.

Immunological response

Results following IHA examination of sera are given in table 48 and those from ocular secretions are given in table 49.

Mean log reciprocal IHA titres from serum samples are illustrated in figure 22. In the control group, mean log serum titres remained low up to day 49, rose to a peak

EXAMINATION TIMES (DAYS)

CALF	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3A	0	0	0	3	7	5	0	11	14	18											
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	14	15	16	16	15	15
5B	0	0	0	0	0	0	0	0	0	0	0										
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	15	14
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	8	14	14	14	14	13	11	13	16	13	12	13	11	11	10	13
16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CALF	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	12	8	7
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3A																				
4	17	17	17	18	18	18	18	14	16	16	14	13	13	15	13	12	7	0	0	0
5B																				
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8C	14	15	15	15																
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	13	13	17	18	16	17	15	16	17	16
11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	9	11	12	12	6	6	12	12	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	13	13	7	7
15	11	13	13	13	12	9	6	7	11	3	8	0	11	12	11	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	4	3	2	2	4	3	2	1	0	0

A Calf 3 slaughtered day 52
B Calf 5 slaughtered day 53
C Calf 8 slaughtered day 67

TABLE 47. Clinical scores from left eyes, vaccination experiment.

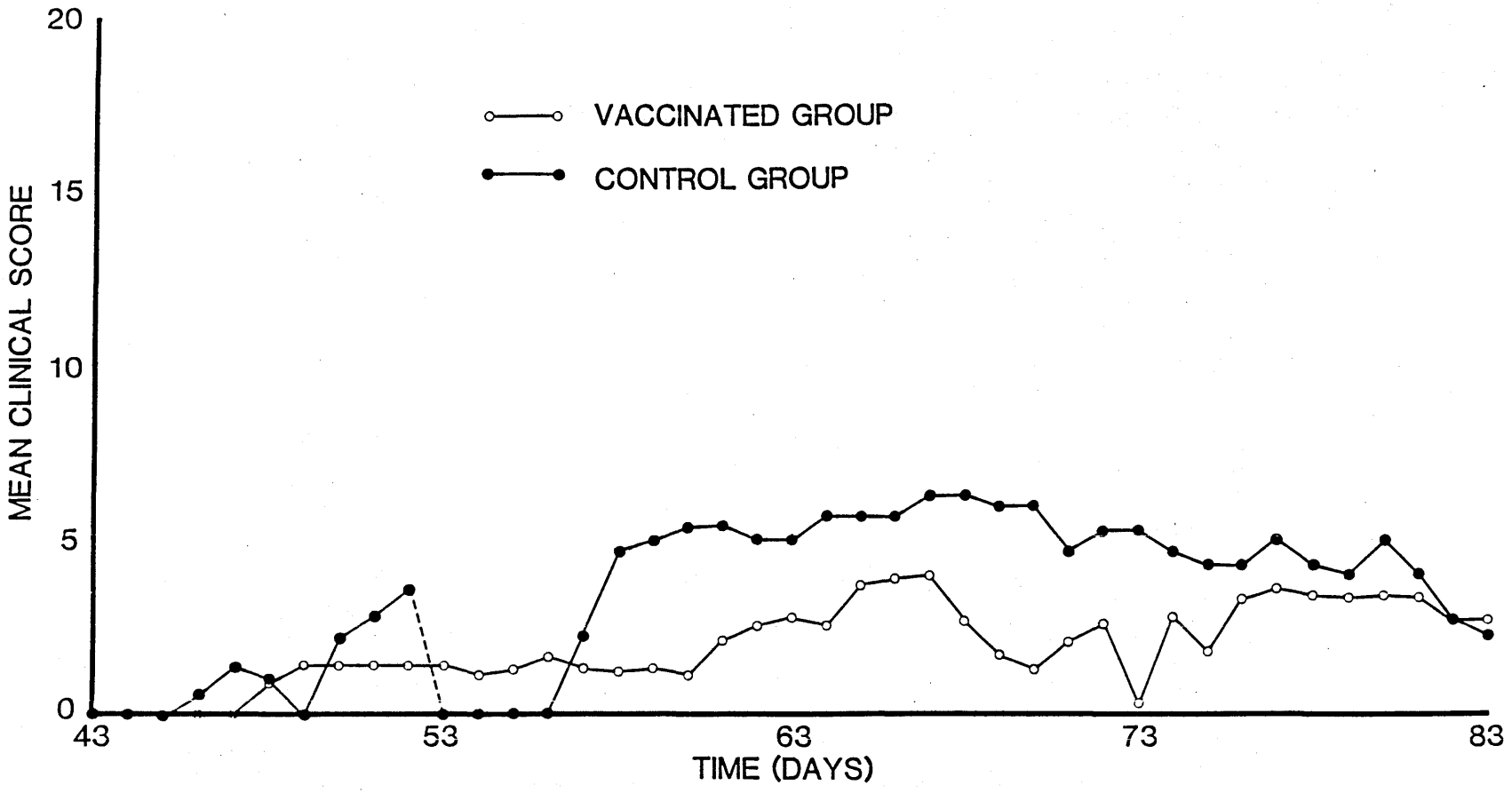


FIGURE 21. Vaccination experiment. Mean clinical scores in the left eyes of vaccinated and non-vaccinated calves following instillation of M.bovis (GS), into each right eye, on day 43.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)									
	0	14	28	35	42	49	56	63	70	77
1L	-	N	-	-	4	2	N	N	4	4
1R		N	N	-	2	N	N	4	8	8
2L	-	-	-	-	2	N	2	N	N	N
2R		-	-	-	2	N	N	N	N	N
3L[A]	-	-	N	N	N	N				
3R		N	N	N	2	-				
4L	-	2	N	N	2	N	N	N	N	N
4R		4	N	N	2	N	N	N	N	N
5L[B]	-	2	N	N	4	N				
5R		N	N	-	4	-				
7L	-	N	N	-	4	N	N	N	N	-
7R		N	N	N	4	-	4	N	N	N
8L[C]	-	N	-	N	2	8	16	N		
8R		N	-	-	2	N	4	8		
9L	-	N	-	N	2	N	N	N	N	N
9R		N	-	-	N	N	N	N	N	N
10L	-	N	N	2	2	N	4	2	N	N
10R		N	N	2	2	N	N	N	N	2
11L	-	2	2	N	4	4	4	2	N	2
11R		N	N	N	2	N	16	4	4	4
12L	-	2	2	N	N	2	-	4	N	2
12R		N	N	N	N	N	N	N	N	2
13L	-	N	N	N	N	2	-	N	-	N
13R		N	N	N	2	N	2	N	N	N
14L	-	N	2	N	N	2	-	N	N	8
14R		N	N	-	2	4	-	N	N	N
15L	-	N	N	-	N	N	-	-	-	N
15R		N	2	-	N	2	2	-	N	N
16L	-	2	-	-	N	2	-	-	N	N
16R		N	-	-	2	2	N	N	N	N

L Left eye
R Right eye
\- No haemagglutination
N Haemagglutination in neat dilutions only

A Calf 3 slaughtered day 52
B Calf 5 slaughtered day 53
C Calf 8 slaughtered day 67

TABLE 48. Reciprocal IHA titres against whole cell *M.bovis* in lachrymal secretions, vaccination experiment.

SOURCE OF SAMPLE	SAMPLING TIMES (DAYS)										
	O	7	14	28	35	42	49	56	63	70	77
1	N	N	N	-	N	N	2	2	16	4	8
2	N	N	N	N	4	N	N	2	4	2	2
3A	N	N	N	-	N	N	N				
4	N	N	N	-	N	N	2	N	8	8	4
5B	N	N	N	N	N	N	N				
7	-	2	2	16	8	128	16	64	64	8	4
8C	N	2	2	16	4	2	4	16	8		
9	-	N	N	2	2	8	4	8	8	4	4
10	-	2	N	2	8	4	4	8	8	4	4
11	2	2	8	4	8	64	128	128	128	32	32
12	N	2	N	N	4	4	8	8	4	4	4
13	N	N	N	2	4	4	4	4	2	8	2
14	N	N	N	2	2	8	64	2	8	16	4
15	N	2	2	2	2	N	4	16	16	8	4
16	N	2	8	2	64	8	16	16	16	8	2

- No haemagglutination
N Haemagglutination in neat dilutions only

A Calf 3 slaughtered day 52
B Calf 5 slaughtered day 53
C Calf 8 slaughtered day 67

TABLE 49. Reciprocal IHA titres against whole cell M.bovis in serum samples, vaccination experiment.

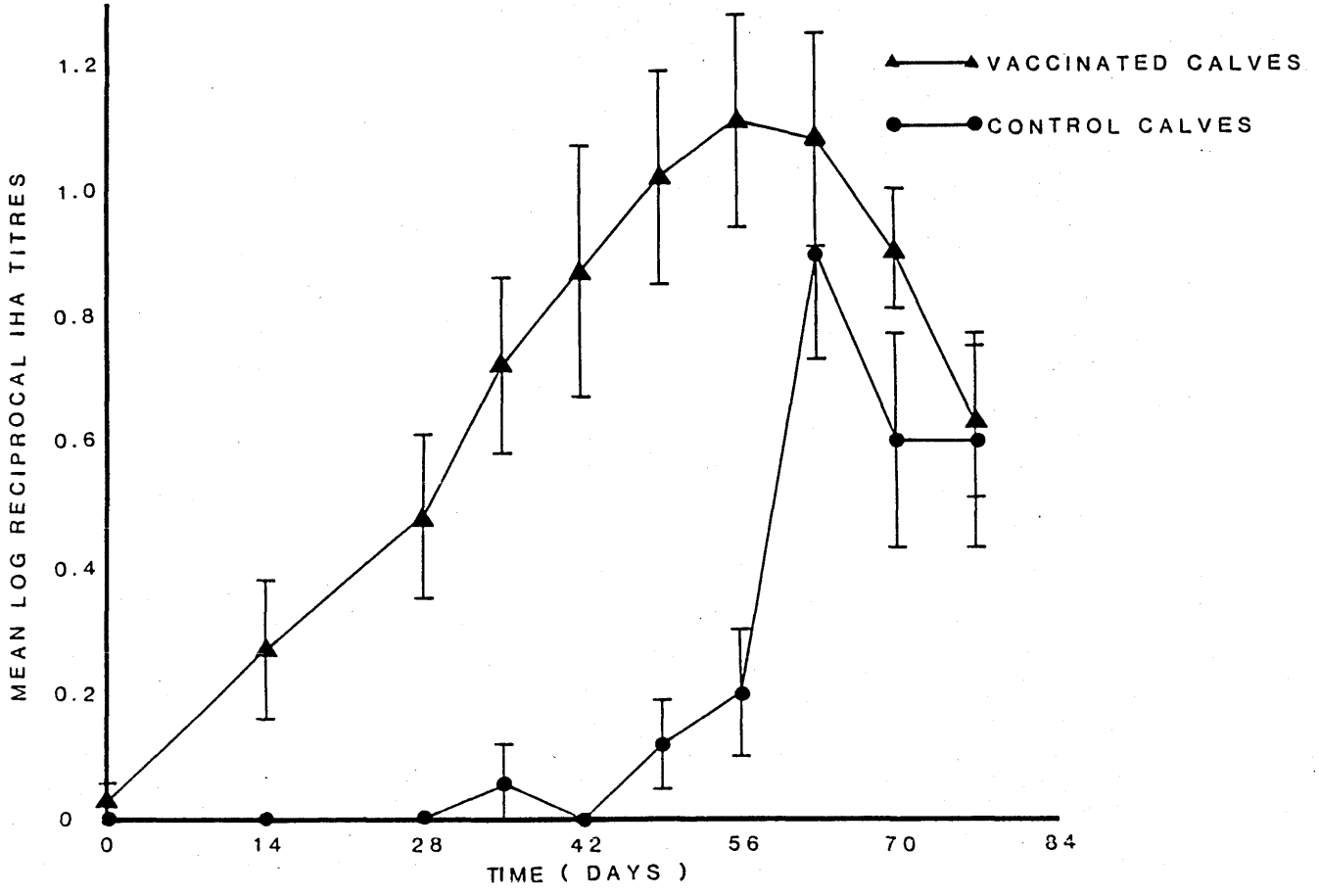


Figure 22. Vaccination experiment. Mean log reciprocal IHA titres. Days 0 to 77.

of 0.9 on day 63 and declined to 0.6 by day 77. In the vaccinated group, mean log titres rose slowly from day 0, reached a peak of 1.11 on day 56 and declined to 0.63 by day 77.

- Control group

Significant antibody titres to M.bovis were not found in any of the serum or lachrymal samples collected prior to challenge.

Following challenge a significant but low reciprocal antibody titre of eight was found on only one occasion, in the serum sample collected from calf 1 on day 63. In lachrymal secretions, significant but low reciprocal antibody titres were found in two samples, collected from the right eye of calf 1 on days 70 and 77, following resolution of severe lesions of IBK in this eye.

- Vaccinated group

Significant antibody titres were not found in any of the serum or lachrymal samples collected prior to initial vaccination.

Significant reciprocal titres were found in calves 11 and 16 on day 14, calves 7 and 8 on day 28, calves 7, 11 and 16 on day 35 and calves 7, 9, 11 and 16 on day 42. Significant levels of antibody were not found in any of the tear samples collected prior to challenge.

Calf 7, which had transient lesions in the left eye on day 65, had significant serum titres between days 42 and

70 although significant lachrymal titres were not found at any time.

Calves 13, 14 and 16 were mildly affected in one or both eyes. Of these, calf 14 had significant serum titres on day 49 and between days 63 and 77 and calf 16 between days 42 and 70; significant titres were never detected in calf 13. Significant lachrymal titres were found in one sample, collected from the left eye of calf 14 on day 77.

Calves 9, 11, 12 and 15 were severely affected in the right eye, with lesions present 24 hours following challenge. Calf 9 had significant serum titres in samples collected on days 42 and 56, calf 11 between days 42 to 77 inclusive, calf 12 on day 49 and calf 14 on days 56, 63 and 70. Significant lachrymal titres were found in one sample, collected from the right eye of calf 11 on day 56.

Calf 8 developed severe, bilateral lesions after a delayed incubation period and was slaughtered on day 67. Significant serum titres were found on days 56 and 63. Significant lachrymal titres were found on days 49 and 56 in the left eye and on day 63 in the right eye.

Calf 10 was mildly affected in the right eye and severely affected in the left eye with lesions being first noted on days 65 and 74, respectively. Significant serum titres were present in one sample only, collected on day 63. Significant lachrymal titres were not found at any time.

- Reaction to vaccination

There was no apparent reaction in any of the calves following initial vaccination on day 0. Seven days after revaccination on day 28 a nodule was palpable at the site of injection in calves 8, 9, 10, 12, 13 and 14 accompanied by enlargement of the ipsilateral prescapular lymph nodes. Fifteen days after revaccination, the lymph nodes were no longer enlarged although subcutaneous nodules 1 to 2 cm in diameter were still palpable at the injection site. These were halved in size by day 57 and were no longer palpable by day 71.

Of the six animals which produced a vaccine reaction three were severely affected in the right eye and three mildly. Two of the left eyes were severely affected, two mildly affected and two remained free from disease. In the right eyes of those which did not react to vaccination, two were mildly affected and two severely. In the left eyes, two remained free from lesions, one was mildly affected and one severely.

DISCUSSION

In the above investigation, the efficacy of a formalin killed, whole cell vaccine prepared from a highly pathogenic strain of M.bovis was assessed, on the basis of immunological, bacteriological and clinical results, following homologous challenge in experimental calves.

A variety of methods have been used to study immune responses in cattle to M.bovis. Agglutination (89)

tests have been shown to be useful in qualitative terms, although the results may be affected by the tendency of strongly fimbriated strains of M.bovis to autoagglutinate (76) and methods to prevent this, such as the addition of 10% magnesium chloride (15%) or removal of agglutinated masses by centrifugation, may affect the immunogenic composition of the test antigens. Other workers have used a GDP test (100) although again, this test is purely qualitative.

More recently, accurate FATs (118,141) and ELISA tests (25,132) have been developed. Unfortunately, it is difficult to quantify serological response with the former due to indistinct end points while the development and standardisation of the latter test was not feasible. Given the facilities presently available, the IHA test was used to quantify the immune response although the poor sensitivity of this test was recognised.

The serological response to M.bovis vaccination in this experiment was poor and did not correlate well with either presence or severity of disease in individual calves. In particular, calf 11, which had the most consistently raised serum (IHA) antibody levels, also had the most severely affected right eye within the vaccinate group while its left eye remained absolutely free of lesions. Similar situations in which measurable serum or lachrymal antibody have failed to protect calves have been reported, particularly in respect of field outbreaks (13, 14, 104, 163). Quite apart from the fact that the majority

of calves did not attain high titres, two calves had failed to develop detectable levels of circulating antibody prior to challenge.

The IHA test was also used to measure the immune response in tears. The IHA response in tear secretions was much lower than that found in serum, with significant titres being found on only seven occasions from four calves. Such a situation was expected, as in the healthy eye, the protein concentration in tears is approximately one tenth of that found in serum (133,151) such levels falling further in inflamed eyes, despite increased conjunctival seepage of serum, due to increased serous tear production by the lachrymal and accessory lachrymal glands (133). In addition, collection techniques may well have exerted a significant effect on tear production rate (197). Methods are available that will not produce a reflex increase in production, such as nasolachrymal catheterisation (95,197) or manual pipetting from the medial canthus (150). However, in the above studies collection was made by sponge strips which could possibly have induced reflex lachrymation with consequent decrease in tear protein concentration. It has been demonstrated in healthy eyes, that the predominant antibody in lachrymal secretions is locally synthesised SIgA although IgG_1 is also present having been preferentially derived from serum (150,151,154). It has also been shown that in diseased eyes, the relative concentration of IgG_2 increased as inflammation of the mucous membrane surfaces allows easier diffusion of plasma proteins from serum onto external surfaces (151). It is

therefore surprising that in normal unvaccinated, recovered cases of IBK, the highest specific titres in lachrymal secretions as measured by FAT have been found in the IgG class of antibody (118). Other workers using a different test system in similar animals found the highest titres occurred in the IgA class of antibody (25,143).

As in previous experiments, BHV1, adenoviruses or PI3 were not isolated from the experimental animals. Mycoplasmas were isolated, mainly from nasopharyngeal swabs and occasionally from ocular swabs but, as discussed above (Chapter 3), the mycoplasmas that were isolated were considered to be common inhabitants of the upper respiratory tract of immature cattle and were probably not significant factors in the production of ocular disease. Myco.bovoculi which has been associated with conjunctivitis (186) and implicated in the pathogenesis of IBK (185) was never isolated during this trial. A large variety of bacteria were isolated and identified from ocular swabs, the most frequently isolated bacteria were those which are normal commensals in mucous membranes of the upper respiratory tract (3). A number of species of bacteria were isolated less frequently, mainly from the Enterobacteriae group, and were probably transient inhabitants as a result of faecal or other contamination of the eyes. It is interesting to note that M.(B.)ovis was isolated from several healthy eyes during the early stages of this experiment but only from one right eye on day 49 following exposure to M.bovis. Strains of this bacterium have been associated with the development of

infectious keratitis in sheep (56,128,204,205,206) and goats (36) while some workers have suggested that it may have a role in the production of IBK (18,56,232). The strain isolated in this experiment appears to have been either non-pathogenic or of low pathogenicity as it was not associated with any clinical signs. Moreover the relatively few isolations and its early disappearance suggests that this organism had no significant role in the development of disease.

Moraxella bovis became established in the right eyes of all animals, vaccinates and controls, within four days following initial exposure and was consistently isolated during the next 40 days. These isolation rates are higher than in other studies using killed vaccine strains (100,101,162,227) and much higher than when live vaccines have been used (99).

Infection was transmitted to the left eyes of both groups of calves in an average time of five days. However, marked differences were found to be present in the establishment of infection since, in the control group, M.bovis became established in all left eyes immediately following the first isolation while, in the vaccinates, infection did not become established in some eyes for a further several weeks. This apparent delay in establishment may reflect either successful clearance of M.bovis by host defences or very low levels of infection (164). However, post-establishment infection rates were similar in both vaccinates and controls although it is difficult to judge

the significance of this due to the reduced size of the control group following elective slaughter of two calves.

Frequency of isolations from all eyes remained consistently high in both groups until day 78, and then decreased by approximately 50% between days 79 and 83. However, this rate of decline was exaggerated by losses due to a contaminated batch of agar affecting results on days 79, 70 and 81. These results contrast with those of previous workers (101,103,162,176) who found that the duration of infection in vaccinated calves was decreased to between 30 and 50% of that of the control calves, although in the same studies the mean duration of infection in the control groups was 35 days or less. These differences are explicable on the basis of experimental design, choice of challenge organism and methods of isolation. In the present studies both control and vaccinated calves were maintained as a single large group; thus, vaccinated calves may have been subjected to higher continuing challenge from non-vaccinated calves in contrast to previous studies, where vaccinated and control calves were maintained in isolation (101,103,162,176). In addition, infection and disease was readily produced without recourse to UV irradiation which suggests that the strain of M.bovis used was of higher pathogenicity. Finally, initial isolations were made on Tween 80 agar which appears to be a more sensitive isolation medium than standard BAPs.

Differences were found between control and vaccinated calves in terms of incubation period and severity of disease. Moreover, as in previous studies, it was agreed that any animal developing severe bilateral lesions or corneal perforation would be slaughtered. In the event, this policy resulted in the slaughter of two of five control and only one of ten vaccinated calves.

All five calves in the control group developed severe lesions of IBK in their right eyes, following exposure to M.bovis. While all ten right eyes in the vaccinated group developed lesions, only five were severe and five mild. In addition over the initial 25 days post-challenge the mean clinical score of the vaccinated calves was almost 30% less than that of the controls. Clearly, it is possible that the actual reduction is greater due to bias within the scoring system, which allocates equal weighting to signs of irritation and degree of corneal damage; moreover no allowance was made for calves 3, 5 (controls) and 8 (vaccinated) which were slaughtered. Similar results have been reported by other workers using homologous challenge, although in these cases challenge was preceded by UV irradiation and protection against the disease has mainly been measured by reduced prevalence rather than by a reduction in clinical severity (99,100, 103). Webber and Selby (227), however, using a modified scoring system, originally devised to grade lesions in field outbreaks (116), found a reduction in severity of between 42.2% and 80.4% dependent upon whether the vaccine was administered subconjunctivally or s.c.

Vaccination resulted in a similar reduction in disease in the left eyes of the calves in this experiment, the average clinical score over the 40 day period being reduced by 32.1%. However, these results must be treated with some caution due to the very small numbers of eyes affected in both groups of calves thereby allowing the severity of lesions in individual eyes to produce extreme influences on the mean clinical score. In this situation, the challenge doses in the left eyes were probably comparable to the challenge to healthy eyes during field outbreaks of IBK. The results of the present study clearly demonstrate that the protection afforded was less than total.

The efficacy of vaccines against homologous challenge as judged by protection against the development of clinical signs under laboratory conditions has been shown to vary with the type of vaccine used, route of administration and timing between vaccine doses following intramuscular injection. Live vaccines produced better protection than formalin killed vaccines (99,100), which in turn have a higher efficacy than heat killed vaccines (100). However, suspension of the antigen in an oil or aluminium hydroxide adjuvant may, at least with formalin killed vaccines, increase efficacy (162). Under similar circumstances vaccines prepared from purified pili combined with diphtheria-tetanus toxoids and B.pertusses vaccine have also produced such improved effects (173). Again with pili vaccines, failures to increase efficacy have been reported using Freunds incomplete adjuvant (162) and

Mycobacterium paratuberculosis bacterin (178). Vaccination by the i.m. approach has been associated with adverse reactions (99) and in the majority of vaccine trials the s.c. vaccination route has been used. This latter approach has also been shown to be superior to local periocular vaccination which not only resulted in poorer protection but in some circumstances has induced severe granulomatous reactions (227), although other workers have reported contradictory results (174).

Timing between vaccine doses affects the development of an immune reaction and resistance to the disease, and vaccine doses given three weeks apart are recognised as being more efficacious than those one week apart (101).

The use of autogenous vaccines in the field (i.e. strains isolated from early cases in the same herd) has produced equivocal results, with similar rates of infection and disease in both vaccinated and control animals (13,104, 172). This may reflect either failure of these vaccines to protect cattle against immunologically identical strains or the infiltration of fresh immunological variants of M.bovis into the protected herd.

The above factors must be considered if attempts are to be made to develop a commercial vaccine. The use of a systemically administered live vaccine is unlikely to be viewed as an acceptable option due to the known production of a wide range of toxins (93,99,147,169,189) which have been shown to produce severe local and systemic

reactions, in some cases leading to sudden death. While an effective formalin killed, whole cell vaccine is economically justifiable the use of a pure pili vaccine is to be preferred despite the currently expensive purification processes required (83). Indeed, it may be that the latter approach, combined with the use of a suitable adjuvant, will prove to be the way forward in the prevention of IBK.

CHAPTER 5

PATHOLOGICAL AND MICROSCOPIC STUDIES

CHAPTER 5

PATHOLOGICAL AND MICROSCOPIC STUDIES

INTRODUCTION

The anatomical position and nature of the tissue affected by IBK allows detailed examination in the live animal of the gross pathological changes induced and these are well documented in descriptions of the clinical signs (18,21,58,67,134,229,230). However, despite the obvious importance of such changes in the elucidation of the early pathogenesis and subsequent development of lesions, it has been noted that few papers have been published describing the histological and ultrastructural changes induced by IBK (41,180,230).

Chandler and others (41) described the fine structure of the normal bovine cornea, finding it to be similar to that reported for other mammalian species. In the same study the changes associated with the ulcerative stages of IBK were described. However, little information was provided regarding the earlier and non-ulcerative stages. Furthermore, it would appear that little is known about changes which may arise in closely related tissues. In the following chapter the histological changes induced in the ocular tissues by infection with M.bovis are described.

Isolates of M.bovis which are pathogenic in experimental infections have been shown to be strongly fimbriated when examined by electron microscope (39) or have colony characteristics suggestive of a high degree of

fimbriation (76,153). In vitro experiments using corneal cells have demonstrated that fimbriation is required for adhesion of the bacteria to the cells and that non-fimbriated bacteria are easily removed by gentle washing (6,40).

This has encouraged the testing of vaccines prepared from purified pili of M.bovis (161,162,173,174, 178) and although these vaccines have been shown to provide some degree of protection the precise mechanism of action has not yet been defined. In any event, the protection obtained appears to be less than that provided by previous exposure to the disease (104).

The second study described in this chapter was designed to investigate the effect of immune sera on the ability of fimbriated strains of M.bovis to adhere to corneal cells in vitro and also the possibility that previous disease might produce an increase in the ability of the cornea to resist adherence of M.bovis.

MATERIALS AND METHODS

Pathological and histological examination of bovine corneas

- Source of samples

The eyes were obtained from four calves (3,5,8 and 9) slaughtered at varying times following experimental infection with strain GS of M.bovis. Calves 3 and 5 were slaughtered, on humane grounds, following complete perforation of the cornea of their right eyes nine and ten days after initial exposure, respectively. The cornea of

the left eye of calf 3 contained a two day old ulcer and that of calf 5 was scarred as a result of healing of a previous corneal ulcer considered to be of probably traumatic origin. Calf 8 was slaughtered, again on humane grounds, due to the development of bilateral blindness, the cornea of the right eye being affected by a 12 day old ulcer and that of the left by a four day old ulcer. Calf 9 was electively slaughtered specifically to study the pathological changes found in the early stages of the disease, the cornea of the left eye having been affected by an ulcer of less than 24 hours duration while the right eye was clinically normal despite being infected by M.bovis.

Immediately following slaughter, the eyes and surrounding tissues were removed by careful incision round the rim of the orbit, severing the extraocular muscles and optic nerve and then immersed in 10% buffered formal saline for 24 hours prior to dissection and post-fixing in corrosive formal. Samples were processed by standard methods for histopathological examination and stained with haematoxylin and eosin. Additional samples were stained with Martius scarlet blue, phosphotunstic acid and haematoxylin and carbol chromotrope.

Samples were examined for the presence of M.bovis by indirect immunofluorescence in trypsinised paraffin embedded sections (211).

Studies on adherence of M.bovis to corneal samples

- Collection of corneal samples

Corneas were obtained from nine of the ten experimental animals in groups 1 and 2 and from one control animal not exposed to experimental infection. The eyes were removed immediately after slaughter and placed in sPBS. The corneas were then dissected from the eye within ten minutes and cut into three, triangular sections, each of approximately 0.5 cm^2. Each section was then placed in individual multi wells (Gibco Bio-cult, Paisley, Scotland), suspended in 2 ml of sPBS at room temperature and washed three times with sPBS. One cornea from each of calves 5, 7 and the control animal were placed in formol saline for histopathological examination.

- Preparation of bacterial suspensions

Three strains of M.bovis were used, the inoculating strain, GS, a low passage strain reisolated from the left eye of calf 10, GS (RI), and a low passage strain, GVS, isolated from a mildly affected field case. All three strains were highly fimbriated when examined by electron microscopy as described in Chapter 2, section G.

The bacteria were grown overnight at 35°C on blood agar plates and harvested using a sterile inoculating loop. Six suspensions of M.bovis each using the growth of one BAP per 10 ml of suspension were prepared as follows; Suspension A - live strain GS in sPBS; Suspension B - strain GS in sPBS and heat killed at 56°C for 30 minutes;

Suspension C - live strain GVS in sPBS; Suspension D - strain GVS in sPBS and heat killed at 56°C for 30 minutes; Suspension E - live strain GS in serial dilutions of immune rabbit serum in sPBS ranging from 1:10 to 1:1280 and incubated at 35°C for 30 minutes; Suspension F - live strain GS(RI) in sPBS.

The wells containing the corneal samples were drained immediately prior to use and each corneal piece was exposed to 1 ml of one of the previously prepared suspensions and incubated at room temperature for 15 minutes before being washed three times in sPBS and fixed in 2 ml of 10% formal saline.

Histological sections were prepared and stained using indirect immunofluorescence and examined "blind" under a fluorescent microscope (211).

RESULTS

Pathological and histological examination of bovine corneas

- Calf 3

The right eye contained an eight day old corneal ulcer which extended over 80% of the corneal surface and which contained a central perforation 2 mm in diameter (figure 23). The base of the ulcer was covered by an exudate of neutrophils and macrophages and these cells were present in large numbers throughout the corneal stroma. Vascularisation extended from the corneoscleral junction into the superficial layers of the cornea to the ulcer edge. The iris and ciliary body were heavily infiltrated

FIGURE 23. Vaccination experiment, calf 3R, perforated ulcer, H & E staining x 4.

The cornea contains a perforated ulcer with prolapse of the iris across the perforation. The surrounding corneal stroma is thickened due to oedema and vascular tissue is present in the anterior layers extending from the corneoscleral junction.

with neutrophils, the iris having fallen forward to obliterate the anterior chamber of the eye thereby sealing the perforation. The conjunctivae were oedematous and infiltrated by many neutrophils. There were many large subepithelial lymphoid aggregates present with the formation of germinal centres.

The left eye contained a two day old shallow, anterior polar ulcer 5 mm in diameter (figure 24). Histologically there was a shallow erosion of the substantia propria at the ulcer base with marked infiltration of neutrophils and macrophages. Large numbers of Gram-negative bacteria were present at the base of the ulcer penetrating the corneal stroma with extensive lateral spread. The corneoscleral junction, scleral spur and the base of the ciliary body were heavily infiltrated by neutrophils and occasional macrophages and vascularisation extended 2 mm from the corneoscleral junction in the superficial layers of the corneal stroma. The conjunctiva was infiltrated by many neutrophils and many large subepithelial lymphoid aggregates were present.

- Calf 5

The right eye contained a nine day old, 1 cm diameter, anterior-polar, corneal ulcer with a white, dry base and a central perforation. Microscopically (figure 25), while there was complete loss of epithelium over the ulcer base the remaining epithelium was firmly attached at the edges with no under-running. There was extensive vascular tissue development throughout the depth of the

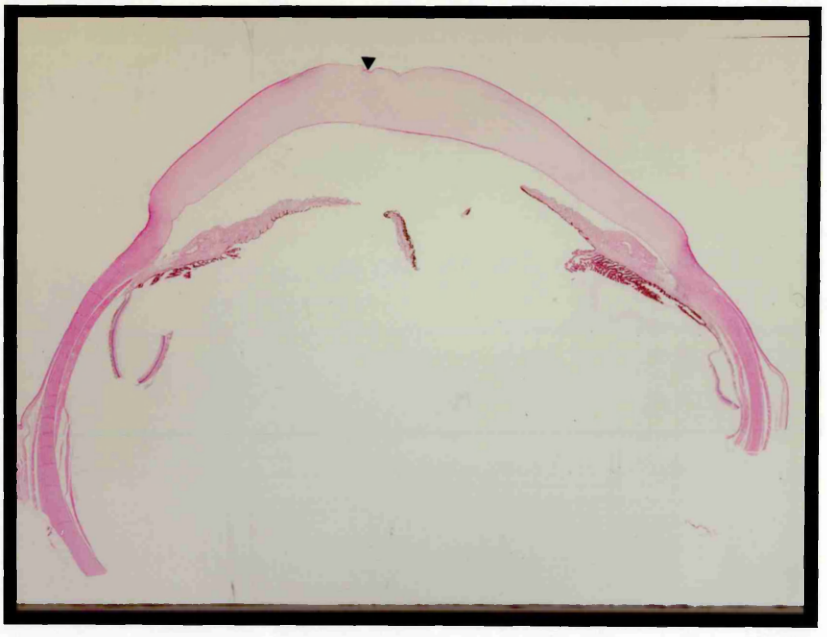


FIGURE 24. Vaccination experiment, calf 3L, 2 day old ulcer, H & E staining x 4.

There is oedema of the corneal stroma resulting in increased corneal thickness over the entire cornea. An ulcer is present at the anterior pole with shallow erosion of the underlying stroma and extensive loss of epithelium.

FIGURE 25. Vaccination experiment, calf 5R, perforated ulcer, H & E staining x 4.

There is complete corneal perforation with prolapse of the iris▲. The cornea is markedly thickened due to oedema and vascular tissue extends from the corneoscleral junction to the ulcer edge▲▲.

cornea, extending under the ulcer edge. The ulcer base contained granulation tissue infiltrated by large numbers of macrophages and neutrophils. The conjunctivae were oedematous with extensive subconjunctival infiltration by neutrophils and small numbers of plasma cells with well developed lymphoid aggregates (figure 26). There was mild neutrophil infiltration of the scleral spur, iris and ciliary body.

The left eye contained a small white opacity that had arisen subsequent to the healing of a, possibly traumatic, corneal ulcer; otherwise it appeared normal. Microscopically (figure 27), the scar was marked by slight disruption of the normal cellular architecture with a small number of capillaries present in the superficial layers of the substantia propria. A very mild neutrophilic reaction was present subconjunctivally with no evidence of lymphoid hyperplasia.

- Calf 8

In the right eye there was a 12 day old central corneal ulcer approximately 1 cm in diameter with a dense white base with only a small degree of surface erosion. The surrounding corneal tissue was opaque and vascularisation extended approximately 1 cm from the corneoscleral junction. The third eyelid was granular in appearance. Microscopically (figure 28), the ulcer consisted of oedematous tissue infiltrated by large numbers of neutrophils with extensive loss of epithelium. There was a moderate neutrophilic reaction throughout the

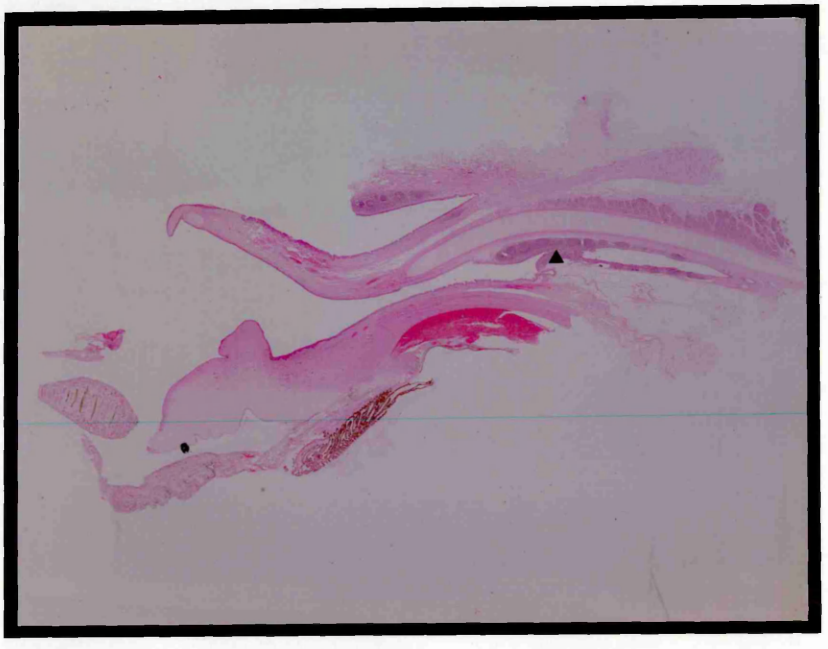


FIGURE 26. Vaccination experiment, calf 5R, perforated ulcer, H & E staining x 4.

Enlarged subepithelial lymphoid aggregates with germinal follicles are present at the base of the nictitating membrane▲

FIGURE 27. Vaccination experiment, calf 5L, healed ulcer, H & E staining x 4.

The cornea is of normal thickness with vascular tissue present in the anterior layers extending from the corneoscleral junction to the anterior pole.

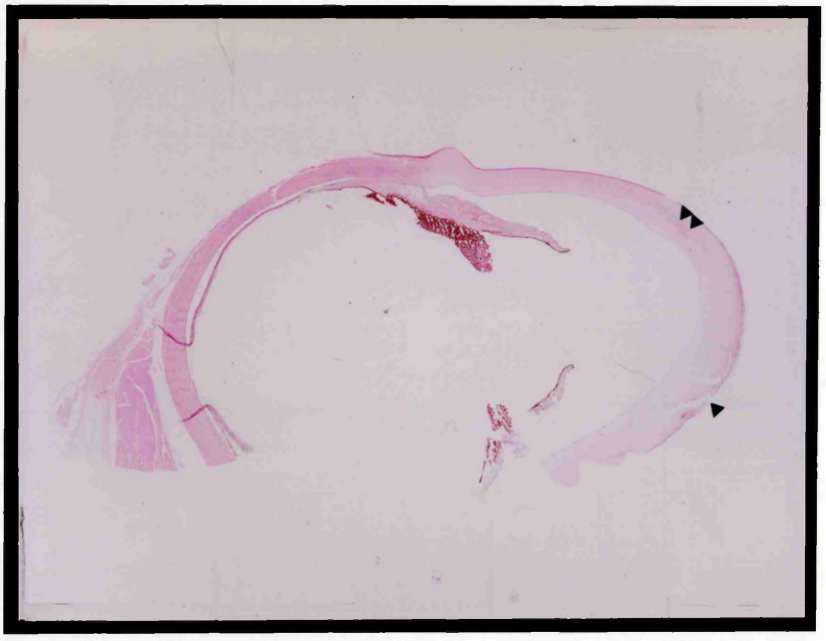


FIGURE 28. Vaccination experiment, calf 8R, 12 day old ulcer, H & E staining x 4.

There is erosion of the corneal stroma at the ulcer site with loss of overlying epithelium, with surrounding epithelium oedematous and thickened▲. The cornea is oedematous with vascular tissue in the anterior layers extending 1cm from the corneoscleral junction▲▲

substantia propria and an extensive reaction at the corneoscleral junction and in the region of the scleral spur. Vascular tissue was visible in the superficial corneal stroma extending from the corneoscleral junction. The iris and ciliary body were normal and there were many small subepithelial lymphoid aggregates in the conjunctiva.

The left eye was similar to the right although the lesion was less advanced with a smaller, four day old corneal ulcer present. Vascular tissue was again clearly visible in the superficial layers of the cornea extending 2 mm from the corneoscleral junction. Again the iris and ciliary body were normal.

- Calf 9

In the right eye, there were no histological changes present in the cornea, iris or ciliary body but a mild inflammatory reaction was noted affecting areas of the conjunctiva and small subepithelial lymphoid aggregates were also noted. The mild opacity noted in the left eye immediately prior to slaughter was, on microscopic examination, attributed to mild oedema with slight separation of corneal cells in the stroma with infiltration of low numbers of eosinophils. The overlying corneal epithelium appeared normal. In contrast there was complete disruption and loss of the corneal epithelium at the edge of the ulcer with slight separation and under-running from the stroma at the level of Bowman's membrane. Small aggregates of bacteria were present on the inner surface of the epithelium. There was slight erosion of the

superficial layers of the stroma at the base of the ulcer with marked disruption of the normal cellular architecture. Small amounts of debris were present overlying the ulcer base associated with small aggregates of bacteria which were also present within the substance of the stroma. Significant histological changes were present at the corneoscleral junction with oedema and infiltration of many neutrophils and to a lesser extent eosinophils and macrophages around the canal of Schlemm and the scleral spur with localised infiltration of the overlying epithelium. Similar histological changes were noted affecting the conjunctiva, particularly the palpebral conjunctiva where the squamous epithelial cells were pale and sloughing from the surface with many adherent Gram-negative bacteria, identified as M.bovis by immunofluorescence (figure 29). The iris and ciliary body appeared to be normal.

Studies on adherence of M.bovis, to corneal samples

The results from this experiment are summarised in table 50.

Live strain GS (suspension A) adhered to the cornea most consistently, numerous bacteria being present in eight of the nine corneas exposed but only small numbers adhering to the ninth. In only one sample were the bacteria clumping together. Live strain GS (RI) (suspension F) gave similar results with numerous bacteria adhering to six out of eight corneas, with few and no bacteria each present on one occasion. Clumping was again present in one sample

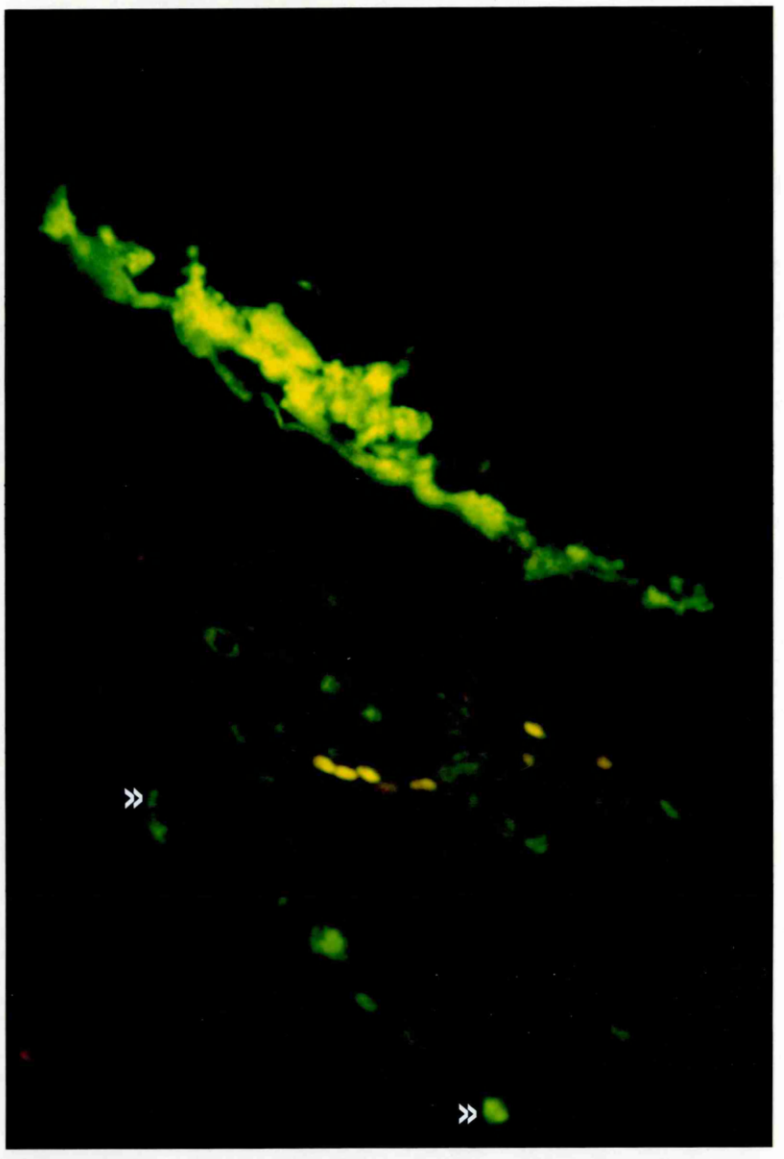


FIGURE 29. Experiment 2, calf 9, conjunctiva, day 3. Fluorescent antibody staining x 1000.
Aggregates of M.bovis are adherent to the superficial epithelial cells which are sloughing. Occasional fluorescing bacteria are present sub-conjunctivally.

CORNEAL SOURCE	STRAIN OF M.BOVIS USED: GS	KILLED GS	GVS	CORNEAL SOURCE	KILLED GVS	GS + ANTI SERUM	REISOLATED GS 1OL 15/6
1L	+++e	-	+c	1R	-	+++cc(1:10)*	+++e
2L	+e	-	+++e	2R	-	+++cc(1:20)	+++c
3L	+++e	-	+++cc	3R	-	+++c(1:40)	+++e
4L	+++e	-	+++c	4R	-	+++cc(1:80)	+++e
5L	+++e	-	+	5R	ND	ND	ND
6R	+++e	-	+e	6L	-	-(1:160)	-
7R	ND	ND	ND	7L	-	+++cc(1:320)	+++e
8R	+++cc	-	+e	8L	-	-(1:640)	+e
1OR	+++e	-	+++e	1OL	-	+cc(1:1280)	+++e
CONTROL	+++e	ND	-		ND	+++cc(1:640)	ND

+++ Numerous bacteria adhering to cornea
+ Few adherent bacteria
- No bacteria found
cc Most bacteria found in clumps
c Some bacteria found in clumps
e Bacteria evenly distributed

ND Sample not tested
* Dilution of antiserum used

TABLE 50. In vitro adhesion of M.bovis to corneal pieces.

only. In corneas exposed to live strain GS which had been incubated in immune antiserum (suspension E), numerous bacteria were found adhering in six out of nine samples, a few in one and none in two. In six of the adhering samples the majority of bacteria were found in clumps and in the remaining sample a minor degree of clumping was present. In samples exposed to the GVS strain (suspension E), numerous adherent bacteria were found on only four out of nine corneas, a few bacteria were found in four other corneas and none on the remaining, control, cornea; clumping was present on two occasions. Killed strains GS (suspension B) and GVS (suspension D) both failed to adhere to any of the corneas exposed.

DISCUSSION

The histological changes found in the corneas affected by non-perforating ulcers are similar to those recorded in both gnotobiotic and conventional calves following experimental infection and with those reported from field cases (41). In the ulcers that were examined, there was complete loss of epithelium and Bowman's membrane over the base of the ulcer with infiltration of the cornea by large numbers of neutrophils. Significant numbers of macrophages were also present underlying the base of the ulcers. In early ulcers, bacteria could be seen associated with Bowman's membrane and within spaces formed by separation of the epithelium. Healing of the ulcers followed the development of granulation tissue over the ulcer base and infiltration of capillaries, principally

within the superficial layers of the cornea.

Iridospasm is a consistent clinical feature present in the early stages of IBK and histopathology of samples obtained from an abattoir has shown the presence of iriditis (41). However, in the present studies inflammatory changes were only present in those cases with perforating corneal ulcers. It seems therefore more likely that the iridospasm arises as a result either of pain reflexes or local diffusion of inflammatory by-products from the corneal or scleral spur where neutrophil infiltration was consistently noted.

In one infected, albeit clinically normal eye, minor inflammatory changes were noted affecting the conjunctiva although the cornea appeared to be normal. Thus, it would seem that, at least in experimental IBK, conjunctivitis precedes keratitis, which is in agreement with most previous workers (17,21,57,62,82). However, in more severely affected eyes the pathological changes recorded in the conjunctiva were less severe than those affecting the cornea. In early cases, the cells of the conjunctival epithelium appeared pale with marked sloughing of the superficial squamous cells. These cells were noted to have adherent bacteria, which were identified as M.bovis by immunofluorescence, although the basal cell layers appeared normal and free from bacteria. Large numbers of infiltrating neutrophils were found in the subconjunctival tissues which were oedematous and vascular injection was noted. The cellular infiltration was most

marked at the region of the corneoscleral junction and canal of Schlemm. In more advanced lesions, marked lymphoid hyperplasia was present subepithelially with formation of germinal centres.

Sections stained by Gram's stain and by immunofluorescence methods revealed the distribution of M.bovis within the tissues. Although the former test is limited to defining the general morphology of bacteria present, the FAT and related tests have been demonstrated to have a high degree of specificity to M.bovis (118,130, 219). In areas with intact corneal or conjunctival epithelium, the bacteria were restricted to the superficial squamous cell layers and found to be adherent to the cell surface. However, at the site of ulceration bacteria were found deep within the corneal stroma, lying between collagen fibrils as described previously (41,44). These organisms were exclusively Gram-negative, morphologically similar to M.bovis and gave a positive reaction to immunofluorescence. Gram-positive bacteria and Gram-negative bacteria morphologically dissimilar to M.bovis were present in low numbers, but confined to the superficial debris present at the ulcer base. This pattern of bacterial infiltration is similar to that reported for experimental infections using mice (44) suggesting that extension of the lesion can be induced by M.bovis alone without the involvement of secondary pathogens.

Two of the isolates used in the in vitro experiment were GS strains of known experimental

pathogenicity. The third was of unknown experimental pathogenicity, having been isolated from a mildly affected field case of IBK. All three isolates were shown by EM to be strongly fimbriated and had colony morphologies comparable to the SC type (28).

While it has been noted that small numbers of non-fimbriated isolates of M.bovis may adhere to corneal cells (6) only fimbriated strains have been found adhering in large numbers (6,42). This situation is comparable to the adherence of fimbriated strains of N.gonorrhoea to cell cultures (35,121,210).

The method by which fimbriated strains of M.bovis attach to cells is poorly defined. Scanning EM studies have revealed a distinct pattern of adhesion with bacteria mainly being associated with lightly stained "dark" cells which contain many microplicae on their surface while few bacteria are found on the rougher surface of heavily stained "light" cells (40). In this present study, the degree and pattern of adherence produced by all three live isolates was similar with numerous, evenly-distributed bacteria present in the majority of cases. However poor adhesion was noted in samples from the right eyes of calves 6 and 8. This did not correspond to severity of, or resistance to, infection in life and it is possible that this was due to the physiologic status of these corneas i.e. possibly due to preponderance of light cells present or else damage of the corneal surface during preparation.

Even with the aid of EM it has not so far been possible to determine whether fimbrial attachment is by penetration of the cell membrane or simple association with the cell surface (6,40,210). There is, however, evidence that specific receptor sites exist on the bovine corneal cell surface (6). Since heat treatment would destroy specific binding sites it is interesting to note that there was no adherence present in any of the samples challenged by the heat killed M.bovis, a treatment that has also been shown to reduce the antigenicity of M.bovis pili proteins (100). It is obvious that further EM studies require to be carried out on heat killed M.bovis to determine whether the structural integrity of the pili is maintained. Similarly, the above, in vitro, approach would seem to provide an ideal opportunity for studies aimed at defining the adherence potential and, perhaps, pathogenicity of different strains of M.bovis.

Although incubation of M.bovis with specific antisera did not appear to affect the proportion of bacteria which adhered to the corneal surfaces, a marked difference in the pattern of adherence was noted with bacteria being found in clumps rather than evenly distributed as in samples to which no sera had been added. However, since the antiserum used in this experiment had been raised against whole cells of a fimbriated isolate of M.bovis such clumping may have been due to the combined effects of both fimbrial and somatic antibody.

CHAPTER 6

AN INVESTIGATION INTO THE USE OF FENVALERATE IMPREGNATED EAR TAGS IN THE CONTROL OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS UNDER FIELD CONDITIONS

CHAPTER 6

AN INVESTIGATION INTO THE USE OF FENVALERATE IMPREGNATED EAR TAGS IN THE CONTROL OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS UNDER FIELD CONDITIONS

INTRODUCTION

Although outbreaks of IBK can occur throughout the year the disease demonstrates a marked seasonal pattern with the highest incidence being recorded during the late summer months of July to September (13,18,33,55,98,160,180, 198,228,232). This has been associated with the period of peak solar radiation which occurs from June to July (105, 109) and with periods of peak fly infestation of grazing animals during July and August (32,46,49,207).

It has been demonstrated that the incidence of fly infestation in grazing cattle can be reduced by use of frequent topical applications of insecticides (15,72,216, 238) or by the use of insecticide impregnated ear tags (49, 72,94,127,238). Trials specifically designed to test the efficacy of insecticide treatment using cypermethrin impregnated ear tags in the control of IBK have given equivocal results with similar low incidences of disease occurring in both treated and control groups (49,238). However, regular topical treatment with tetrachlorvinphos or tagging with fenvalerate or tetrachlorvinphos impregnated ear tags have been shown to exert a marked effect upon the incidence of M.bovis isolations and disease (72).

The following experiment was designed to attempt to test the efficacy of a commercialy available insecticide impregnated ear tag in reducing the incidence of IBK in a herd, of spring calving beef cows and their calves, which had consistently suffered from a high incidence of severe IBK during previous summers.

MATERIALS AND METHODS

Experimental animals and their management

The herd used in this study consisted of 26 spring calving beef suckler cows of various breeds and their 25 calves, born between March 23 and May 5, 1984.

The herd had been overwintered from October to May in traditional byre-type accommodation, each being tied up in tandem stalls, and identified by numbered plastic neck bands. As each calf was born it was identified by the same number as its dam using individual plastic ear tags in the right ear. The calves were tied up behind their corresponding dams but were freed twice a day to allow suckling.

The calves were weighed and the herd was turned out on May 14 into a single, 10 hectare, south-facing field consisting mainly of rough grazing with areas of marshland and a long, overgrown hedgerow. Two sides of the field were bordered by a belt of trees and two sides by pasture belonging to a neighbouring farm. During June and July the herd ran with a Hereford bull and Charolais bull, respectively.

The herd remained in this field throughout the summer until October 9 when the cows were housed and the calves weighed and sold.

Sampling of animals

- Virology

Unilateral ocular (L) and nasopharyngeal swabs were taken from all animals in the group on the following dates - May 3,4, July 5, September 4 and October 9 and processed for the isolation and identification of viruses as described in Chapter 2, Section B.

- Mycoplasmology

Unilateral ocular (L) and nasopharyngeal swabs were collected from all animals in the group on the following dates - May 3,4, June 7, August 2 and October 9 and processed for the isolation and identification of mycoplasmas as described in Chapter 2, Section C.

- Bacteriology

Bilateral ocular and nasopharyngeal swabs were collected from all animals in the group on the following dates - May 3,4, June 7, July 5, August 2, September 4 and October 9. Additional ocular swabs were collected between these dates from animals showing signs of ocular irritation or which were diagnosed as having IBK. The swabs were returned to the laboratory within two hours of collection, immediately streaked onto Tween 80 plates and BAPs and, due to the number of samples involved, processed for the isolation and identification of M.bovis only as described

in Chapter 2, Section D.

- Serology

Serum samples were collected from all cows and calves on May 3,4, July 5, September 4 and October 9. Antibody levels against M.bovis were measured using an IHA test as described in Chapter 2, Section E. Comparisons of serological titres were made by converting to log reciprocal titres prior to statistical analysis by Student's "t"test. Samples in which no antibody was detected were given a nominal reciprocal titre of one.

Treatment of ocular disease

Treatment was given to all cases in which IBK was diagnosed (i.e. those showing signs of ocular irritation in conjunction with grossly visible corneal lesions). Such cases were treated by a single subconjunctival injection of 0.5 to 2 ml of 5% oxytetracycline hydrochloride injectable solution ("Engemycin 5%", Gist-Brocades, Braintree, Essex, UK) into the upper eyelid of both eyes. The dose given was dependent upon the relative size of the eyes and in the majority of cases was administered immediately following diagnosis. Treatment was repeated in those cases in which signs of ocular irritation persisted for two days.

Allocation to groups and insecticide treatment

At turnout the calves were allocated into one of two groups, A and B, of 13 and 12 calves respectively, balanced as close as possible for age, weight and sex.

The calves in group A were designated as the treatment group and they and their respective dams were each tagged in the left ear with a slow release ear tag impregnated with 8.5% W/W fenvalerate (Tirade, Hoechst UK Ltd., Milton Keynes, Buckinghamshire, UK). Neither the calves in group B nor their dams were tagged and were designated as the control group. Unfortunately, the weight of calf 22 in group A was not recorded at turnout and cow 16 and its calf in group B had to be moved to a separate field in late May due to the dam's lameness. The single barren cow had been given an ear tag impregnated with 8.5% cypermethrin (Flectron, Deosan Ltd., Northampton, UK) at an earlier date as part of routine husbandry procedure and this was not removed.

Clinical examination

The animals were examined daily by an experienced stockman who walked through the herd looking, among other things for signs of excessive epiphora and blepharospasm which are suggestive of IBK. When such cases arose, the affected animals were herded as soon as practicably possible into a mobile pen situated in one corner of the field and a detailed examination carried out. All examinations were carried out in daylight, usually in strong sunlight and supplementary light sources were not used.

At monthly intervals, during routine sampling periods, the eyes of all animals were examined with the aid of a torch and any abnormalities were noted.

RESULTS

Microbiology

- Virology

An adenovirus was isolated on one occasion only from an ocular swab collected from calf 9 on July 5. No other viruses were isolated.

- Mycoplasmology

Mycoplasmas were not isolated from any of the ocular or nasopharyngeal swabs submitted for examination.

- *Moraxella bovis* isolations

Isolations of *M.bovis* made from ocular swabs taken during routine sampling of the herd are summarised in table 51.

Isolations of *M.bovis* from samples collected from calves affected by ocular irritation or IBK and during routine sampling periods are illustrated in table 52. One hundred and thirty four ocular swabs were taken from the calves between July 15 and September 8. Of these, 79 (59.0%) were collected from clinically normal eyes, 32 (40.5%) of which were positive for *M.bovis*. Thirty-eight (28.4%) swabs were collected from eyes showing signs of ocular irritation only with 23 (60.5%) positive for *M.bovis*. Seventeen (12.7%) were taken from eyes diagnosed as having IBK, of which 14 (82.4%) yielded *M.bovis*. Isolations of *M.bovis* from samples collected from adults affected by ocular irritation or IBK and during routine sampling periods are illustrated in table 53. One hundred

SAMPLING DATE	TAGGED CALVES L	TAGGED CALVES R	TAGGED COWS L	TAGGED COWS R	NON-TAGGED CALVES L	NON-TAGGED CALVES R	NON-TAGGED COWS L	NON-TAGGED COWS R	CALVES TOTAL L	CALVES TOTAL R	COWS TOTAL L	COWS TOTAL R	TAGGED TOTAL L	TAGGED TOTAL R	NON-TAGGED TOTAL L	NON-TAGGED TOTAL R
3/5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7/6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5/7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2/8	7	6	8	4	3	6	2	1	10	12	11	10	15	10	5	7
4/9	2	2	3	3	3	3	2	4	5	5	5	7	5	5	5	7
4/10	-	-	-	-	1	2	-	-	1	2	-	-	-	-	1	2

- M.bovis not isolated

L left eye
R right eye

TABLE 51. Number of isolations of M.bovis during routine sampling periods, fly control trial.

TAGGED GROUP	CALF NUMBER	15/7 -21/7	22/7 -28/7	29/7 -4/8	5/8 -11/8	12/8 -18/8	19/8 -25/8	26/8 -1/9	2/9 -8/9	9/9 -15/9	16/9 -22/9	23/9 -29/9	30/9 -4/10
		SAMPLING DATES											
	2^{L}_{R}	1(-)	1(-)	(-)		1			(-)				1(-)
		(-)	(-)	(-)					2(-)				1(-)
	3^{L}_{R}			(-)					(-)				(-)
				(+)					(-)				(-)
	7^{L}_{R}		(-)	(+)				2(+)	1(-)				(-)
			1(+)	2(+)			2	(+)	1(-)				(-)
	9^{L}_{R}			(-)					(+)				(-)
				1(-)					(-)				(-)
	10^{L}_{R}		(-)	(-)			2	(+)	2(+)	1	1		(-)
			1(-)	(+)	1	1	2	1(+)	2(+)	2	1	1	(-)
	18^{L}_{R}			(+)			1	1	(-)				(-)
				(+)			1		(-)				(-)
	19^{L}_{R}			(+)					(-)				(-)
				1(-)					(-)				(-)
	21^{L}_{R}			(-)		1(+)	1	2	1(+)				(-)
				(-)	2	2(+)	1	2	2(+)				(-)
	22^{L}_{R}			2(-)	2(+)			1(+)	(+)				(-)
				(-)	(+)			2(+)	2(+)	1	1	1	1(+)
	23^{L}_{R}			(+)			1	1(+)	(-)				(-)
				(+)				(+)	(+)				(-)
	28^{L}_{R}		1(+)	(+)				1	(+)				(-)
			1(+)	(+)				1	(-)				(-)
	29^{L}_{R}			1(+)					(-)				(-)
				(-)	1				(-)				(-)
	35^{L}_{R}		2(-)	1(+)	1	1	2	1(+)	1(-)				(-)
			1(+)	(+)	2	2	1	1(+)	(-)				(-)

TABLE 52.

	CALF NUMBER	15/7 -21/7	22/7 -28/7	29/7 -4/8	5/8 -11/8	12/8 -18/8	19/8 -25/8	26/8 -1/9	2/9 -8/9	9/9 -15/9	16/9 -22/9	23/9 -29/9	30/9 -4/10
		SAMPLING DATES											
CONTROL GROUP	1^{L}_{R}			(-)					(+)				(-)
				(-)					(-)				(-)
	4^{L}_{R}			(-)					(-)				(-)
				(+)		1			(-)				(-)
	6^{L}_{R}			1(-)	(+)			1	1(+)				(-)
				1(-)	2(+)				2(+)				(-)
	8^{L}_{R}	1(+)	1(+)	(-)					(-)				(-)
		(+)	2(+)	(-)					(-)				(-)
	11^{L}_{R}			(+)		1	1	(+)	1(+)				1(+)
				1(+)	1	1	1	2(+)	1(+)				(+)
	13^{L}_{R}			(-)		1			(-)				(-)
				(+)	1	1			(+)				(-)
	14^{L}_{R}			(+)					(-)				(-)
				(-)				1	(-)				(-)
	15^{L}_{R}		1(-)	(-)		1			(-)				(-)
			1(-)	(-)		1			(-)				(-)
	16^{L}_{R}												
	20^{L}_{R}		(+)	1(+)	1	1		1	1(-)	1			1(-)
			1(-)	(+)	1	1	1	1	1(+)	2	1	1	1(+)
	24^{L}_{R}			1(-)		1		2	(+)				(-)
				1(+)					(-)				(-)
	26^{L}_{R}		1	(-)		1	1		2(+)				(-)
			1	1(+)	1	1	1	1	(+)				(-)

( ) Sample collected
\+ M.bovis isolated
\- M.bovis not isolated

1 Ocular irritation present
2 IBK diagnosed

TABLE 52. contd. Isolations of M.bovis and dates of observations of signs of ocular irritation and diagnosis of IBK in calves, fly control trial.

TAGGED GROUP

COW NUMBER		SAMPLING DATES 15/7 -21/7	22/7 -28/7	29/7 -4/8	5/8 -11/8	12/8 -18/8	19/8 -25/8	26/8 -1/9	2/9 -8/9	9/9 -15/9	16/9 -22/9	23/9 -29/9	30/9 -6/10
2^{L}_{R}				(+)					(-)				1(-)
				(-)					(-)				1(-)
3^{L}_{R}				(-)					(-)				(-)
				(-)					(-)				(-)
7^{L}_{R}				(-)				1(+)	(-)				(-)
				(-)				(+)	(-)				(-)
9^{L}_{R}				(-)					1(-)				(-)
				(-)					1(-)				(-)
10^{L}_{R}				(-)					(-)				(-)
				(-)					(-)				(-)
18^{L}_{R}			1	1(-)	1(+)				1(-)				(-)
			2	1(-)	(-)				(-)				(-)
19^{L}_{R}				(+)					(-)				(-)
				(-)					(-)				(-)
21^{L}_{R}			1	(+)		1	1	1(+)	1(+)				(-)
			1	(+)		1	1	1(+)	1(-)				(-)
22^{L}_{R}				(+)	2(-)				(-)				(-)
				(+)	(-)				1(-)	2			(-)
23^{L}_{R}		2(+)	2(-)	(+)					(-)				(-)
		(+)	(+)	(-)					(-)				(-)
28^{L}_{R}				(+)					1(-)				1(-)
				(+)					1(+)				1(-)
29^{L}_{R}				(+)					(+)				(-)
				(-)					(+)				(-)
35^{L}_{R}				(+)					(+)				(-)
				(+)		1			(+)				(-)
27^{L}_{R}	*			(-)					(-)				(-)
				(-)					(-)				(-)

TABLE 53.

	COW NUMBER	15/7 -21/7	22/7 -28/7	29/7 -4/8	5/8 -11/8	12/8 -18/8	19/8 -25/8	26/8 -1/9	2/9 -8/9	9/9 -15/9	16/9 -22/9	23/9 -29/9	30/9 -6/10
		SAMPLING DATES											
CONTROL GROUP	1^{L}_{R}			(+)					(-)				(-)
				(-)					(-)				(-)
	4^{L}_{R}			(-)					(-)				(-)
				(-)					(-)				(-)
	6^{L}_{R}			(-)					(-)				(-)
				(-)					(-)				1(-)
	8^{L}_{R}			(-)					(+)				(-)
				(-)					(+)				(-)
	11^{L}_{R}			(+)					(+)				(-)
				1(+)					(+)				(-)
	13^{L}_{R}			(-)					(-)				(-)
				(-)					(-)				(-)
	14^{L}_{R}			(-)					(-)				(-)
				(-)					(-)				(-)
	15^{L}_{R}			(-)					(-)				(-)
				(-)					(+)				(-)
	20^{L}_{R}			(-)					1(-)				(-)
				(-)					2(-)				(-)
	24^{L}_{R}		2(-)	2(-)					1(-)				(-)
			2(-)	1(-)					1(-)				(-)
	26^{L}_{R}			(-)					(-)				(-)
				(-)					(+)				(-)
	$BULL^{L}_{R}$			(-)									
				(-)									

( ) Sample collected
\+ M.bovis isolated
\- M.bovis not isolated

1 Ocular irritation present
2 IBK diagnosed

TABLE 53. contd. Isolations of M.bovis and dates of observations of signs of ocular irritation and diagnosis of IBK in adults, fly control trial.

and fourteen ocular swabs were taken from the adults between July 15 and September 8. Of these 88 (77.1%) were collected from clinically normal eyes, 26 (29.5%) of which were positive for M.bovis. Nineteen (16.7%) swabs were collected from eyes showing signs of ocular irritation only with seven (36.8%) positive for M.bovis. Seven (6.1%) were taken from eyes diagnosed as having IBK, of which only one (14.3%) yielded M.bovis.

Clinical features

All of the cattle were found to be free from active ocular lesions when examined at turnout although faint white corneal opacities, suggestive of healed corneal lesions, were noted in seven cows (2L,10R,18L,19L, 23L,24L,26R). All eyes remained healthy until July 18 when the left eye of one of the tagged cows (23) was seen to have epiphora and blepharospasm. This animal was caught and found to have a faint diffuse corneal opacity but no ulceration. Ocular swabs were taken and M.bovis isolated from both left and right eyes, IBK was diagnosed and the cow was treated on July 23. The first signs of IBK among the calves were noted on July 20, affecting one tagged (8) and one untagged animal (2). The subsequent development of additional cases of IBK within the herd was summarised in tables 52 and 53. No fresh cases of IBK were diagnosed after September 23 although lesions had recurred in the left eye of one calf (22) by October 9.

IBK was diagnosed in six eyes (27.3%) from six calves in the control group and 11 eyes (45.8%) from six calves in the tagged group. In the adults, IBK was diagnosed in the control group in two eyes (9.1%) of two cows and in the tagged group it was diagnosed in five eyes (19.2%) of three cows.

The peak incidence of clinical IBK in the adults occurred during the first four weeks of the outbreak (figure 30) while that of the calves was in the second four week period. In 11 instances the mothers of calves with signs of ocular irritation or IBK were similarly affected, of these, lesions were noted in the calf prior to those of its corresponding dam on two occasions, vice versa on four occasions and simultaneously on five occasions.

Immune events

Reciprocal IHA titres against M.bovis for both cows and calves are given in table 54 and illustrated in figure 31. Mean log reciprocal IHA titres (figure 32) for the 24 adults (ie. excluding cows 15 and 27) in samples collected on May 3-4, July 5, September 9 and October 4 were found to be 1.06, 0.90, 0.96 and 0.76, respectively, corresponding to geometric mean reciprocal titres of 11.5, 7.9, 9.1 and 5.8. From the calves, mean log reciprocal IHA titres on the same dates were found to be 0.17, 0.44, 0.64 and 0.80 corresponding with geometric reciprocal titres of 1.5, 2.8, 4.4 and 6.3.

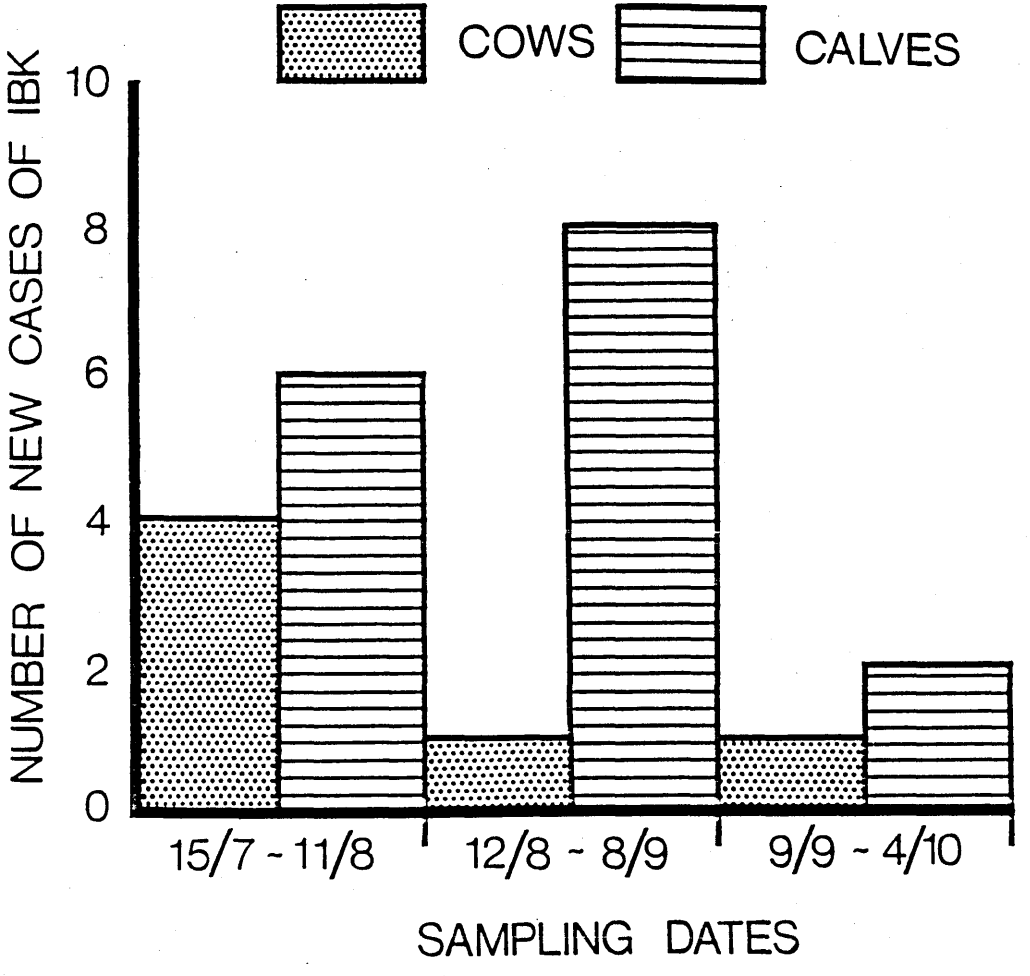


Figure 30. Fly control trial. Incidence of IBK in cows and calves during summer grazing.

	IDENTIFICATION NUMBER	DATE OF SAMPLING AND SOURCE							
		3/5/84		8/7/84		4/9/84		18/10/84	
		COW	CALF	COW	CALF	COW	CALF	COW	CALF
TAGGED GROUP	2	32	N	8	2	16	16	4	8
	3	16	N	16	4	8	8	4	N
	7	2	-	4	8	16	2	2	16
	9	8	-	16	2	32	4	4	4
	10	4	-	8	2	64	32	16	32
	18	64	8	32	2	8	4	8	N
	19	8	N	4	4	2	8	4	4
	21	128	4	64	-	16	4	16	8
	23	8	N	8	8	4	16	16	16
	28	32	-	8	4	2	4	4	8
	29	32	N	4	-	4	4	8	16
	35	2	-	4	4	2	4	4	32
	22	16	-	64	-	8	N	16	2
CONTROL GROUP	1	4	-	8	2	64	4	4	16
	4	4	2	2	2	16	-	4	16
	6	4	-	4	N	16	8	4	32
	8	4	-	4	8	32	2	16	N
	11	16	2	4	8	4	2	2	4
	13	8	-	4	2	8	8	2	16
	14	16	N	4	8	8	16	2	32
	15	32	8	16	2	4	2	8	8
	16x	4	-						
	20	32	16	8	4	16	2	16	4
	24	8	-	8	4	4	2	8	2
	26	16	N	8	N	8	4	8	2

- \- No haemagglutination
- N Haemagglutination in neat dilutions only
- x Cow and calf 16 removed due to lameness

TABLE 54. Reciprocal IHA titres against whole cell *M.bovis* in serum samples, fly control trial.

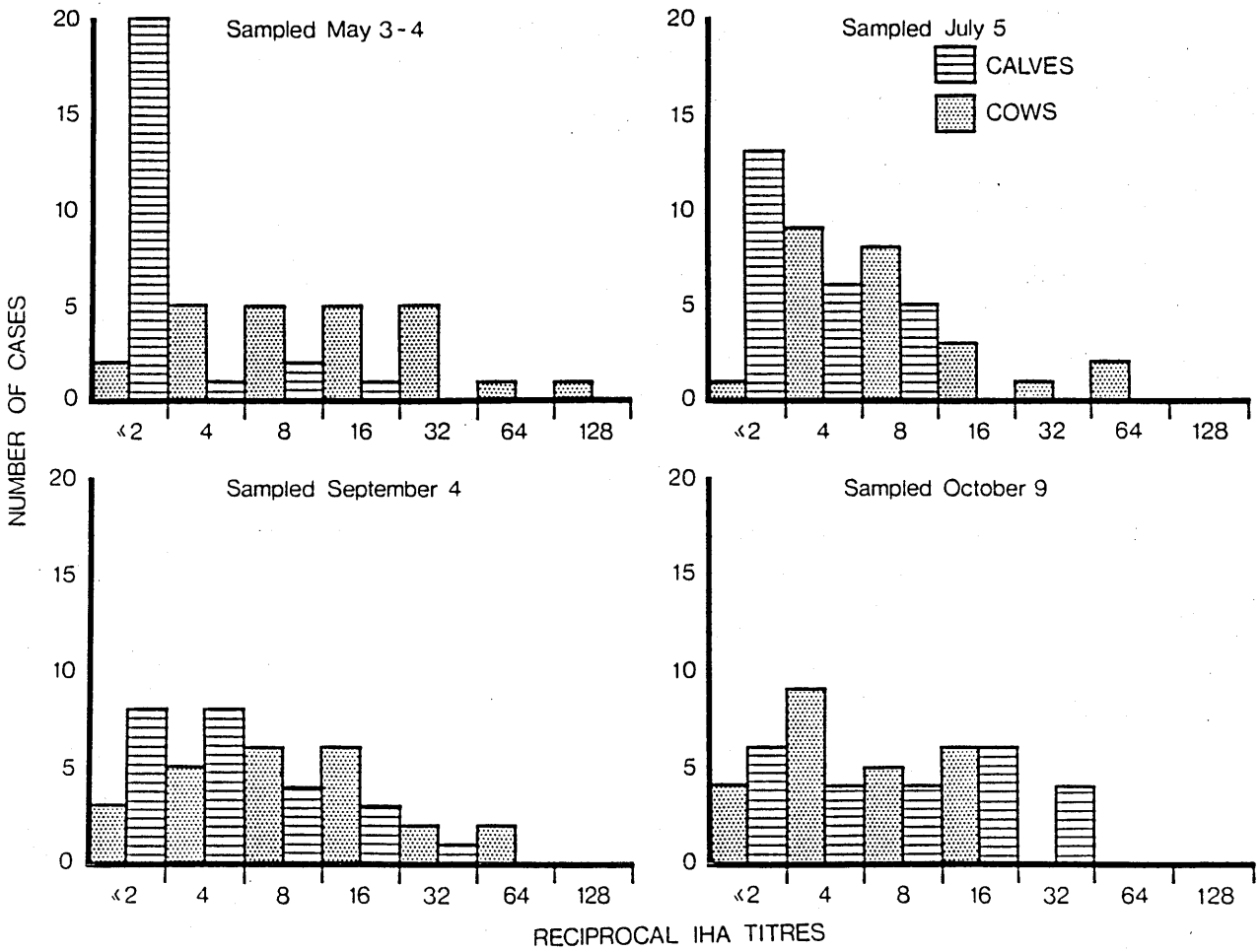


Figure 31. Fly control trial. Changes in serum antibody to M.bovis during summer grazing.

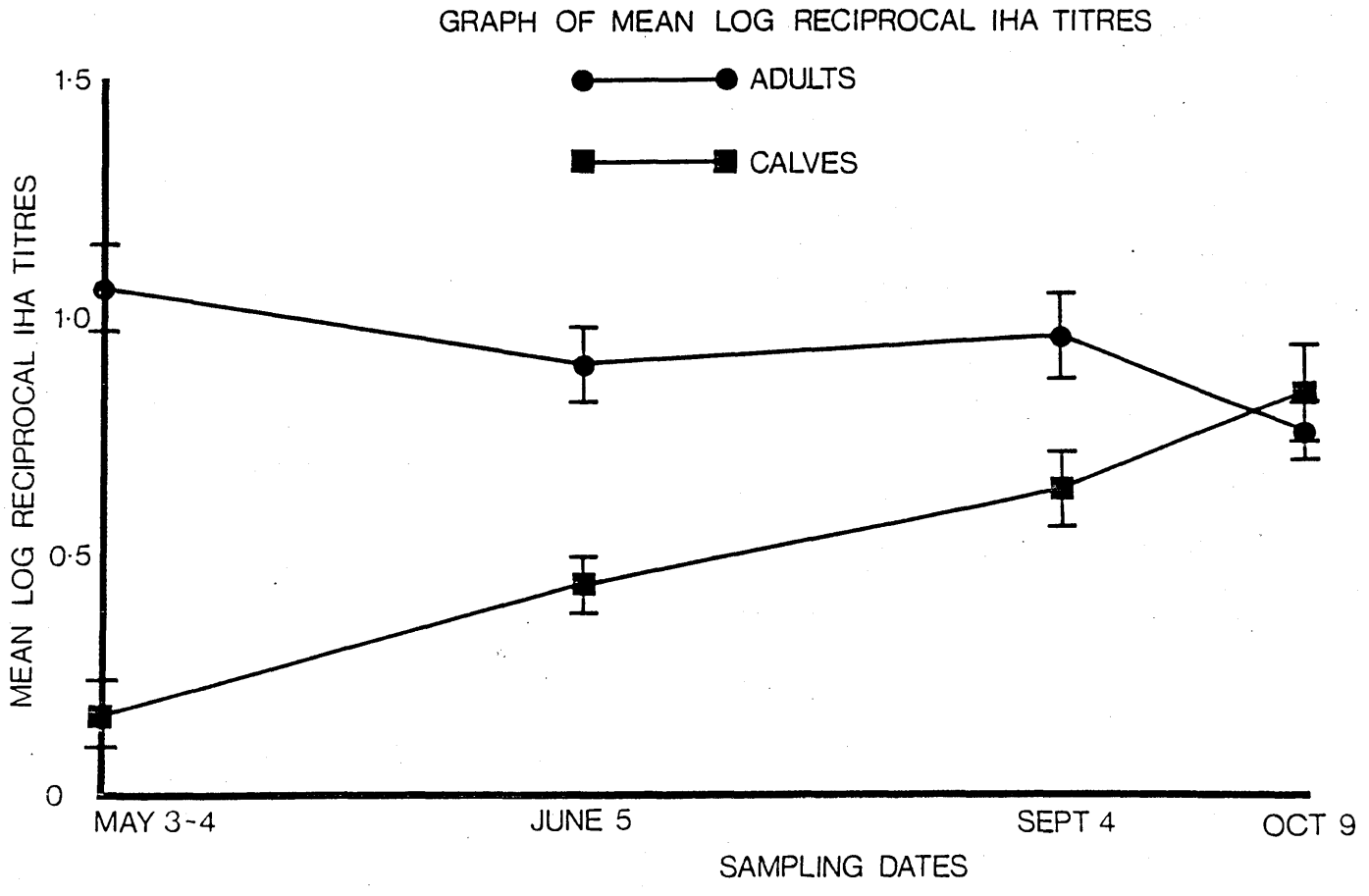


Figure 32. Fly control trial. Mean log reciprocal IHA titres in cows and calves.

Weight gain

The weight gains of calves in control and treated groups are illustrated in table 55 including details of sex, age and diagnosis of IBK.

At turnout, the mean weight of the tagged group was 54.5 kg and the mean weight of the control was 54.9 kg. At weaning the tagged calves had gained a mean of 128.5 kg while those in the control group had gained 136.2 kg, the differences in weights between the two groups were not significant ($p > 0.05$).

Calves which had been affected by IBK during the summer gained 126.2 kg and those which were unaffected gained 137.3 kg, although again, this result was not significant ($P > 0.05$). However, as a result of a slight bias in the sex of calves affected, the female weights were adjusted by a factor of 1.2 (being equal to the mean weight gain of male calves divided by that of the females), resulting in adjusted weight gains of 149.8 kg in the IBK free group and 149.2 kg in the affected group. Hence the differences in weight gain referred to above were definitely not due to IBK.

DISCUSSION

Outbreaks of IBK are often related by farmers to periods of peak fly infestation (87,198) and subsequent research has centred upon infestation with the face fly M.autumnalis (32,46,207,209). This fly has been shown to be able to act as a mechanical vector for M.bovis (11,12,

CALF No.	SEX	AGE AT TURN-OUT (Days)	WEIGHT AT TURN-OUT (Kg.)	WEIGHT GAIN TO WEANING (Kg.)	ADJUSTED WEIGHT GAIN (Kg.)	IBK DIAGNOSED
2	M	32	69	163	163	+
3	F	19	44	120	144	-
7	F	43	63	121	145	+
9	F	50	69.5	134.5	161.4	-
10	M	36	60	135	135	+
18	M	12	45	141	141	-
19	F	37	55	97	116.4	-
21	F	23	36	113	135.6	+
23	F	46	64	130	156	-
28	M	37	55.5	112.5	112.5	-
29	F	9	42	135	162	-
35	M	34	51	140	140	+
22x	F	ND	ND	ND	ND	+
1	M	31	59	159	159	-
4	M	11	45	162	162	-
6	F	33	56.5	121.5	145.8	+
8	F	48	63.5	122.5	147	+
11	F	11	39.5	116.5	139.8	+
13	M	36	53	151	151	-
14	F	49	65	139	166.8	-
15	M	15	47	153	153	-
16x	M	47	69	154	154	-
20	F	6	32	114	136.8	+
24	F	41	59	108	129.6	+
26	F	52	70	134	160.8	+

x Calves 16 and 22 are not included in statistical analysis.

M Male
F Female

TABLE 55. Effect of sex, age and fly control upon the weight gain of calves between turnout and weaning, fly control trial.

22,32,79,80,81,207) and is able to produce ocular lesions which may predispose the eyes to the development of the disease (32,194). However, this fly is not found in Scotland (216,217,237) and the fly most commonly found feeding on facial secretions is the sheep head fly H.irritans (217). Although this latter fly may assume the role of M.autumnalis in this area, M.bovis has not been successfully isolated from flies caught during outbreaks of IBK (237).

Topical application of synthetic pyrethroids either by repeated dusting or spraying or by the use of insecticide-impregnated ear tags has proven to be effective in reducing total fly infestation of grazing cattle (15,49,72,127,148). Such measures are particularly effective against biting flies (15,94,127) although less so against the non-biting species (15,94,238). The use of two tags per animal has been shown to be more effective than using one in reducing fly numbers (94).

In this trial, the use of fenvalerate-impregnated ear tags did not reduce the infection rate by M.bovis, clinical IBK or affect the weight gain in comparison to untagged control animals. This contrasts markedly with the results reported in one American trial (72) in which only five out of 134 treated animals were positive for M.bovis and of those only two developed clinical disease compared to 60 positives of 123 non-treated animals with 41 cases of IBK. However, there were marked differences in the designs of these two trials which could account for this discrepancy.

In designing this trial, it was decided to run the insecticide treated and control groups together as it is recognised that there can be distinct differences in the incidence of IBK between different geographical areas and even between adjacent fields. However, this had the disadvantage that the control animals may have been afforded a degree of protection from flies, especially during periods when the animals are congregated in one place, and the treated group were subjected to a higher fly challenge due to the untreated controls acting as a reservoir. In contrast, in the American trial (72), where the two groups were maintained separately, M.bovis infection was present in the untreated group prior to treatment but not in the treated group; infection did not appear in the latter animals until after the seasonal peak of fly infestation and incidence of IBK in August/ September.

The differences in levels of control are due to the nature of the insecticide, which does not produce a rapid knock down effect, and the relatively short exposures endured by the non-biting species of flies. It is therefore possible that in this trial, treatment with one tag per animal was insufficient to provide adequate protection against non-biting fly challenge, and thus transmission of IBK.

The initial source of M.bovis infection in this trial is unknown. There is a possibility that the infection could have been started by the activation of a "carrier animal" (160) this being supported by the initial

development of the lesions in an adult cow with a pre-existing corneal scar. On the other hand, there were no isolations of M.bovis from any of the samples taken during the three months preceding this case. The other possible source of infection is transmission by flies from infected animals in adjacent fields. Whether these animals had been infected with M.bovis could not be determined however some signs suggestive of IBK had been seen in these animals during late July and August.

In 14 out of the 15 eyes that were treated, subconjunctival injection of a 5% oxytetracycline hydrochloride injectable solution resulted in successful resolution of clinical signs within 24 hours by which time signs of ocular irritation such as blepharospasm, conjunctivitis and epiphora had resolved. Examination of the cornea revealed small facets at the site of ulceration and in some mild cases there was no evidence at all of previous corneal pathology. One calf which developed lesions during the early stages of the outbreak was, inexplicably, not treated and mild lesions persisted in that eye for a period of just under three weeks. Treatment was not effective in another calf which eventually developed severe lesions and a shallow 1 cm diameter corneal ulcer. The disease recurred in six of the 15 treated eyes (40%), one to four weeks after treatment which suggests that the initial infection had not adequately stimulated immunity to the disease. Serial serology demonstrated a rise in IHA titres among the calves, particularly after the first cases of IBK

were detected, and by the end of the grazing season, the cows and calves had similar titres.

No significant differences were found between the weight gain of treated and untreated calves, this result being in agreement with that of Wright and others (238) who found no difference between treated and untreated calves kept in separate fields. There were also no significant differences in weight gain found between those calves which were affected by IBK and those which remained IBK free. This result differs from those of previous studies in beef calves in America (117) which probably reflects the different husbandry methods involved. In the American studies the calves were not treated and IBK was diagnosed by the presence of corneal scars at weaning. Thus, mildly affected cases, which would have completely healed at weaning, would be missed. In this trial, all cases of IBK were recorded and treated early in the course of the disease thereby preventing the development of severe lesions in the majority of cases and decreasing the duration of the disease.

GENERAL DISCUSSION AND CONCLUSIONS

An examination of the relevant literature revealed that IBK is prevalent in all of the major cattle rearing areas of the world (18,230) with a reported annual incidence of between 10 and 20% (198,222). The disease results in serious economic losses, primarily as a result of decreased growth performance in young stock (213,214) and, less importantly, depressed milk yield in dairy cattle (198) with additional costs due to extra handling and treatment of affected animals (199).

Although there are many descriptions of IBK in the literature there are many discrepancies and contradictions present and it is probable that more than one disease is being described (18). This is reflected by the range of bacterial, mycoplasmal, viral and parasitic organisms implicated in the pathogenesis of IBK. However, M.bovis is now recognised as the primary aetiological agent in IBK and, where this bacteria has been isolated, there is a greater degree of consistency in clinical descriptions. On the other hand, there is evidence that combined infection, with either mycoplasmas, particularly Myco.bovoculi (185) or BHV1 (138), will produce more severe lesions than M.bovis alone.

The seasonal variation in incidence and severity of IBK indicates the presence of environmental predisposing factors such as trauma, from dust or grass awns (198), fly infestation (32,46) and UV irradiation (105), of which only the latter two have received detailed investigation.

Indeed, high doses of UV irradiation have been used in the experimental production of the disease to enhance the pathogenicity of M.bovis (106).

The success of the present study clearly indicates that M.bovis may act as a primary pathogen in IBK. It was possible, using two different strains of M.bovis, to produce lesions, comparable in severity with those recorded from field outbreaks of the disease, although marked variations in pathogenicity were noted. The development of such an experimental system made it possible to monitor clinical changes induced by M.bovis infection and obtain an accurate description of sequential lesion development. This clinical picture, correlated with M.bovis isolation, has confirmed the descriptions of others (17).

A variable, occasionally lengthy, incubation period was noted despite regular isolations of M.bovis and the apparent absence of either circulating or local antibody, which indicates that carrier animals may develop following primary infection, with the risk of subsequently developing the disease. Furthermore, persistent infections, up to 70 days, were found during convalescence in some animals.

The results of attempts to reinfect eyes show that most eyes had become resistant. This, combined with high systemic antibody titres in samples collected during reinfection, indicates that the resistance noted in adults is acquired and not age related. In support of this, there was apparently less resistance to heterologous than homologous reinfection in calves of approximately equal age.

The availability of the above experimental system made it possible to study the effect of vaccination on local and systemic antibody and the subsequent clinical and immune responses to challenge by M.bovis. Vaccination produced a rise in serum titres prior to challenge but had little effect upon lachrymal titres. On the other hand, evidence to support the efficacy of the vaccine was provided by lower morbidity, less severe disease, fewer isolations, longer transmission times to left eyes and lower mortality in the vaccinated animals. These results compare favourably with other vaccine trials which used artificial predisposing factors to help initiate the disease (100). In view of the fact that in the present study the vaccine was tested against homologous challenge only and that less than complete protection was provided, more work is required to make vaccination effective.

It has been recognised that only limited pathological studies have been performed in IBK (18,180, 230). However, the limited studies carried out in the present study confirm the views of others in reference to the pathological changes induced. Furthermore, the in vitro studies tended to confirm the importance of fimbriation to adhesion and thus pathogenicity.

In the UK, prevention and control of IBK is limited to treatment of cases and control of fly infestation by the use of insecticide impregnated ear tags or "pour-on" formulations. The development of the model allows for the testing of treatment regimes under controlled conditions in small numbers of animals with

known disease histories. Although control animals were not used in the present studies, it is interesting to note that there were no recurrences following treatment of experimental disease while there were in the field. In the latter situation this may reflect either higher continuing challenge or else treatment of early cases preventing the development of immunity.

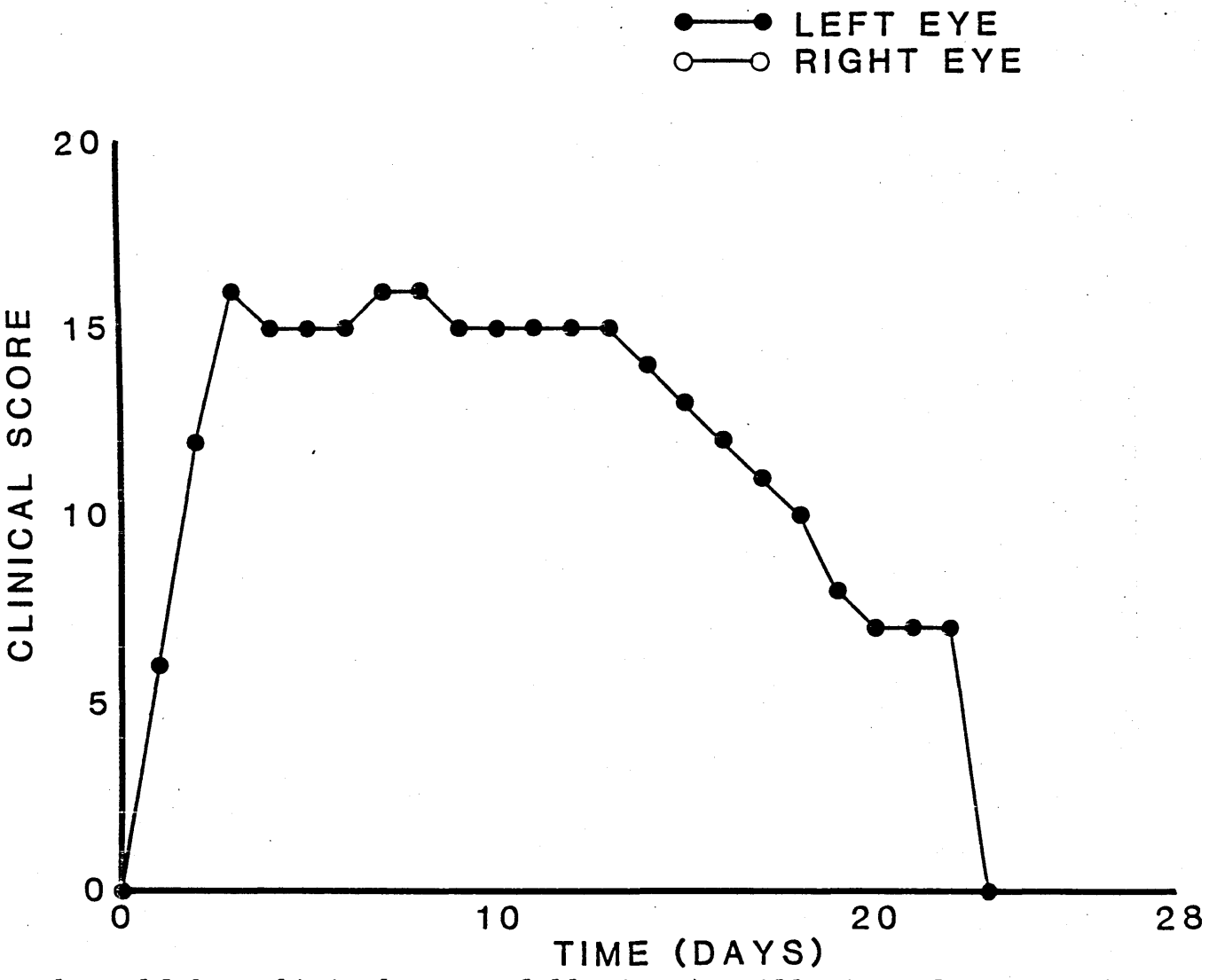
The results of the above field trial indicate that ear tagging was not successful in preventing IBK. This might suggest that the insecticide used did not produce adequate fly control or, conversely, that the importance of flies as vectors of M.bovis has been over-emphasised in the past. The results obtained from the horizontal transmission study make it quite clear that the organism in question may be transmitted relatively quickly, in the absence of significant fly numbers, by close contact during social, grooming and feeding activities.

APPENDIX I: Calf 1

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. On day 1, epiphora, blepharospasm, increased blinking and mild conjunctivitis were noted; severe conjunctivitis, iridospasm and a marked diffuse corneal opacity were present on day 2 and a 3mm anterior-polar ulcer was first noted on day 3. By day 5, the ulcer had attained its maximum diameter of 8mm and, on day 6, vascularisation of the cornea was first noted, as a solid band 1mm wide, at the corneoscleral junction. Vascularisation reached the ulcer edge on day 13, followed by a gradual decrease in intensity of signs of ocular irritation, corneal opacity and ulcer size. Signs of ocular irritation were not noted after day 21.

- Right eye. This eye remained normal throughout this experiment.



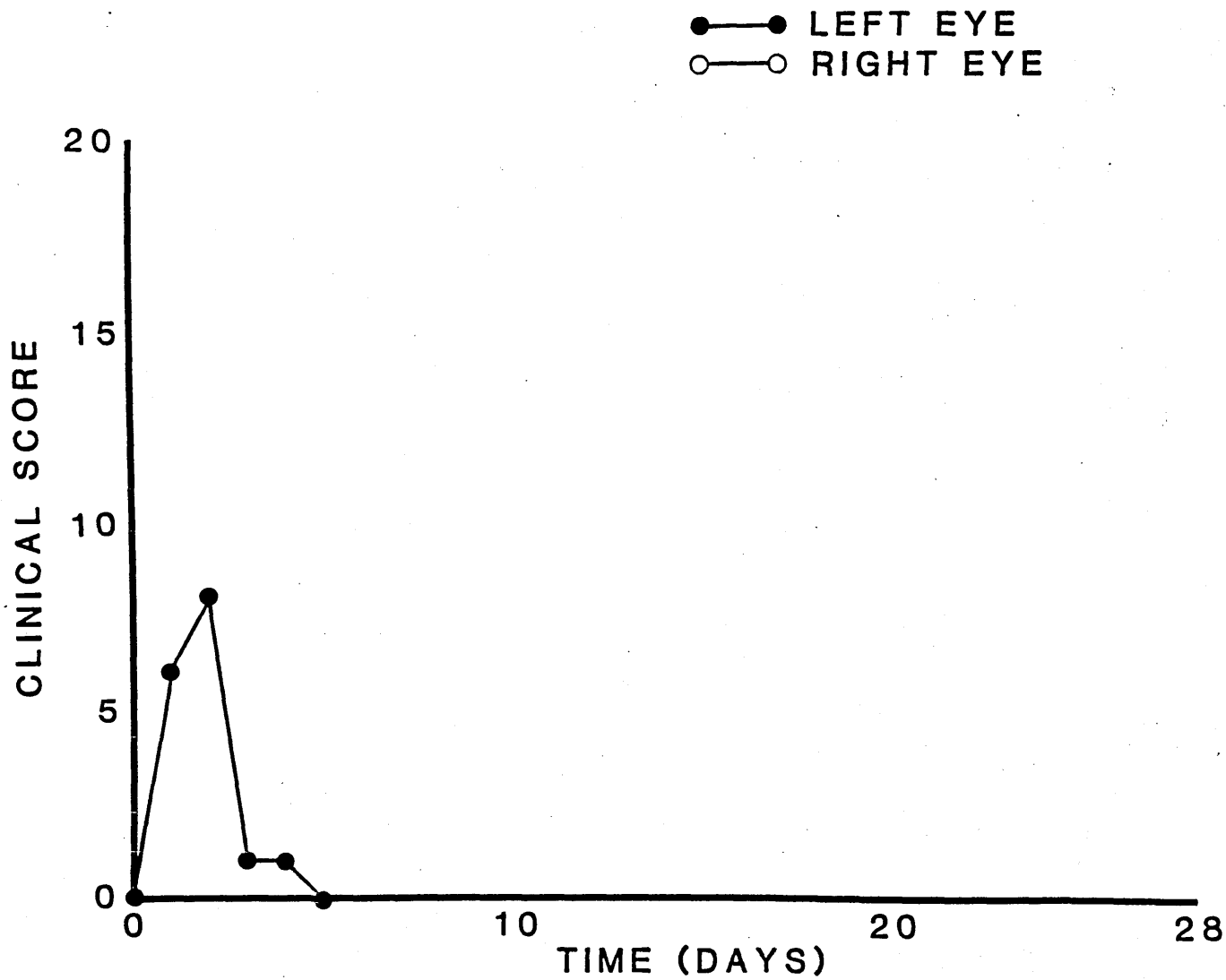
Experiment 1, calf 1. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 2

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. On day 1, there was marked epiphora, mild blepharospasm, increased blinking, conjunctivitis and iridospasm were noted: there were no corneal changes. On day 2, a shallow corneal ulcer, 3mm in diameter, was noted in the upper lateral quadrant and in the absence of corneal opacity. On day 4, there were no signs of ocular irritation and, at the site of the ulcer, there was a very shallow facet, 2mm in diameter, which had resolved by day 5.

- Right eye. This eye remained normal throughout this experiment.



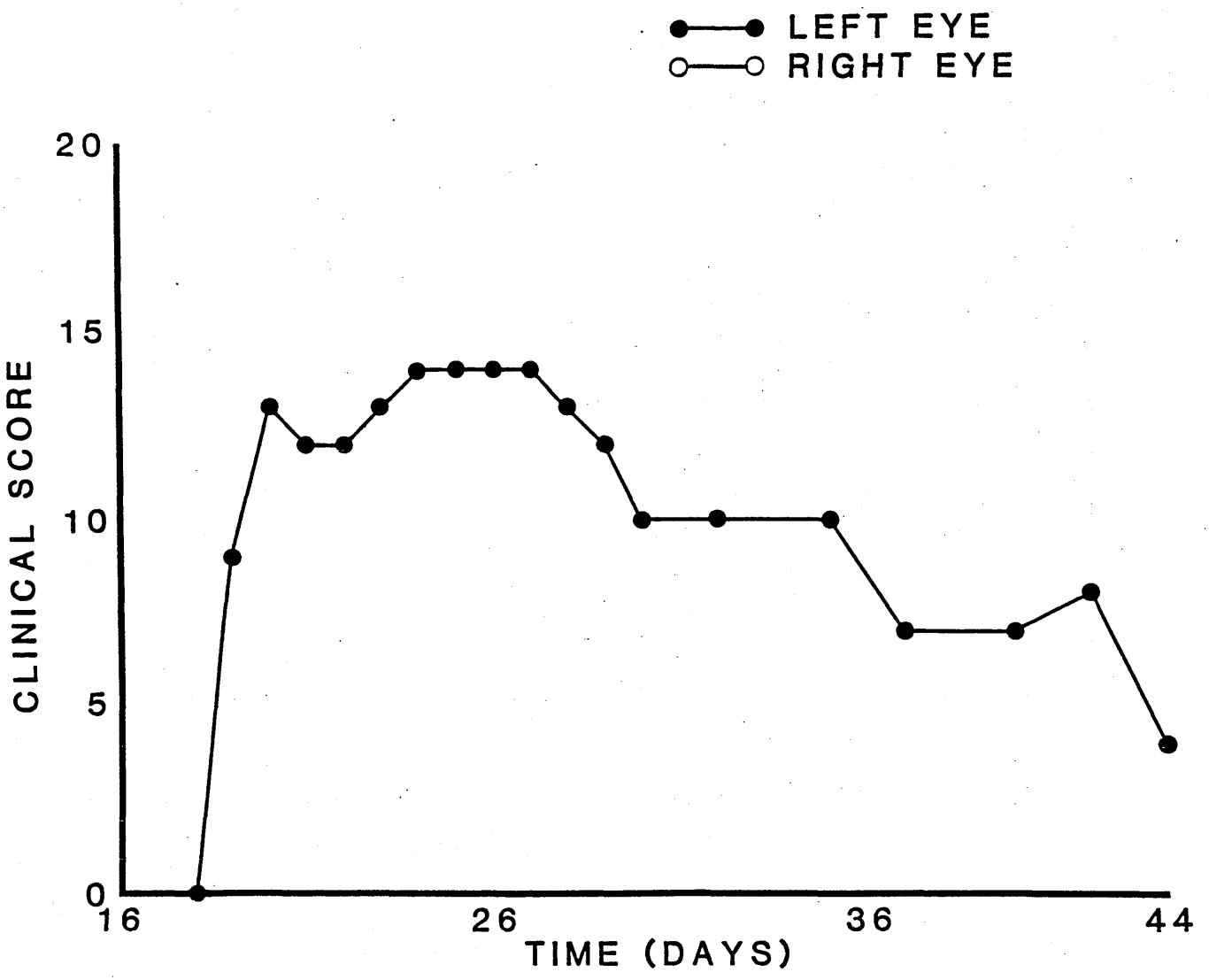
Experiment 1, calf 2. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 3

The clinical scores from this calf for the period from day 16 to day 44 are illustrated overleaf.

- Left eye. On day 18, epiphora, mild blepharospasm, increased blinking and severe conjunctivitis were noted: there was a 2mm anterior-polar ulcer surrounded by a diffuse opacity which was visible under oblique lighting only. The ulcer attained a maximum diameter of 4mm on day 19 although the surrounding cornea continued to become progressively more opaque until day 24. Iridospasm was first noted on day 19 and signs of ocular irritation were most severe on days 25 to 26. Vascularisation was first noted on day 28, along the entire corneoscleral junction, at which time signs of ocular irritation decreased gradually in intensity. Capillaries reached the ventral and dorsal edges of the ulcer on day 37 and, by day 40, had completely vascularised the ulcer floor with overlying granulation tissue projecting 2mm. Signs of ocular irritation were absent by day 44, by which time the granulation tissue had resolved, leaving a faint, pink scar confluent with the surrounding cornea.

- Right eye. This eye remained normal throughout this experiment.



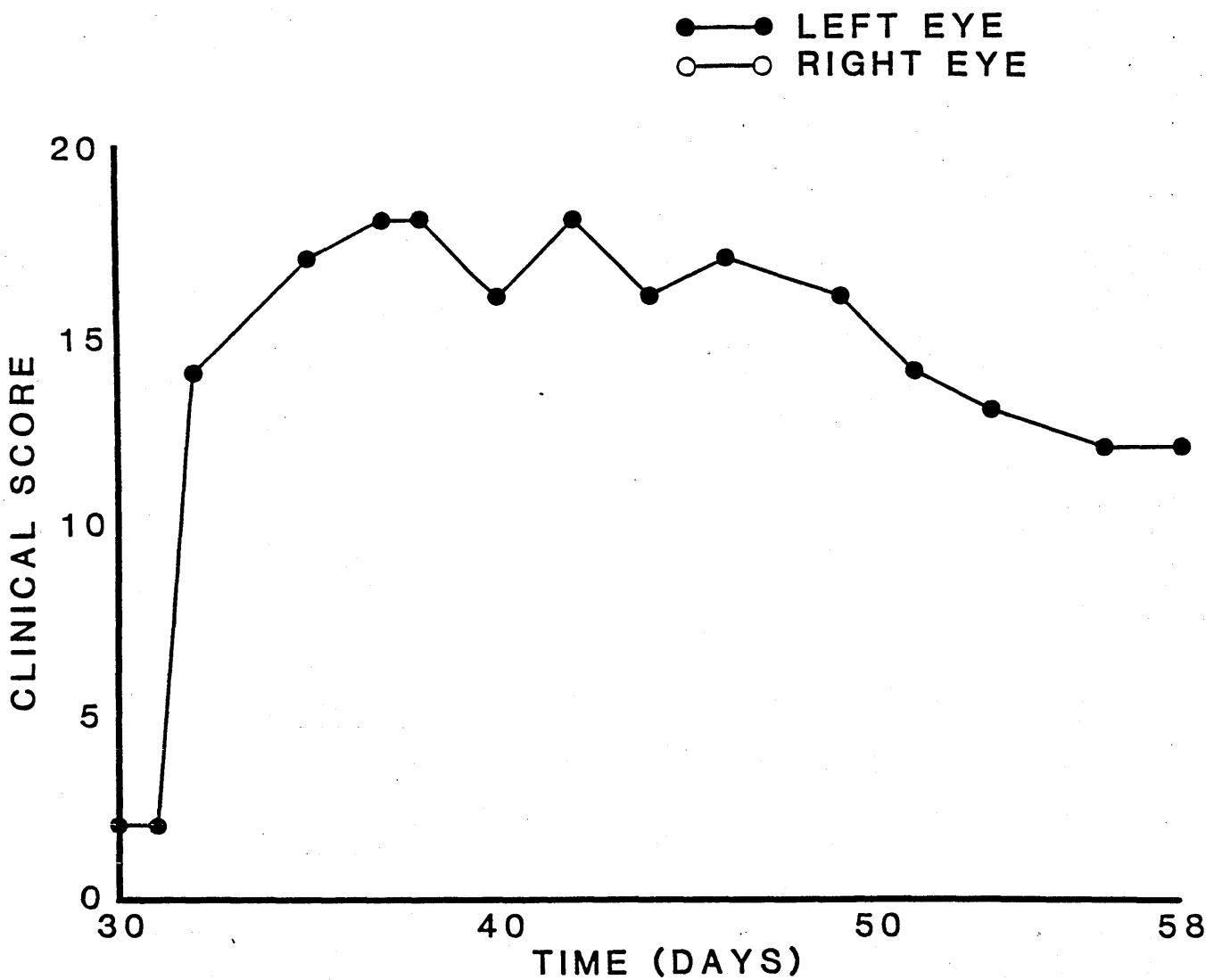
Experiment 1, calf 3. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 4

The clinical scores from this calf for the period from day 30 to 58 are illustrated overleaf.

- Left eye. On day 0, slight tear staining was noted on the left cheek. On day 1, there was increased lachrymation, with severe epiphora, although the eye was otherwise normal. Epiphora persisted as the sole sign until day 31. On day 32, severe blepharospasm, increased blinking, conjunctivitis and iridospasm were noted: there was a 3mm diameter, anterior-polar vesicle surrounded by a mild corneal opacity. Flecks of pus were found in the tears from days 37 to 63. By day 35, the cornea was almost totally opaque, an ulcer, 6mm in diameter, was present and a 1mm wide band of capillaries extended into the cornea from the entire corneoscleral junction. The capillaries spread across the cornea at a rate of approximately 1mm per day, reaching the ulcer edge by day 42. Signs of ocular irritation decreased gradually from this point, conjunctivitis and blepharospasm were last noted on day 60, although epiphora persisted until day 75. Granulation tissue was found on the dorsal and ventral ulcer edges on day 46 and, by day 49, covered the ulcer floor, following which the ulcer gradually contracted. By day 63, the granulation tissue had resolved, leaving a dense scar confluent with the surrounding cornea, and vascularisation was markedly reduced, although a single vessel was visible extending from the ventral corneoscleral junction.

- Right eye. This eye remained normal throughout this experiment.



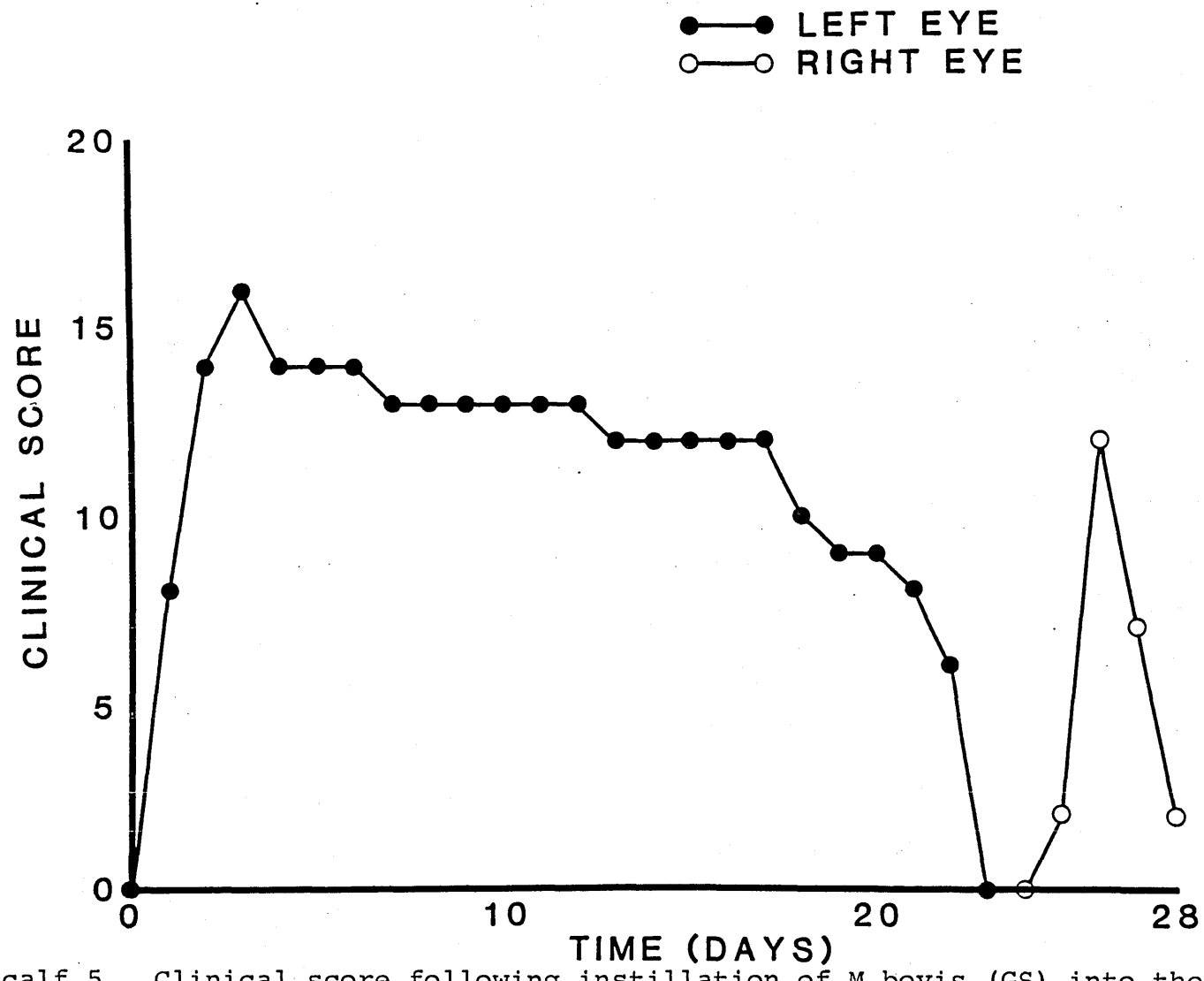
Experiment 1, calf 4. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 5

The clinical scores from this calf for the period from days 0 to day 28 are illustrated overleaf.

- Left eye. On day 1, there was epiphora, moderate blepharospasm, increased blinking, conjunctivitis and iridospasm although no corneal abnormalities could be detected. By day 2, a 4mm diameter, anterior-polar ulcer was noted, surrounded by a faint opacity and, although the ulcer did not expand, the opacity increased in area and intensity such that, by day 3, the entire cornea was diffusely opaque. On day 4, the iris was normal and blepharospasm decreased in intensity; vascularisation of the cornea was first noted on day 6. Signs of irritation decreased in intensity from day 7 onwards and epiphora was last noted on day 19 although conjunctivitis and mild blepharospasm persisted until day 21. Vascularisation reached the ulcer edge on day 15 and had completely vascularised the ulcer floor by day 21; projecting granulation tissue did not form. Vascular tissue faded thereafter such that, by day 35, the ulcer site was marked by a dense, white scar 4mm in diameter.

- Right eye. This eye remained normal until day 24. On day 25, increased lachrymation was noted and on day 26, the eye was severely irritated, with blepharospasm, increased blinking, conjunctivitis and iridospasm also present. The cornea was diffusely opaque and a 1mm diameter ulcer was present at the anterior pole. The lesions did not progress and the eye was normal by day 28 although the site of the ulcer was marked by a slight opaque facet which persisted to day 65.



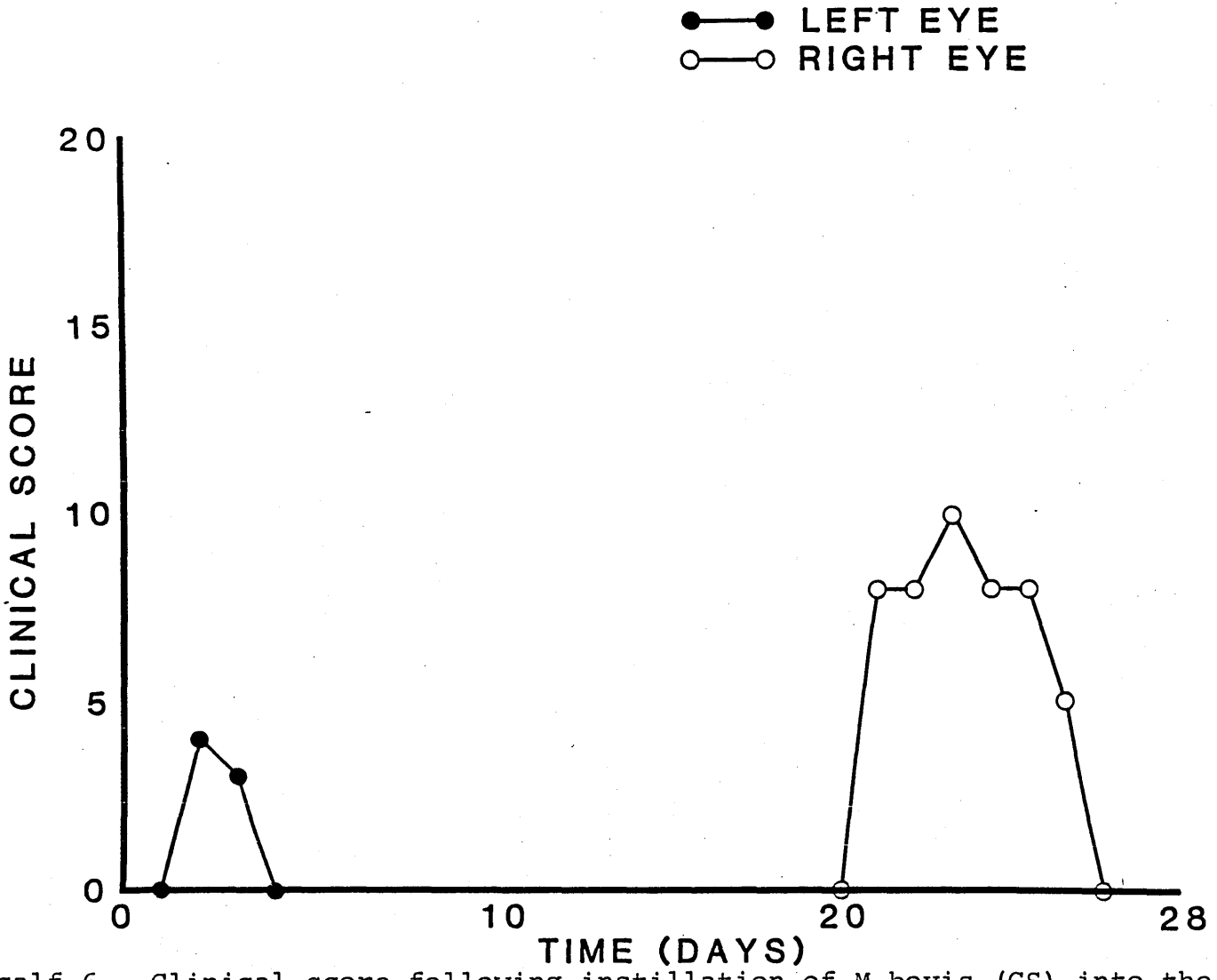
Experiment 1, calf 5. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 6

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. This eye remained normal at all times apart from very mild conjunctivitis and epiphora which were noted on days 2 and 3.

- Right eye. This remained normal up to day 20. On day 21, mild epiphora, blepharospasm, increased blinking, conjunctivitis and iridospasm were noted. On day 22, a pinpoint ulcer and faint corneal opacity were noted. These lesions healed rapidly and, by day 26, the eye was clinically normal apart from slight epiphora which persisted to day 30. On day 44, the eye was noted to be irritated and the cornea was almost opaque. A 2mm ulcer was first noted on day 46. These lesions resolved over a five day period and, by day 51, the eye was normal although a small opacity was present at the ulcer site.



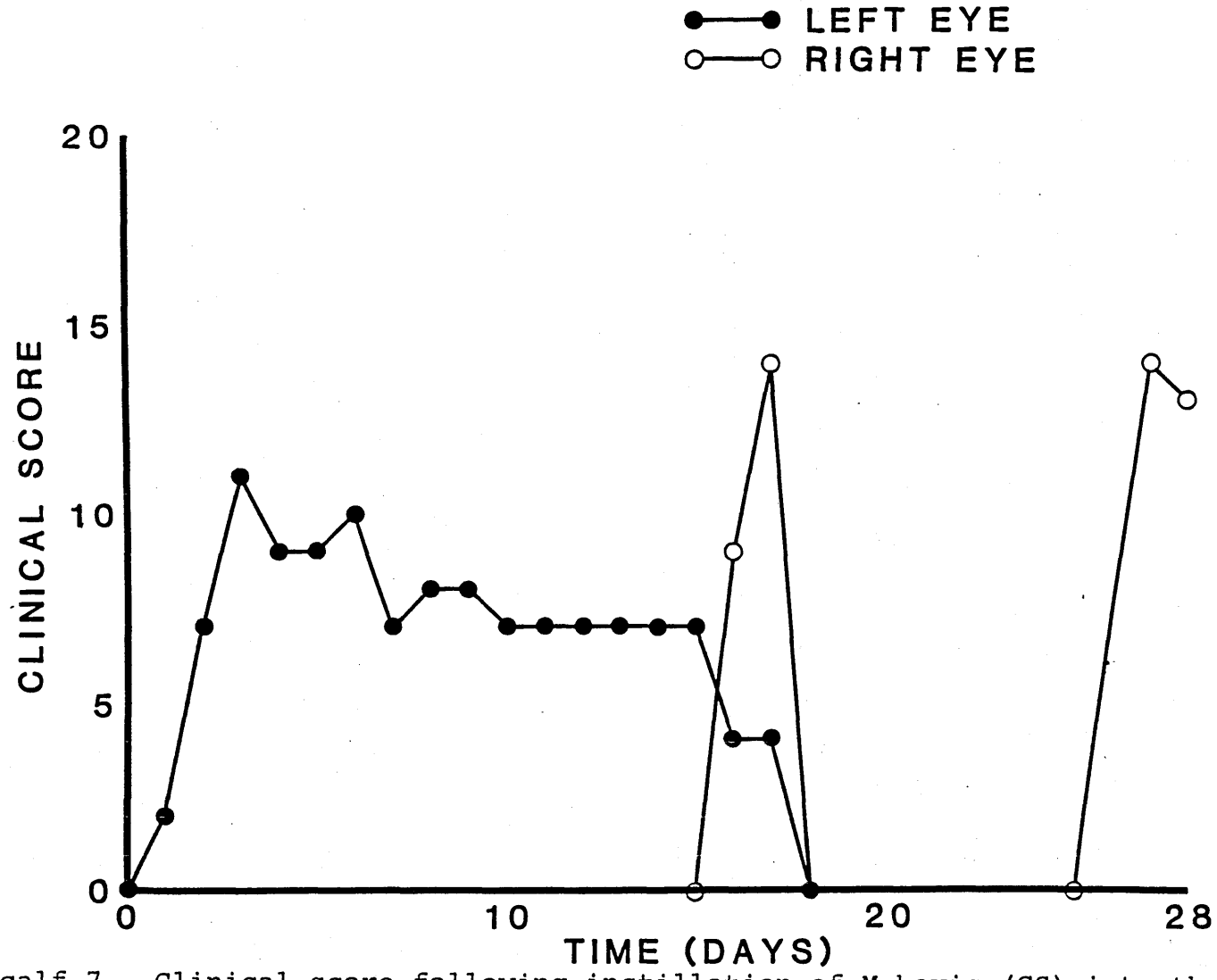
Experiment 2, calf 6. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 7

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. Mild epiphora and conjunctivitis were first noted on day 1. On day 2, these signs were more severe and blepharospasm and mild corneal changes were noted. The ulcer attained a maximum diameter of 3mm by day 7 although, at that point, corneal opacity was not detectable. Mild epiphora and conjunctivitis persisted until day 17 and the ulcer healed without vascularisation although a small facet was still present at the ulcer site on day 30.

- Right eye. Epiphora, blepharospasm, increased blinking, conjunctivitis and iridospasm were first noted on day 16. On day 17, a 2mm diameter, anterior-polar ulcer was noted, surrounded by a hazy opacity. These lesions resolved without corneal vascularisation and, by day 20, the eye was normal apart from a slight depression at the ulcer site. Mild lesions recurred on day 27 and a fresh, pinpoint ulcer was noted in the upper quadrant of the cornea. Signs of mild ocular irritation persisted until day 43 at which time the ulcer site was marked by a small opacity. On day 48, the eye was noted to be irritated for a third time and a third pinpoint ulcer was present. The lesions had resolved completely by day 54.



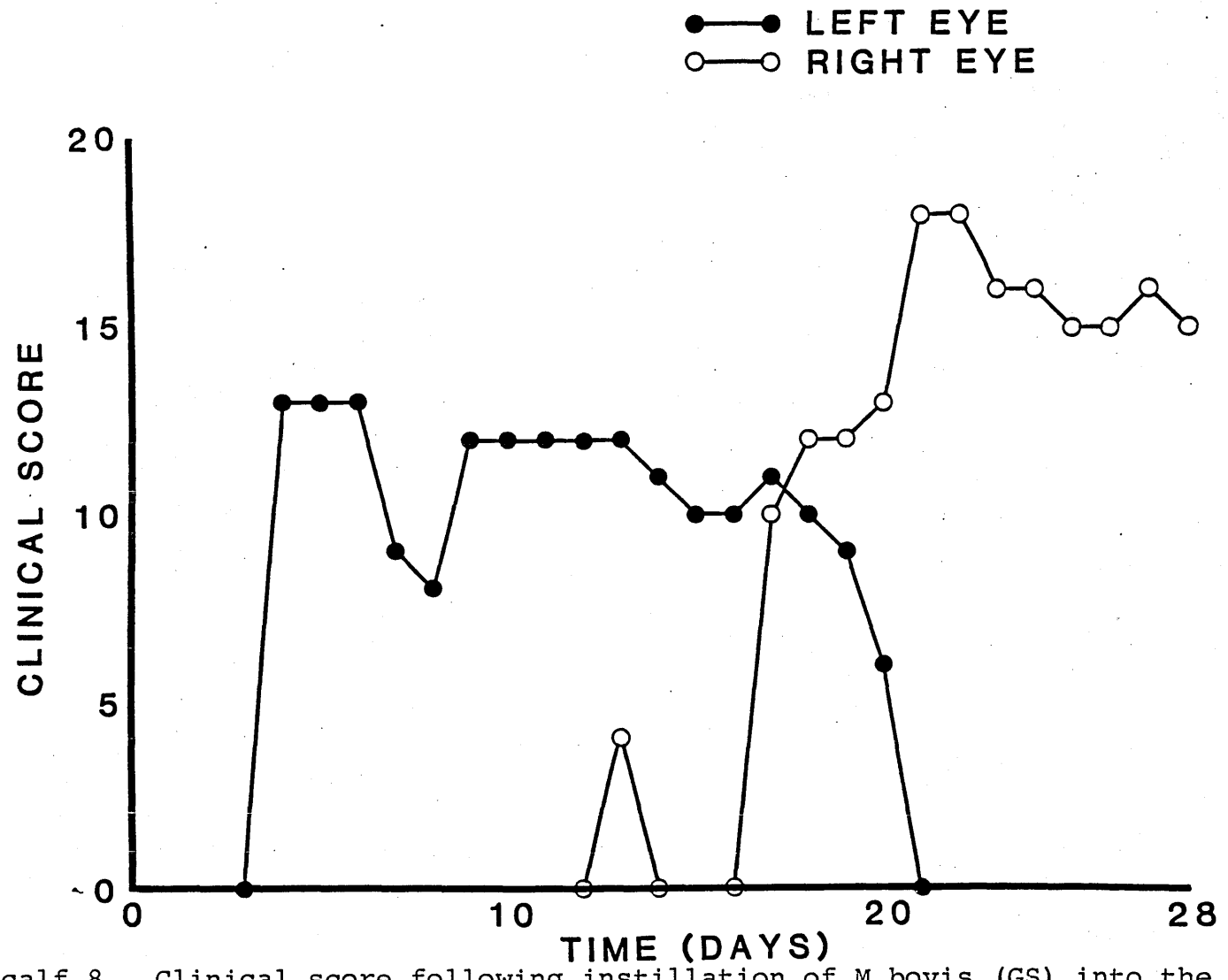
Experiment 2, calf 7. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 8

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. This eye remained normal until day 4 at which point epiphora, blepharospasm, increased blinking, conjunctivitis and iridospasm were noted; a 2mm anterior-polar ulcer was present, surrounded by a mild corneal opacity. The ulcer attained a maximum diameter of 4mm by day 6 and, on days 7 and 8, signs of irritation and the corneal opacity decreased. On day 9, the cornea was noted to be diffusely opaque and signs of severe ocular irritation had recurred. Tenuous vascularisation of the cornea was first noted ventral to the ulcer on day 15. Epiphora and blepharospasm were last noted on day 17 although mild conjunctivitis persisted to day 21. The corneal opacity faded and the ulcer healed without being vascularised, leaving a small facet at the ulcer site; the capillaries which had formed were not visible by day 22.

- Right eye. This eye remained normal up to day 16 apart from transient epiphora, conjunctivitis and iridospasm, noted on day 13 only. On day 17, epiphora, blepharospasm, increased blinking, mild conjunctivitis and iridospasm were noted, and a 2mm corneal ulcer was present although the cornea was completely transparent. A corneal opacity had developed by day 18 and the lesions increased in severity up to day 21, at which point, the cornea was almost opaque, an 8mm ulcer was present and a purulent discharge was noted. Vascularisation of the cornea was first noted on day 21, reached the ulcer on day 27 and had completely vascularised the ulcer floor by day 31, with the development of projecting granulation tissue. Signs of irritation decreased from day 38 and mild epiphora and conjunctivitis were last noted on day 45.



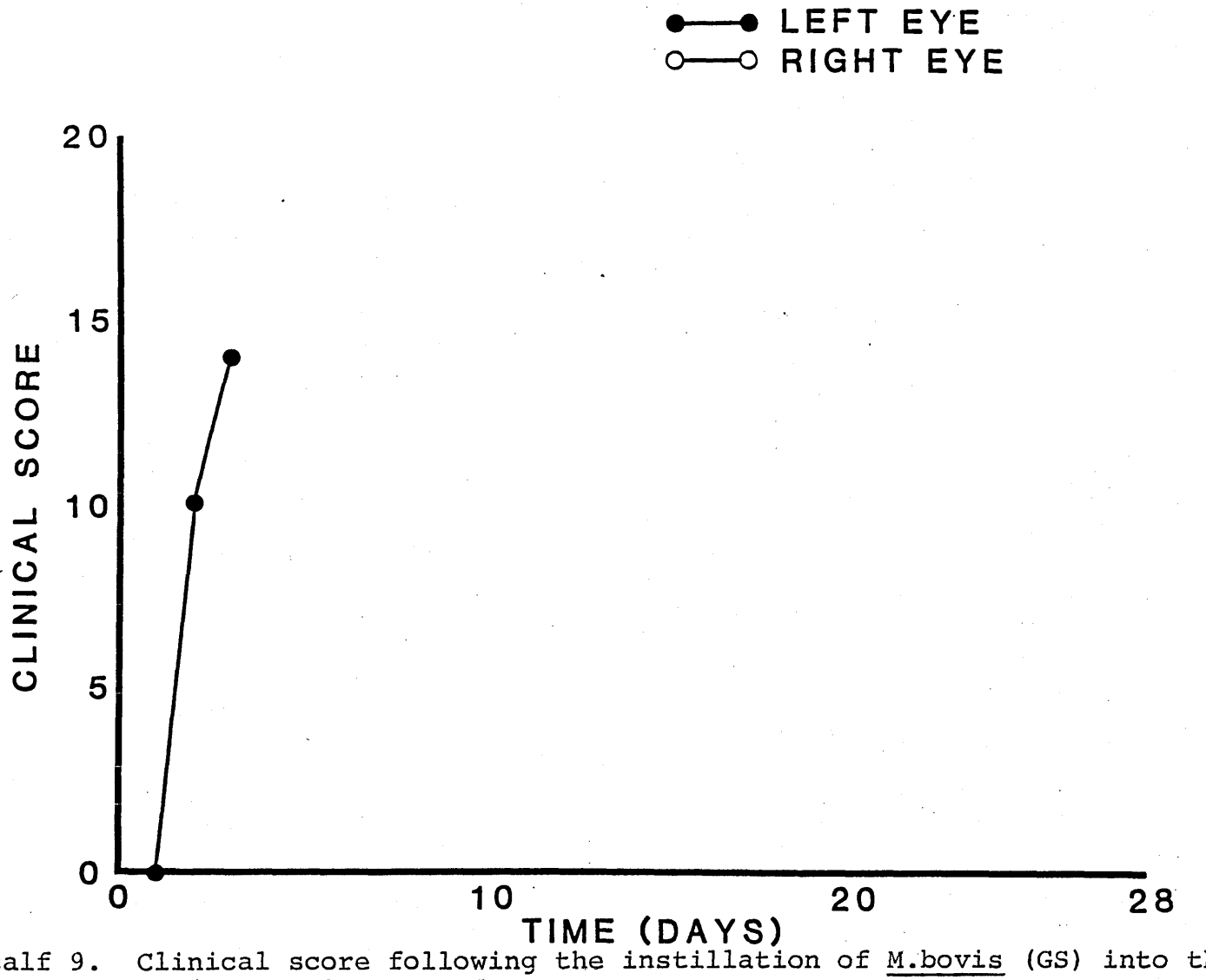
Experiment 2, calf 8. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 9

The clinical scores from this calf for the period from day 0 to day 3 are illustrated overleaf.

- Left eye. On day 2, epiphora, blepharospasm, increased blinking and conjunctivitis were present; a 3mm diameter corneal ulcer was noted and a diffuse corneal opacity could be seen under oblique lighting only. On day 3, there was a 2mm anterior-polar ulcer, the cornea was moderately opaque and iridospasm was noted. The calf was slaughtered on day 3 for histological examination of the lesions.

- Right eye. This eye remained normal throughout this experiment.



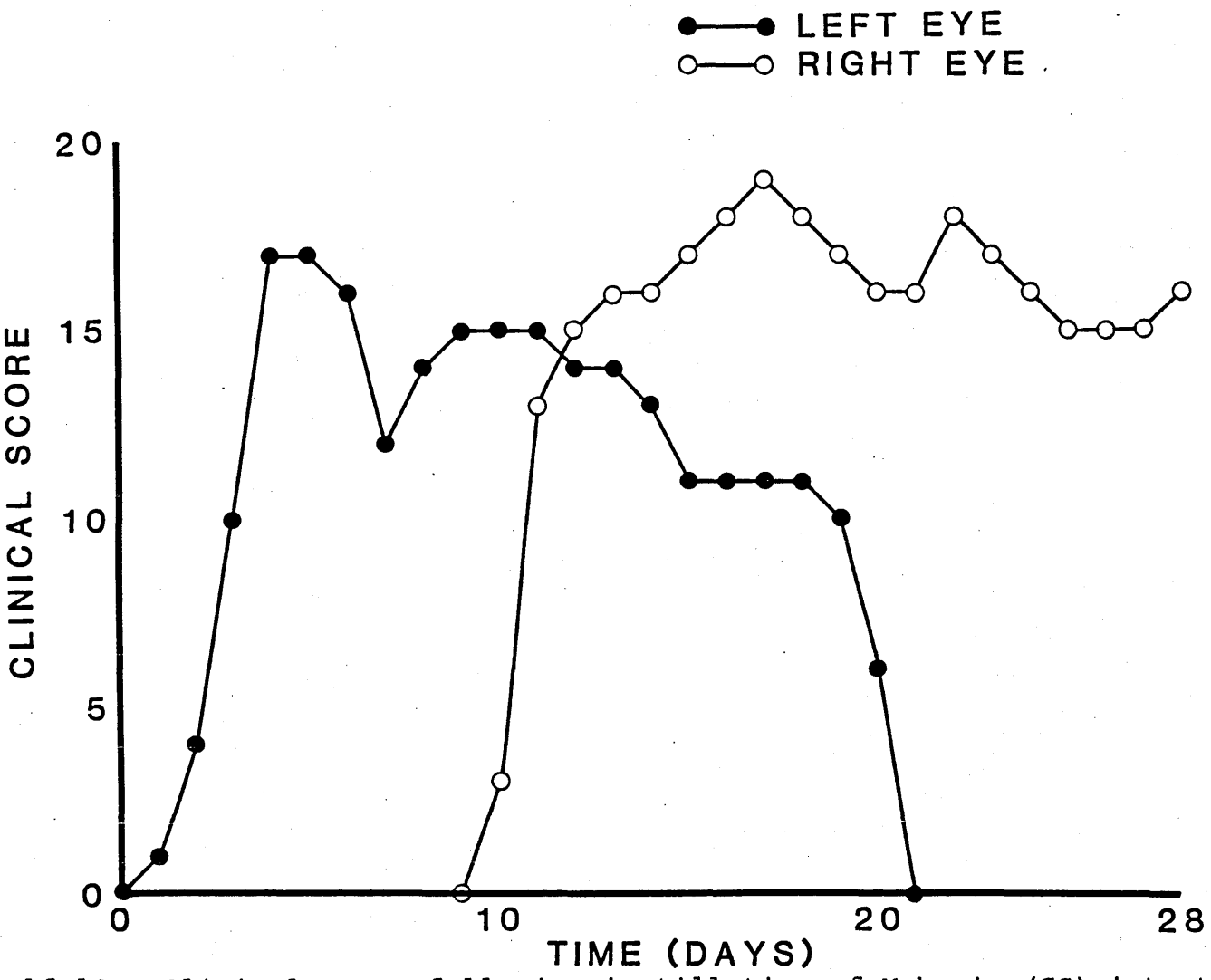
Experiment 2, calf 9. Clinical score following the instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 10

The clinical scores for this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. This eye had developed mild epiphora by day 1, blepharospasm and increased blinking by day 2 and, on day 3, iridospasm, a 2mm corneal vesicle and a diffuse, mild opacity were noted. The lesions were most severe by day 4, the ulcer being 5mm in diameter and the cornea diffusely opaque. Vascularisation was first noted on day 9 and had extended to the bottom rim of the ulcer by day 17. The eye became progressively less irritated from days 11 to 18, although blepharospasm persisted up to day 21. Vascular tissue encroached upon the ulcer floor without the formation of obvious granulation tissue, faded from day 22 onwards and could not be seen by day 30, leaving a small opaque scar at the ulcer site.

- Right eye. Epiphora and conjunctivitis were first noted on day 10. By day 11, severe blepharospasm, increased blinking, iridospasm, a 2mm anterior-polar vesicle and moderate opacity were present. The vesicle became underrun, expanding to a maximum diameter of 10mm before rupturing, on day 17, leaving a large, shallow ulcer; at this point, the cornea was totally opaque and the iris not visible. Vascularisation was first noted on day 14 and reached the ulcer edge by day 22. Dorsal and ventral ridges of granulation tissue formed which coalesced by day 29, forming a single ridge projecting 2mm from the corneal surface. The eye became less irritated from day 23 and, by day 32, was completely free from epiphora and conjunctivitis, although mild blepharospasm was still present. Granulation tissue had resolved by day 45, leaving a dense, white scar at the ulcer site, although a few vessels could still be detected running through the cornea.



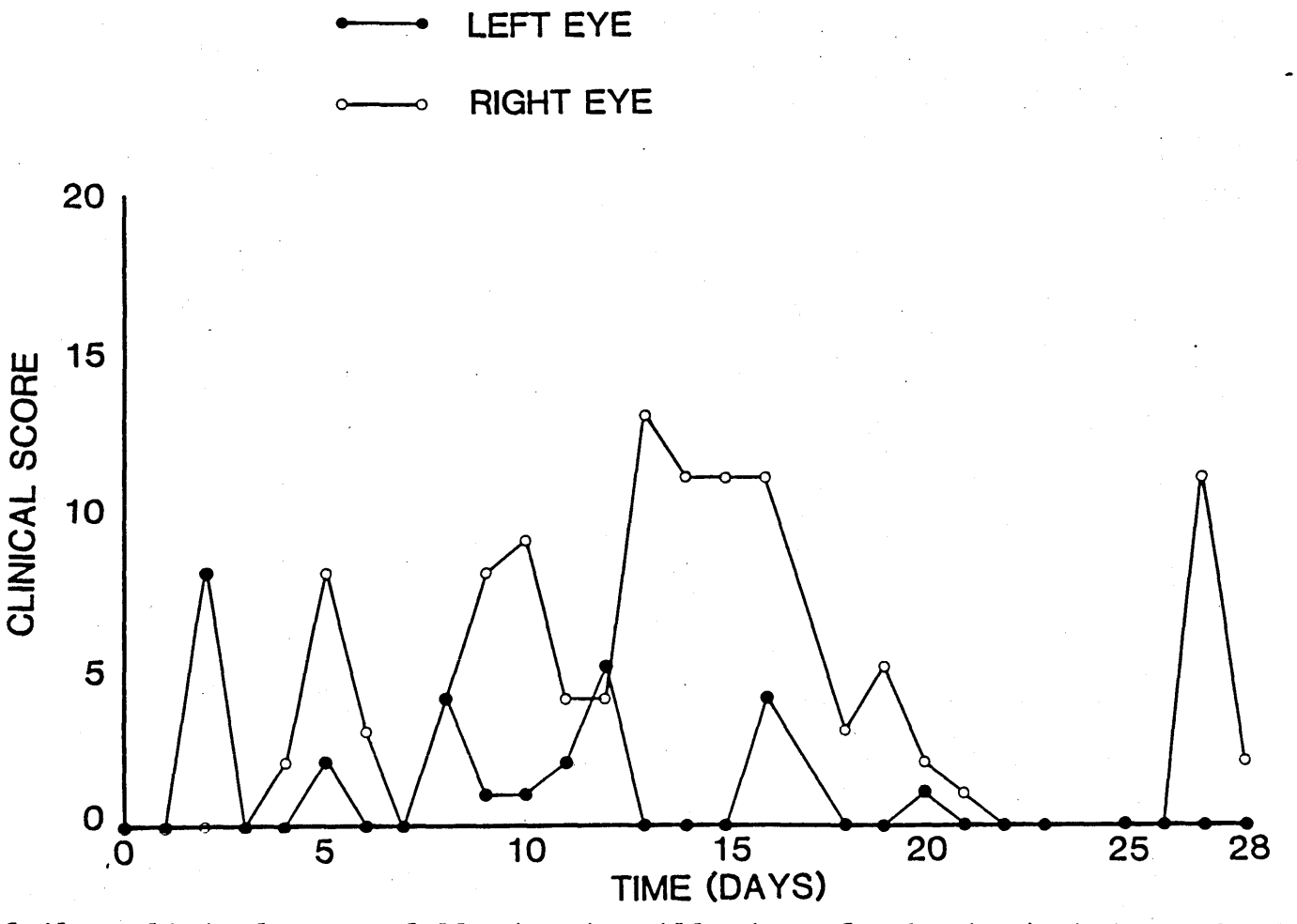
Experiment 2, calf 10. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX I: Calf 41

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. Infectious bovine keratoconjunctivitis was not diagnosed although periods of mild ocular irritation were noted and a slight, transient opacity was present on day 12.

- Right eye. Signs of mild ocular irritation were noted in the absence of corneal changes on days 4 to 6. On day 8, signs of irritation recurred and a pinpoint, anterior-polar ulcer, which healed by day 11, was noted. On day 13, a second 2mm diameter was noted which healed over a period of seven days. The eye remained normal until day 27, when a third pinpoint ulcer was noted, accompanied by severe signs of ocular irritation. These lesions were almost completely resolved 24 hours later.



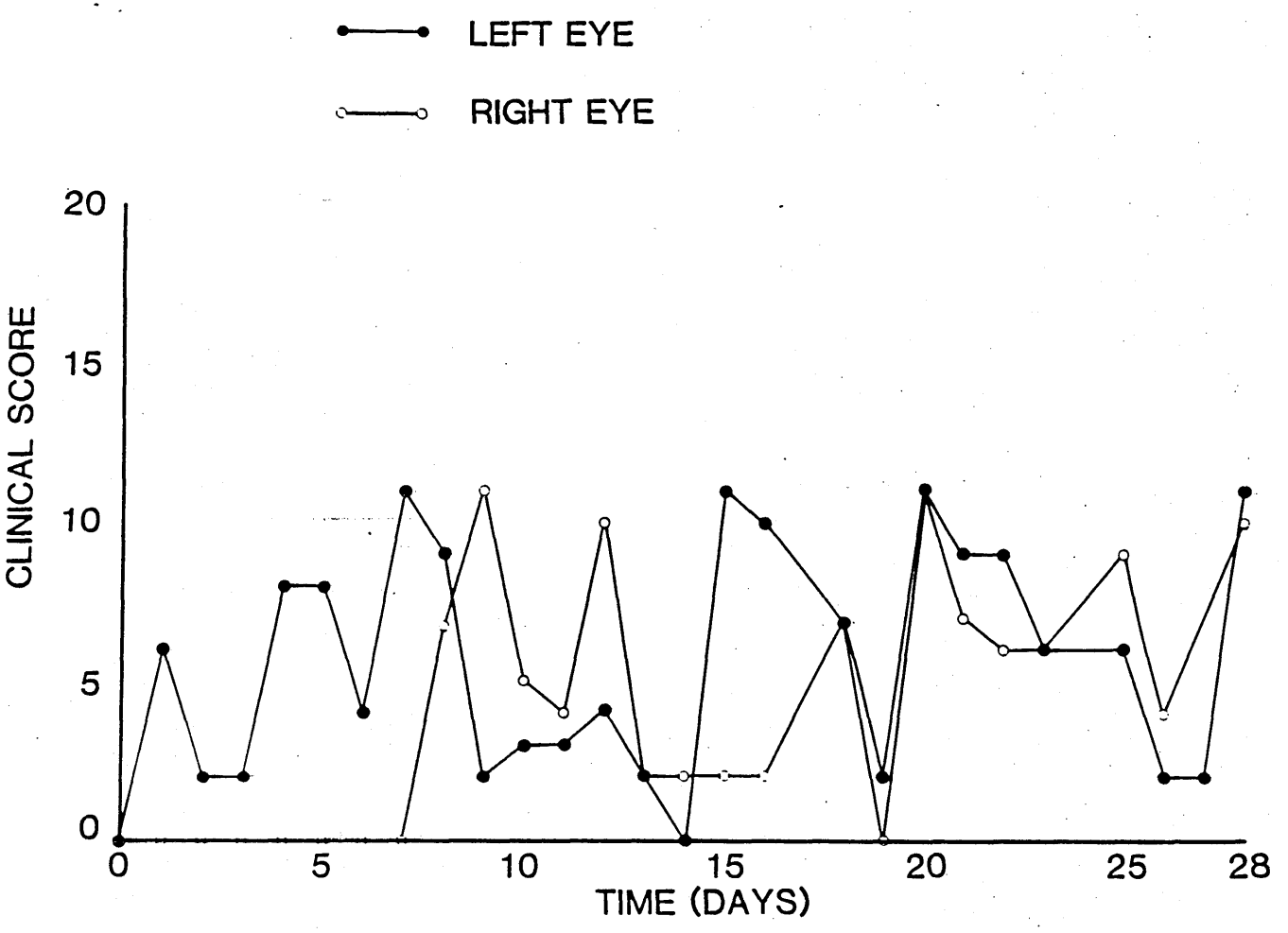
Experiment 3, calf 41. Clinical score following instillation of M.bovis (GM) into the left conjunctival sac on day 0.

APPENDIX I: Calf 42

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. Epiphora, blepharospasm, increased blinking and conjunctivitis were noted on day 1, although conjunctivitis was the only sign present on days 2 and 3. On day 4, epiphora, blepharospasm, increased blinking and iridospasm recurred and, on day 5, two pinpoint ulcers, accompanied by a corneal opacity visible under oblique lighting only, were noted. The corneal ulcers did not progress beyond 1mm diameter and had resolved completely, without corneal vascularisation, by day 14. Signs of ocular irritation remained mild although fluctuating in intensity. On day 15, a fresh 1mm anterior-polar ulcer and severe signs of ocular irritation were noted. These signs decreased in intensity over a period of four days and, on day 19, the only sign present was a faint opacity. Severe signs recurred on day 20 and a fresh 1mm ulcer was still present on day 28, although signs of ocular irritation varied markedly.

- Right eye. This eye remained normal until day 8 when epiphora, blepharospasm, increased blinking and conjunctivitis were noted. A corneal opacity and a 2mm ulcer were noted on day 9 and persisted, without developing further, until day 28. Signs of ocular irritation varied during this period with marked increases in intensity noted on days 18, 20, 25 and 28.



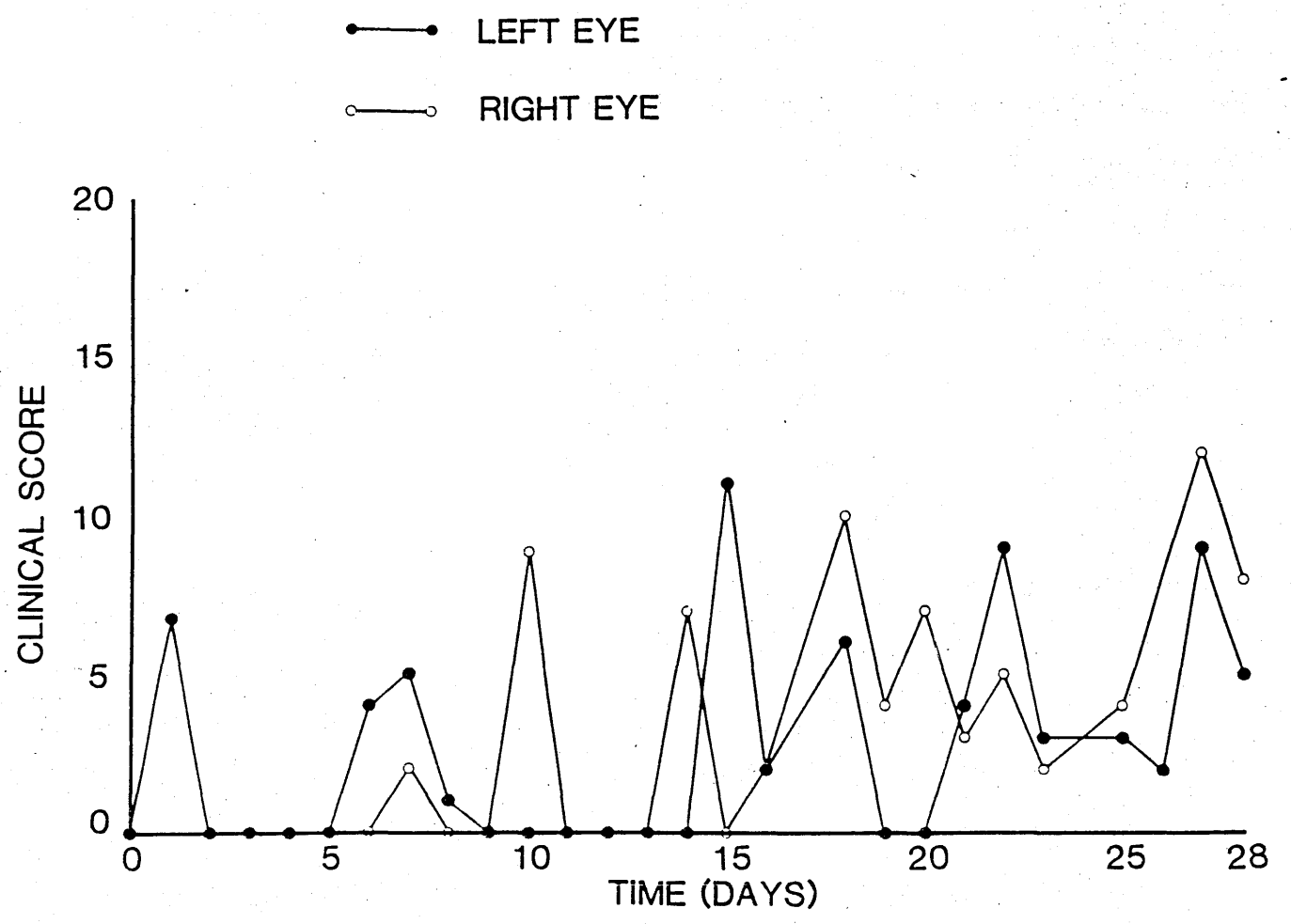
Experiment 3, calf 42. Clinical score following instillation of M.bovis (GM) into the left conjunctival sac on day 0.

APPENDIX I: Calf 43

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eyes. Severe epiphora, blepharospasm, increased blinking and conjunctivitis were present on day 1, although these signs had resolved 24 hours later. Epiphora and conjunctivitis were noted on days 6 and 7 and a small area of corneal opacity was present, on the lateral margin of the cornea, on days 7 and 8. A small ulcer, accompanied by faint corneal opacity and severe signs of ocular irritation, was noted on day 15. The signs of ocular irritation were absent on day 16, were present, but mild, on day 18 and, on day 19, the eye was normal. A fresh 1mm ulcer was noted on the medial margin of the cornea on day 21 which persisted until day 28 although signs of ocular irritation were present only on days 22, 27 and 28.

- Right eyes. Acute severe signs of ocular irritation were noted on days 10 and 14 unaccompanied by corneal changes. Mild corneal lesions, consisting of a 1mm medial ulcer surrounded by a faint opacity, were noted on day 16 and persisted until day 28 although variable signs of ocular irritation were present during only half of the examinations in this period.



Experiment 3, calf 43. Clinical score following instillation of M.bovis (GM) into the left conjunctival sac on day 0.

APPENDIX I: Calf 44

- Left eye. This eye remained normal apart from epiphora and mild blepharospasm on day 20 only.

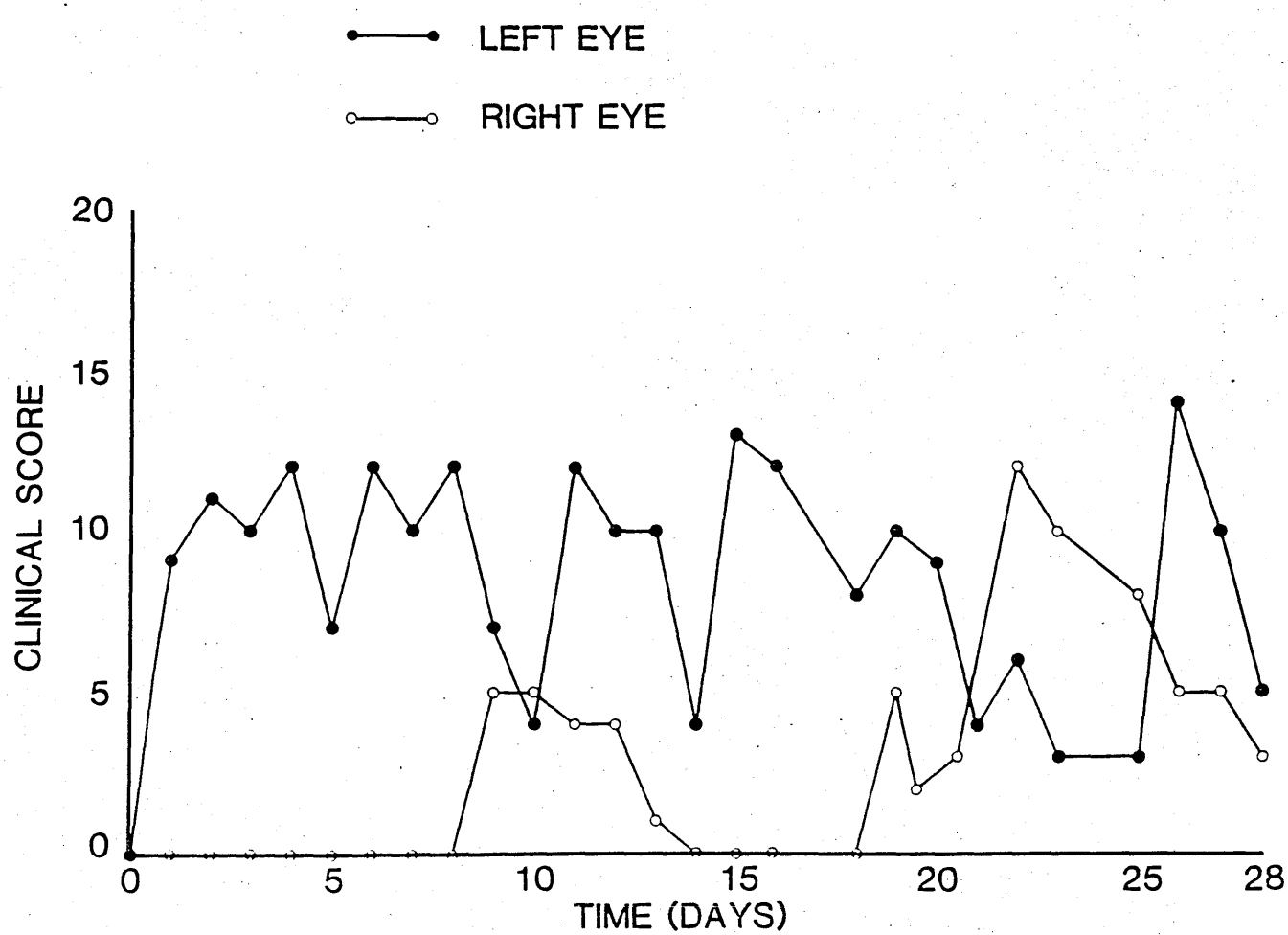
- Right eye. This eye remained normal throughout the experiment.

APPENDIX I: Calf 45

The clinical scores from this calf for the period from day 0 to day 28 are illustrated overleaf.

- Left eye. Signs of mild ocular irritation, a pinpoint ulcer adjacent to the ventro-medial corneoscleral junction and localised corneal opacity were noted on day 1. A fresh, lateral, pinpoint ulcer was noted on day 4. On day 5, three further pinpoint ulcers were found, despite a marked decrease in ocular irritation, and tenuous corneal vascularisation was noted at the ventro-lateral corneoscleral junction. By day 12, the ulcer on the medial corneoscleral junction was completely resolved and vascular tissue had reached the lateral ulcer. Signs of ocular irritation persisted until day 22, at which time the ulcer appeared to be quiescent and vascularisation had faded. On day 26, the eye was severely irritated and the ventral cornea was diffusely hazy with dense white patches at the sites of previous corneal ulcers. By day 28, the corneal irritation was almost completely resolved, apart from slight conjunctivitis, and the corneal opacity was reduced.

- Right eye. On day 9, epiphora, blepharospasm and increased blinking were noted and, on day 10, conjunctivitis and a pinpoint lateral ulcer were present. The ulcer had healed by day 11 and the eye was normal on day 14. On day 19, a fresh ulcer was noted in conjunction with mild epiphora and blepharospasm. The ulcer reached a maximum diameter of 2mm on day 23 and persisted to day 28 although, during this period, signs of ocular irritation were either absent or mild.



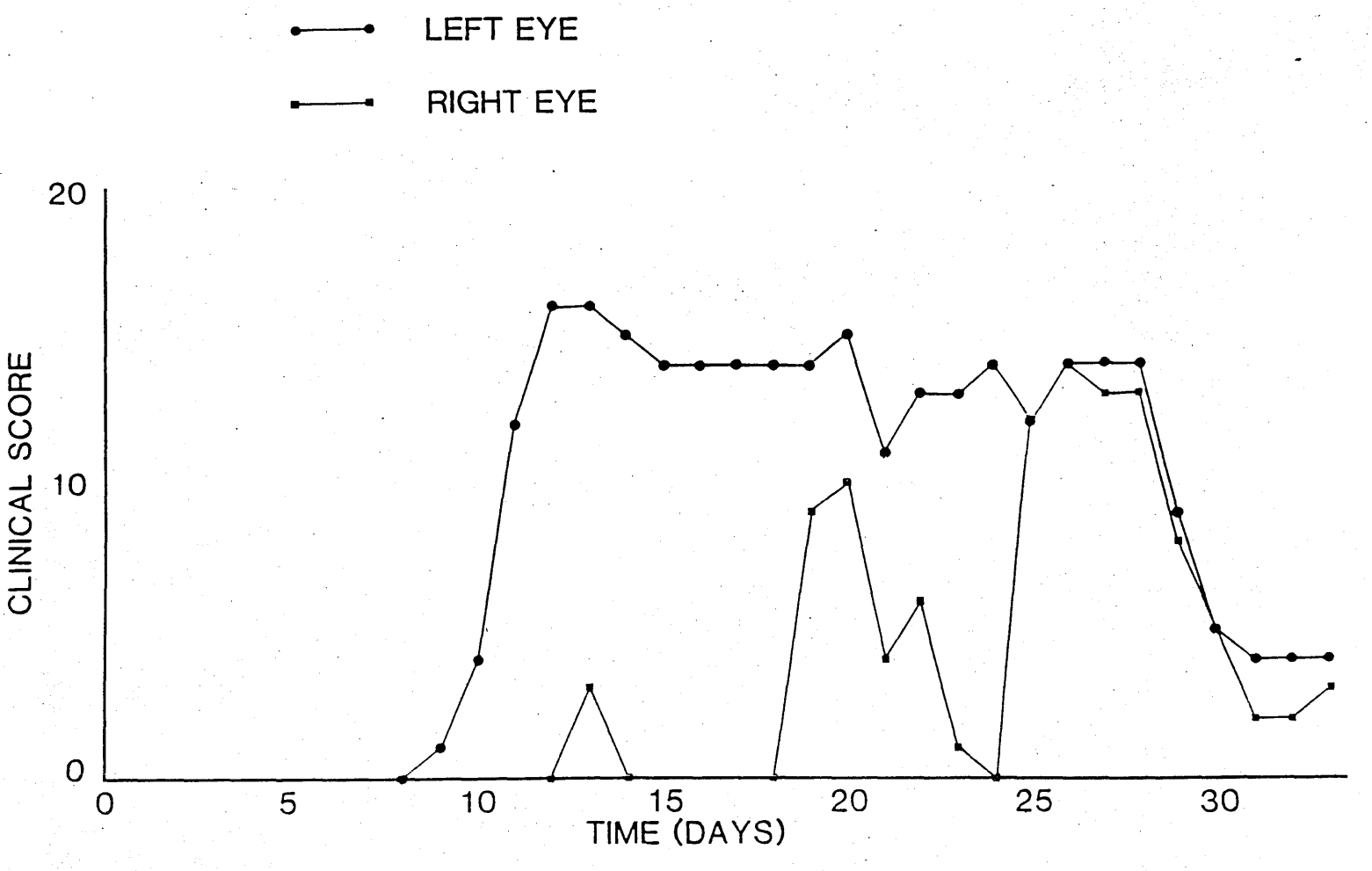
Experiment 3, calf 45. Clinical score following instillation of M.bovis (GM) into the left conjunctival sac on day 0.

APPENDIX II: Calf 11

The clinical scores from this calf for the period from day 0 to day 33 are illustrated overleaf.

- Left eye. Moraxella bovis was first isolated on day 7. Slight conjunctivitis was first noted on day 8, epiphora and iridospasm on day 10 and blepharospasm and increased blinking on day 11; at this point, the calf was dull, movement and blinking were accompanied by a marked rearward jerk of the head, there was a diffuse faint corneal opacity and a 2mm diameter ulcer was noted in the dorso-lateral quadrant of the cornea. By day 13, the vesicle was 8mm in diameter which had ruptured. By day 15, an ulcer 10mm in diameter had formed and corneal vascularisation was first noted as a band 1mm wide along the entire corneoscleral junction. This advanced centripetally, at a rate of 1mm/day, reaching the dorsal edge of the ulcer by day 24. By day 28, the top half of the ulcer was vascularised with an overlying 1.5 mm ridge of granulation tissue and severe signs of ocular irritation were still present.

- Right eye. Moraxella bovis was first isolated on day 14. Signs of irritation were first noted on day 19 with the development of a pinpoint ulcer and surrounding opacity by day 20. These lesions had resolved by day 23 although signs of ocular irritation recurred on day 25 and a 3mm ulcer had developed by day 26, which was still present on day 28.



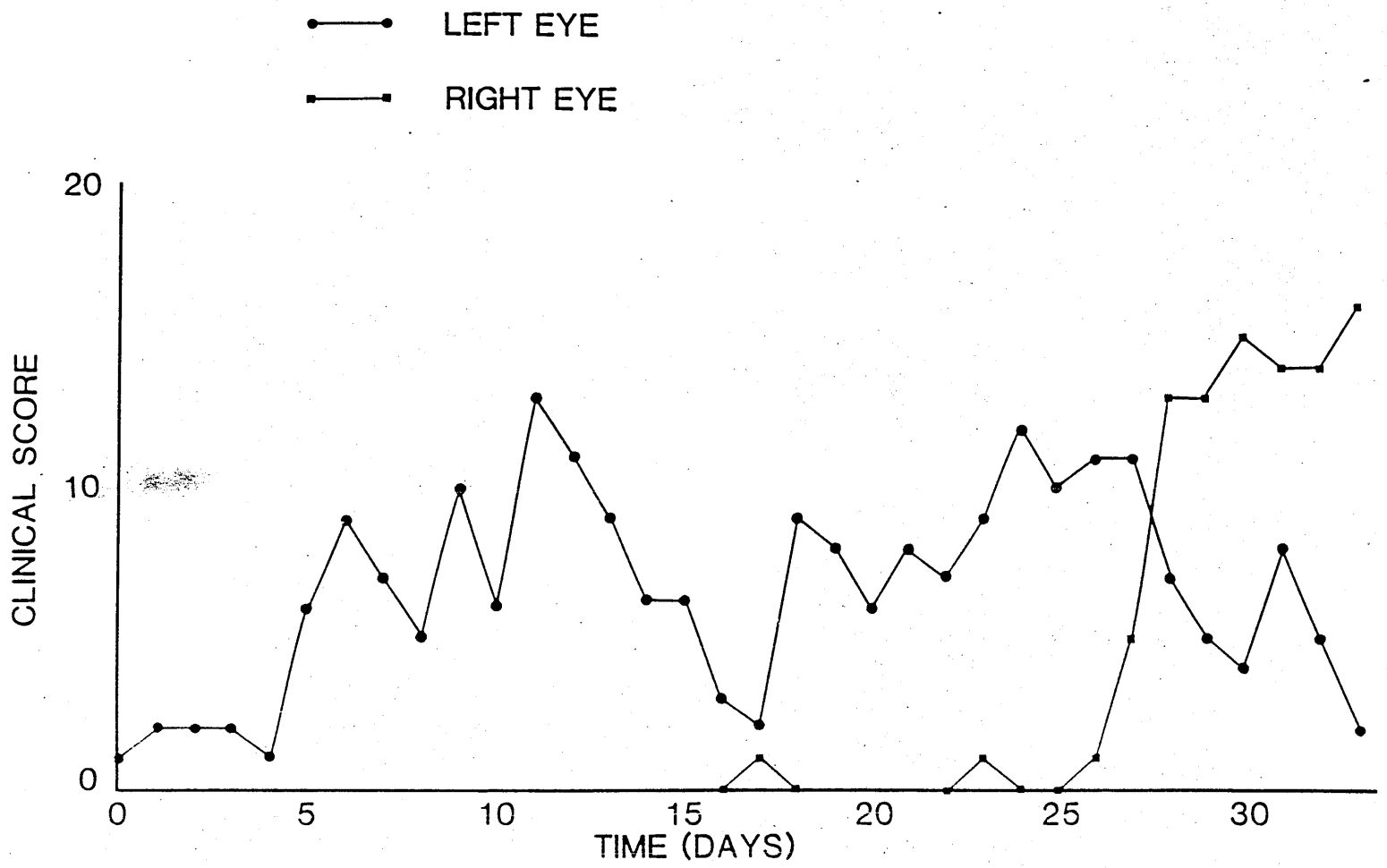
Experiment 4, calf 11. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac of a single calf (12) on day 0.

APPENDIX II: Calf 12

The clinical scores from this calf for the period from day 0 to day 33 are illustrated overleaf.

- Left eye. Moraxella bovis was consistently reisolated following inoculation. Epiphora and conjunctivitis were first noted on day 1, blepharospasm and iridospasm on day 5 and a generalised, hazy corneal opacity on day 6. The cornea did not develop an ulcer at this stage and the corneal haze resolved and was clear by day 10 despite continuing signs of ocular irritation. On day 11, a 2mm anterior-polar vesicle and diffuse corneal opacity were noted. By day 13, an ulcer had formed which healed by day 16, leaving a small facet. On day 15, faint vascularisation was noted at the ventral corneoscleral junction. At this stage, epiphora was marked but other signs of ocular irritation had resolved. The capillary bed, which was visible under oblique light only, advanced across the ventral cornea at a rate of 1mm/day. A second, medially oriented ulcer was found on day 18 and a third on day 25, at which time, the capillary bed became markedly injected. These ulcers had not healed by day 28.

- Right eye. Moraxella bovis was first isolated on day 20. Signs of ocular irritation were noted on day 27 and IBK diagnosed on day 28 when corneal changes and a head jerk were present.



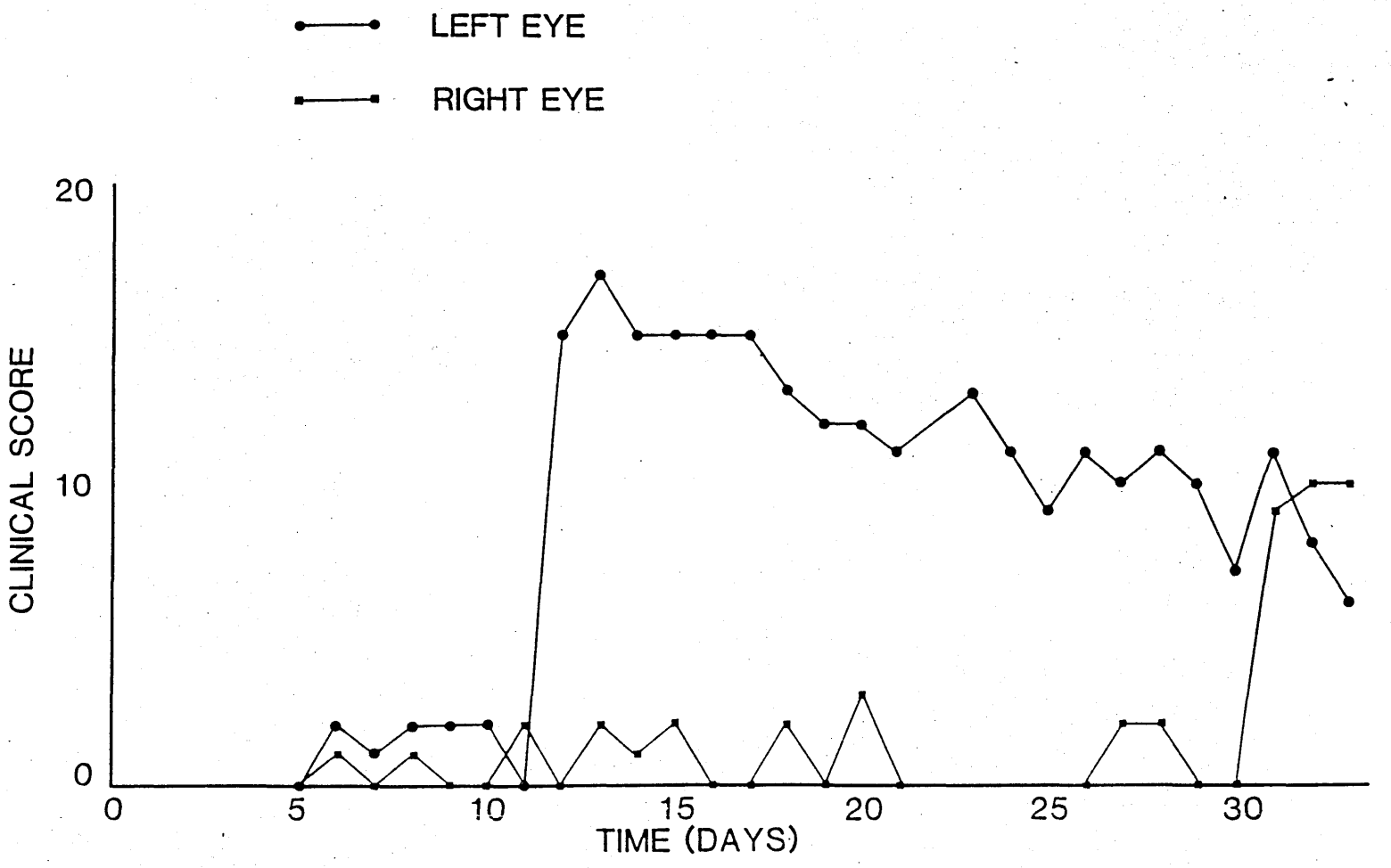
Experiment 4, calf 12. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac on day 0.

APPENDIX II: Calf 13

The clinical scores from this calf for the period from day 0 to day 33 are illustrated overleaf.

- Left eye. Moraxella bovis was first isolated on day 2. Very slight corneal changes were noted, under oblique illumination, on days 6 to 8 and slight epiphora and conjunctivitis on days 8 to 10 but the eyes were free from lesions on day 11. On day 12, signs of ocular irritation were noted with epiphora, blepharospasm, increased blinking, conjunctivitis and iridospasm present. Three ulcers, the largest of which was 4mm in diameter, were present and the cornea was diffusely opaque. On day 15, a single anterior-polar ulcer remained, and vascularisation was noted dorsally. Tenuous vascularisation developed in all areas but was most marked in the dorsal quadrant. Capillaries reached the dorsal ulcer edge by day 23 but, by day 28, mild irritation persisted and the ulcer had not changed.

- Right eye. Moraxella bovis was first isolated on day 9. Signs of ocular irritation were first noted on day 27 and, by day 28, two anterior-polar ulcers had developed.



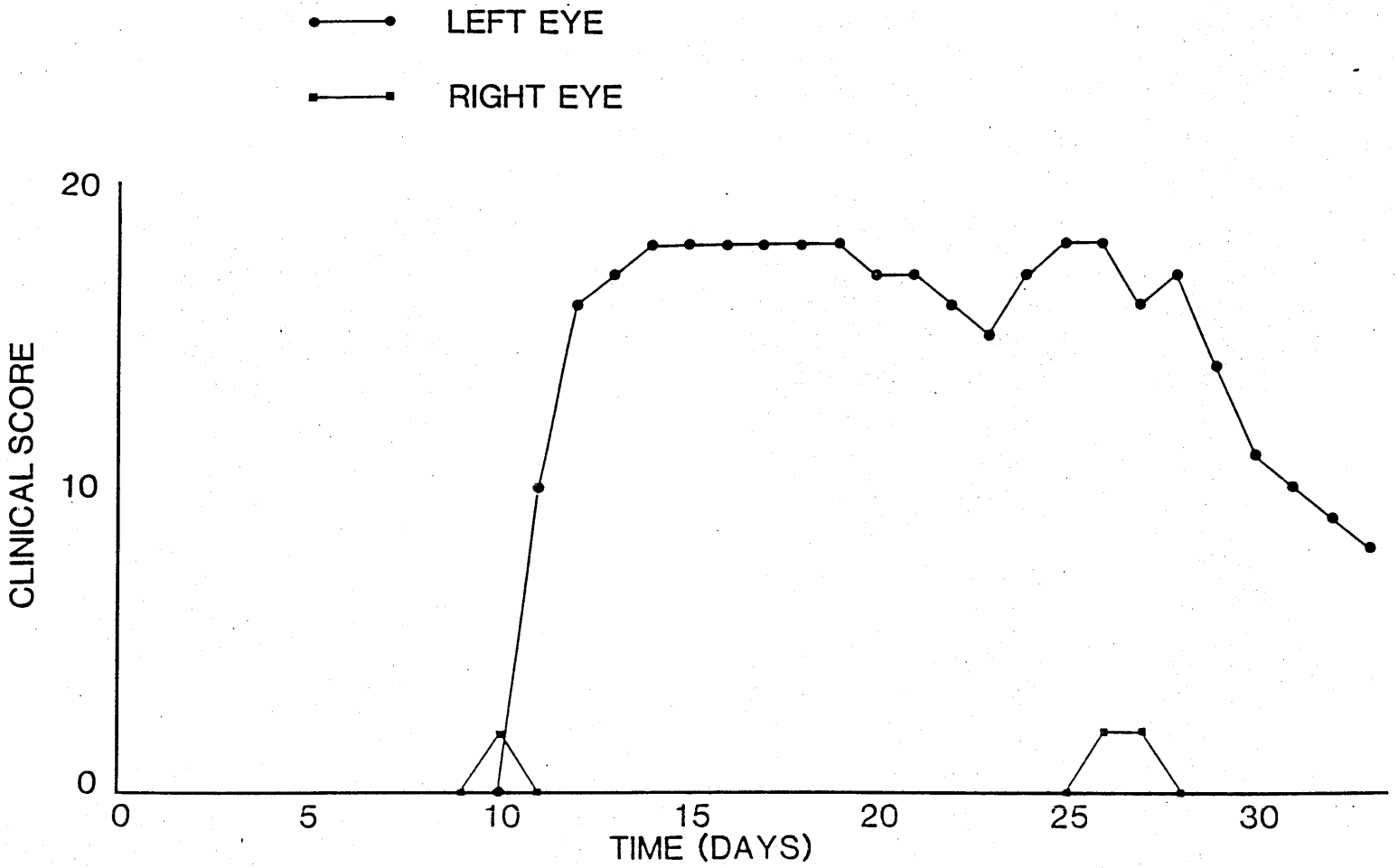
Experiment 4, calf 13. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac of a single calf (12) on day 0.

APPENDIX II: Calf 14

The clinical scores from this calf for the period from day 0 to day 33 are illustrated overleaf.

- Left eye. Moraxella bovis was first isolated on day 9. Signs of ocular irritation were first noted on day 11, with epiphora, blepharospasm, increased blinking, conjunctivitis and iridospasm, and the cornea showed a slight haziness under oblique illumination. On day 12, a 2mm anterior-polar vesicle was noted and the cornea was almost totally opaque. By day 15, the vesicle had expanded to a 10mm x 15mm oval, with a central, deep ulcer of 4mm in diameter. Vascularisation of the cornea was present along the entire length of the corneoscleral junction. The capillary bed reached the vesicle edge by day 22 and a ridge of granulation tissue began to form at the periphery of the ulcer by 25. By day 28, the ulcer was still not fully vascularised and moderate to severe signs of ocular irritation were still present.

- Right eye. Although M.bovis was first isolated on day 10 and consistently thereafter, there were no signs of IBK in this eye at any time.



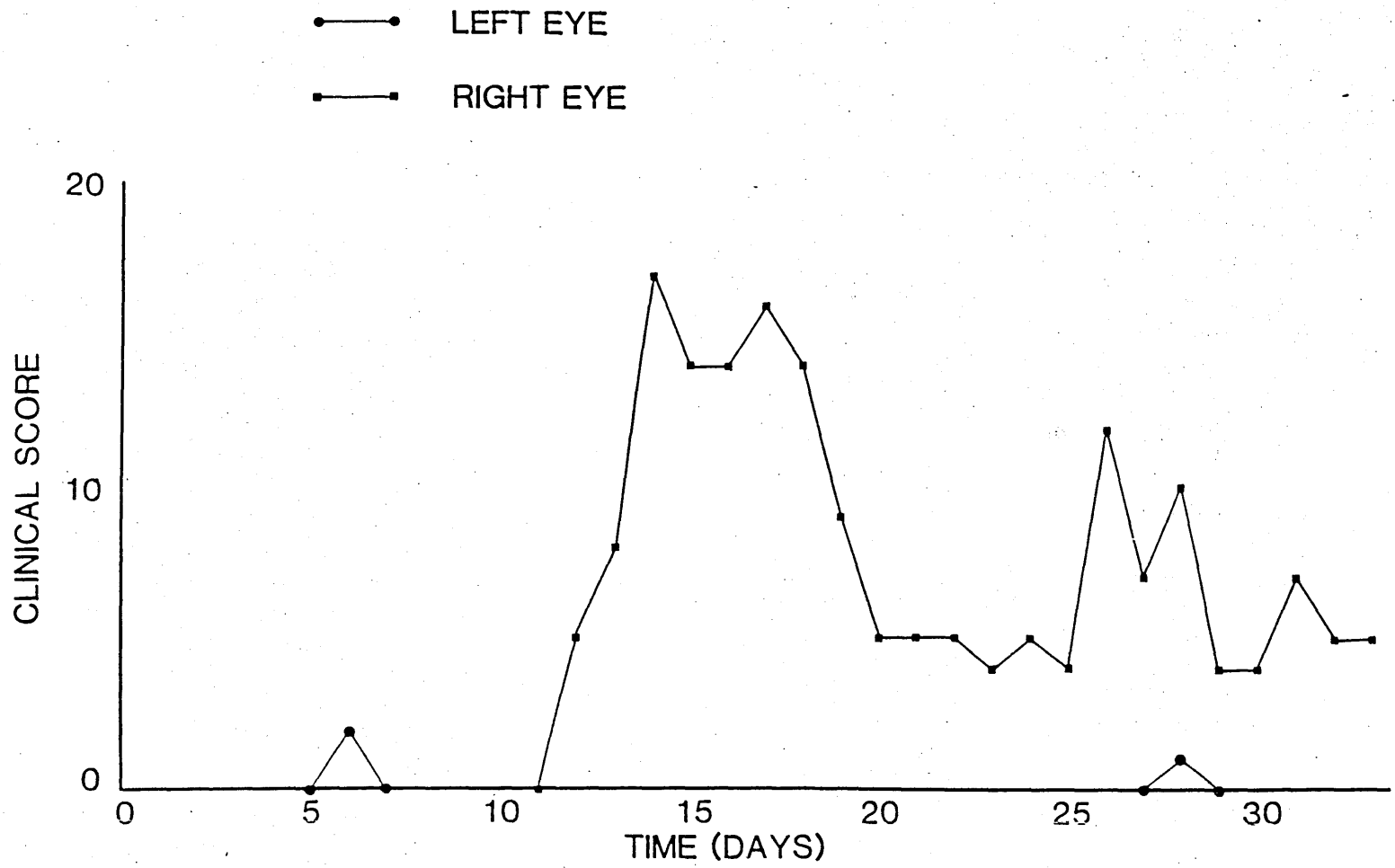
Experiment 4, calf 14. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac of a single calf (12) on day 0.

APPENDIX II: Calf 15

The clinical scores from this calf for the period from day 0 to day 33 are illustrated overleaf.

- Left eye. Moraxella bovis was isolated on days 8 and 9, but was not isolated again until day 21. Signs of IBK did not develop in this eye.

- Right eye. Moraxella bovis was first isolated on day 9. On day 12, epiphora, increased blinking and iridospasm were present although conjunctivitis and blepharospasm were not noted until day 13. On day 14, a 5mm diameter corneal ulcer and a high degree of corneal opacity were noted. Vascularisation of the cornea was first noted on day 16 as a dense band, 1mm wide, along the entire corneoscleral junction. Although the capillary bed advanced at a rate of 1mm/day, from day 17 onwards, it gradually became more tenuous and corneal opacity decreased. Signs of irritation recurred on day 26 followed by marked injection of corneal blood vessels on day 27 although there were no other corneal changes during this period.



Experiment 4, calf 15. Clinical score following instillation of M.bovis (GS) into the left conjunctival sac of a single calf (12) on day 0.

APPENDIX II: Calf 69

- Left eye. Moraxella bovis was consistently isolated following instillation. The eye remained free from disease up to, and including, day 8, demonstrated mild signs of irritation on days 9 to 11, without the development of clinically detectable corneal involvement, and was normal on days 12 to 15. On day 16, epiphora, moderate blepharospasm, increased blinking, conjunctivitis, iridospasm and a 1mm diameter, anterior-polar ulcer, surrounded by a faint opacity, were present. Signs of irritation and the corneal opacity decreased in intensity over a period of six days while the ulcer appeared to have healed by day 19. The eye remained healthy from day 23 to day 27 although mild epiphora was again noted on day 28.

- Right eye. Moraxella bovis was not isolated from any of the samples collected from this eye. The eye remained clinically normal apart from occasional periods of epiphora in the absence of other clinical signs.

APPENDIX II: Calf 70

- Left eye. Moraxella bovis was first isolated on day 20. The eye was healthy on all examinations prior to day 24 apart from occasional periods of epiphora. On day 24, mild signs of ocular irritation, a pinpoint corneal ulcer and diffuse opacity were present. Epiphora and conjunctivitis persisted for three days only and the eye was normal on day 28.

- Right eye. Moraxella bovis was first isolated on day 24. The right eye remained normal apart from mild epiphora and conjunctivitis on one occasion only.

APPENDIX II: Calf 71

- Left eye. Moraxella bovis was first isolated on day 20. The eye remained normal throughout the examination period except for mild ocular irritation on day 21.

- Right eye. Moraxella bovis was first isolated on day 17. The eye remained normal up to day 22 when mild signs of irritation were present and which persisted to day 25.

APPENDIX II: Calf 72

- Left eye. Moraxella bovis was first isolated on day 18. The eye was normal from days 0 to 26, apart from periods of epiphora and mild conjunctivitis, mild signs of ocular discomfort were noted on day 27 and, by day 28, a 2mm, anterior-polar ulcer surrounded by a hazy opacity had developed.

- Right eye. Moraxella bovis was first isolated on day 5. Mild signs of ocular discomfort were noted on day 18 in conjunction with a diffuse corneal opacity, visible under oblique light only. On day 19, the eye was more severely irritated, iridospasm was present and a milky white opacity was noted over the lateral quadrant of the cornea. On day 20, despite the continued presence of severe conjunctivitis and epiphora, the eye lids and iris were fully open and blinking was normal. The cornea was slightly more opaque and a 4mm x 6mm vesicle was noted adjacent to the dorso-lateral corneoscleral junction. On day 21, the vesicle had ruptured, leaving a very shallow corneal ulcer, and, on day 22, faint vascularisation was first noted which, by day 26, had extended across the ulcer floor. By day 28, the site of the ulcer was marked by slight facet, surrounded by a faint ring of corneal opacity.

APPENDIX II: Calf 73

- Left eye. Moraxella bovis was first isolated on day 20. The eye remained normal throughout the 28 day examination period.

- Right eye. Moraxella bovis was first isolated on day 20. On day 18, there were severe signs of ocular irritation, blinking was accompanied by a marked rearward jerk of the head, the cornea was diffusely opaque and a central corneal vesicle, 3mm in diameter, was noted. The corneal vesicle ruptured by day 19, leaving a shallow ulcer which did not change in diameter or depth. Vascularisation of the cornea was first noted on day 22, along the entire corneoscleral junction, and had advanced 6mm across the cornea by day 28.

APPENDIX III: Calf 11

- Left eye. On day 28, there were severe signs of ocular irritation, with profuse lachrymation, blepharospasm, increased blinking and conjunctivitis, and an 18 day old ulcer with projecting granulation tissue at the dorsal and ventral ulcer edges. By day 29, epiphora had ceased, the other signs of irritation were much reduced and were absent from day 30 onwards. Capillary infiltration of the cornea decreased from day 29 to 31, leaving two dorsal and two ventral veins visible supplying a central, slightly projecting, pale pink scar. No vessels were visible from day 37 onwards.

- Right eye. On day 28, there was severe ocular irritation and a 3mm diameter, three day old, corneal ulcer. Irritation was much reduced on day 29 and was absent by day 31. The ulcer did not increase. Very faint neovascularisation was first noted on day 30, reached the ulcer edge on day 34, and remained visible until day 36, at which point the ulcer site was marked by a transparent facet.

APPENDIX III: Calf 12

- Left eye. On day 28, there were advanced healing ulcers of 17 and 10 days duration, both surrounded by facing vascular tissue, and signs of ocular irritation were much reduced from previous levels. By day 29, the ulcers were non-reactive and mild epiphora was the only sign of ocular irritation present although this persisted, with variable intensity, up to day 38.

- Right eye. Signs of ocular irritation had first been noted on day 27 and two corneal ulcers had developed by day 28. Despite treatment signs of severe irritation persisted and the ulcers became underrun - forming a single 8mm diameter corneal vesicle by day 34 which had ruptured, leaving a similar sized ulcer, by day 35. Vascularisation was first noted on day 31, reaching the ulcer edge on day 41. Granulation tissue was first noted on day 43 and the eye was still showing severe signs of irritation on day 44.

APPENDIX III: Calf 13

- Left eye. On day 28, there were mild signs of ocular irritation and a 14 day old corneal ulcer which was partially vascularised. There was no change on day 29, a slight reduction in severity on day 30 but more severe signs of irritation recurred on day 31. These abated following the second antibiotic treatment on day 31 and the eye was not irritated from day 33 onwards despite the continuing presence of the healing ulcer.

- Right eye. This eye had been free from all signs of IBK up to day 31 when epiphora, blepharospasm, increased blinking, conjunctivitis and iridospasm were noted. On day 32, a pinpoint corneal ulcer, accompanied by a diffuse opacity, was noted which reached a maximum size, of 3mm, by day 34, although remaining very shallow, and healed without vascularisation, leaving a slight facet, by day 37.

APPENDIX III: Calf 14

- Left eye. On day 28 there were severe signs of ocular irritation, a 17 day old ulcer with extensive vascularisation of the cornea and granulation tissue at the ulcers edge. Signs of ocular irritation decreased gradually in both number and intensity over a period of five days following initial treatment. Healing of the ulcer was accompanied by a gradual decrease in peripheral corneal opacity and a gradual reduction in vascular and granulation tissue such that, by day 42, a faint pink scar, 10mm by 5mm, supplied by two larger capillaries, remained.

- Right eye. This eye remained normal throughout the experiment.

APPENDIX III: Calf 15

- Left eye. This eye remained free from lesions up to day 34 when severe signs of ocular irritation were present accompanied by a very diffuse corneal opacity. A 3mm anterior-polar vesicle was found on day 35 when the first treatment with cloxacillin benzathine was administered. By day 36, this vesicle had ruptured leaving a 5mm ulcer. Faint vascularisation was first noted on day 37 and signs of ocular irritation continued until day 42 but were absent by day 43.

- Right eye. On day 28, there was a vascularised, 3mm, laterally orientated, 14 day old ulcer. Signs of ocular irritation had been present from days 12 to 19 and had recurred on days 26 to 28. Following treatment signs of irritation were absent on days 29 and 30 but recurred, in a mild form, on days 31 to 33. By day 42, the eye appeared normal apart from a slight corneal opacity at the site of the ulcer.

APPENDIX III: Calf 41

- Left eye. There was transient, very mild, ocular irritation on day 41 but the eye was free from lesions from day 42 to 44. On day 45, there were signs of moderate ocular irritation and a 3mm, shallow corneal ulcer was present at the anterior pole. The ulcer did not increase in size or depth and signs of irritation persisted until day 49 only. By day 54 the eye appeared healthy apart from a transparent facet present on the anterior pole.

- Right eye. This eye remained normal apart from the development of a small corneal opacity which was present on day 42 and 43 at the site of a previous corneal ulcer.

APPENDIX III: Calf 42

- Left eye. Small, pinpoint opacities were noted, scattered over the lateral quadrant of the cornea, on days 41 to 49. Conjunctivitis was noted on day 46 and signs of severe ocular irritation were noted on day 49. On day 50, the cornea had developed a uniform opacity and a 3mm, shallow, anterior-polar ulcer was present. By day 53, the eye was completely free from irritation, the corneal opacity was much reduced and the ulcer had contracted slightly and appeared to be quiescent.

- Right eye. This eye remained normal apart from small dense white corneal opacities present from day 41 to the end of the experiment.

APPENDIX III: Calf 43

- Left eye. On day 41, there were signs of moderate ocular irritation and a pinpoint ulcer on the lateral corneal border which was surrounded by a dense, opaque halo. These signs resolved rapidly and, by day 45, the eye appeared to be normal. Very mild epiphora, conjunctivitis and a slight lateral corneal opacity were noted on days 48 to 51. These signs resolved completely but, on day 54, signs of severe ocular irritation and a fresh 2mm ulcer were noted.

- Right eye. This eye remained normal apart from several dense white opacities 0.5mm in diameter which were found scattered over the cornea from day 41 until the end of the experiment.

APPENDIX III: Calf 44

- Left eye. On day 41, conjunctivitis, mild blepharospasm, increased blinking and a small corneal opacity in the upper quadrant were noted. On day 42, only mild conjunctivitis remained and the opacity had decreased. A fresh area of opacity was noted on day 43 in the lateral quadrant but the eye was normal by day 45.

- Right eye. This eye remained healthy throughout this part of the experiment.

APPENDIX III: Calf 45

- Left eye. On day 41, conjunctivitis, mild epiphora and small, dense corneal opacities were noted. The former two signs had resolved by day 43 but, on day 45, signs of severe irritation recurred. By day 46, there was a 3mm, anterior-polar ulcer, and a diffuse, ventral opacity with underlying vascularisation which covered 50% of the corneal area. Signs of irritation decreased gradually and were absent by day 50, at which time, the ulcer appeared to be quiescent, the corneal opacity was much reduced and vascular tissue was not visible.

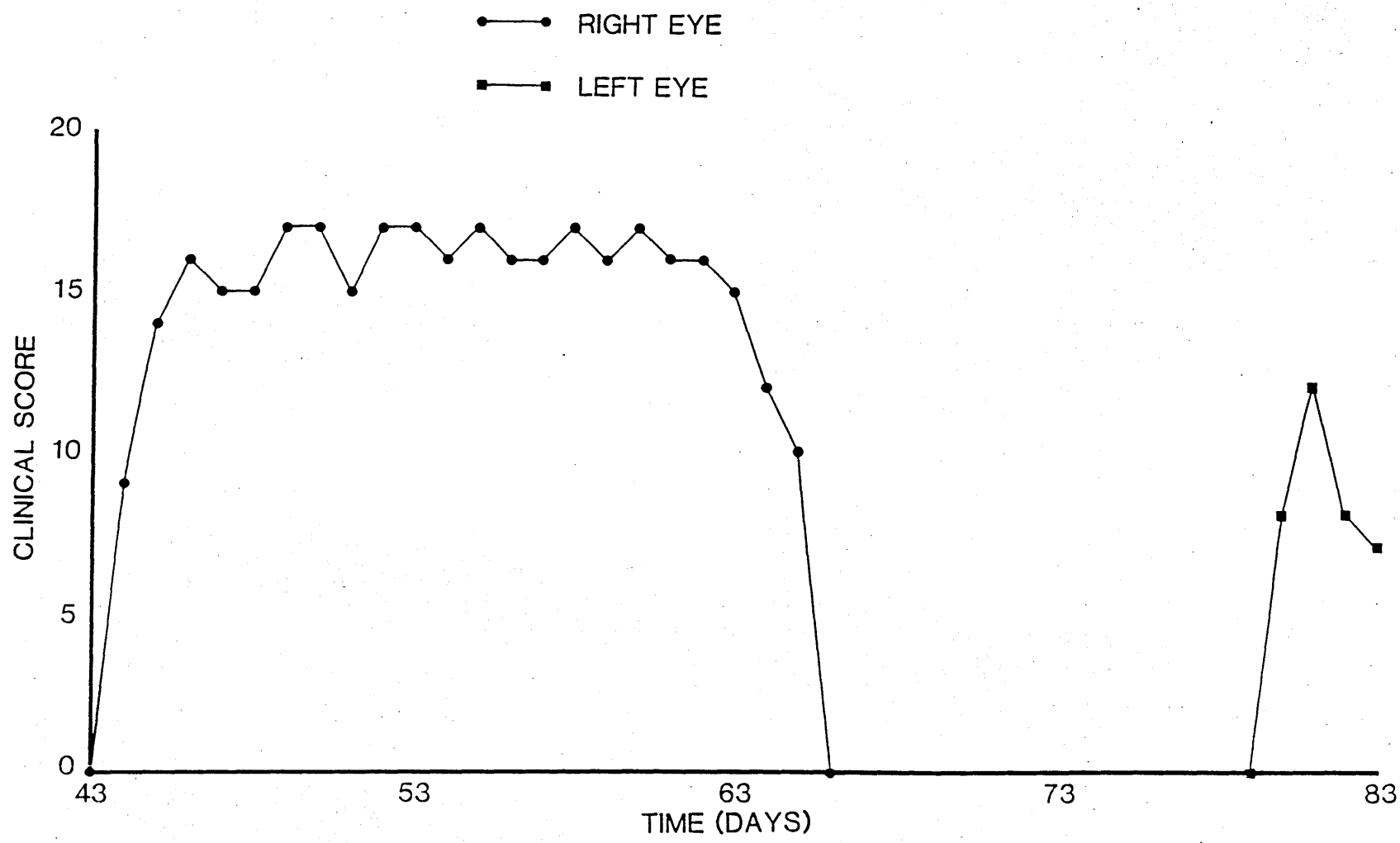
- Right eye. This eye remained normal apart from a small dense white corneal opacity present on the anterior pole from day 41 to the end of the experiment.

APPENDIX IV: Calf 1

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. On day 44, epiphora, conjunctivitis, blepharospasm, increased blinking, iridospasm and a 1mm, anterior-polar ulcer, were noted. Corneal opacity was not noted until day 45, but increased gradually until, by day 52, the cornea was totally opaque and the iris was not visible. The ulcer expanded to a maximum size, by day 47, of 7mm x 10mm, oriented horizontally, at which time vascularisation was first noted as a 1mm rim at the corneoscleral junction. The vascular rim advanced at a rate of approximately 1mm per day and reached the dorsal edge of the ulcer by day 55 and the ventral edge by day 56. Reorganisation of the capillary bed resulted in a decrease in corneal opacity in areas which had been vascularised and the iris became clearly visible by day 63. The capillary bed advanced over the ulcer floor at a rate of 0.5mm per day, forming dorsal and ventral ridges of granulation tissue over the ulcer floor. By day 63, a single ridge, 6mm long, 4mm wide and raised by 3mm, supplied by 11 main vessels had formed. All signs of ocular irritation persisted until day 64 and slight blepharospasm and iridospasm only were present on day 65. On day 66, the peripheral cornea was almost totally clear with linear opacities around the blood vessels supplying the granulation tissue, which at that time, covered an area of 4mm by 3mm, projected 3mm above the corneal surface, and was situated centrally in an opaque scar which measured 7mm x 8mm. This granulation tissue resolved such that by day 72 the area covered was 3mm x 2mm and raised by less than 1mm. At the end of the daily observation period on day 83, a pale pink, corneal scar, 4mm x 5mm, and confluent with the corneal surface was still present with two blood vessels visible ventrally.

- Left eye. This eye remained free from lesions until day 80 when epiphora, conjunctivitis, blepharospasm, increased blinking and iridospasm were noted. A pinpoint ulcer and diffuse corneal opacity were noted on day 81. These lesions did not progress and by day 83, the signs of irritation and corneal opacity were less severe.



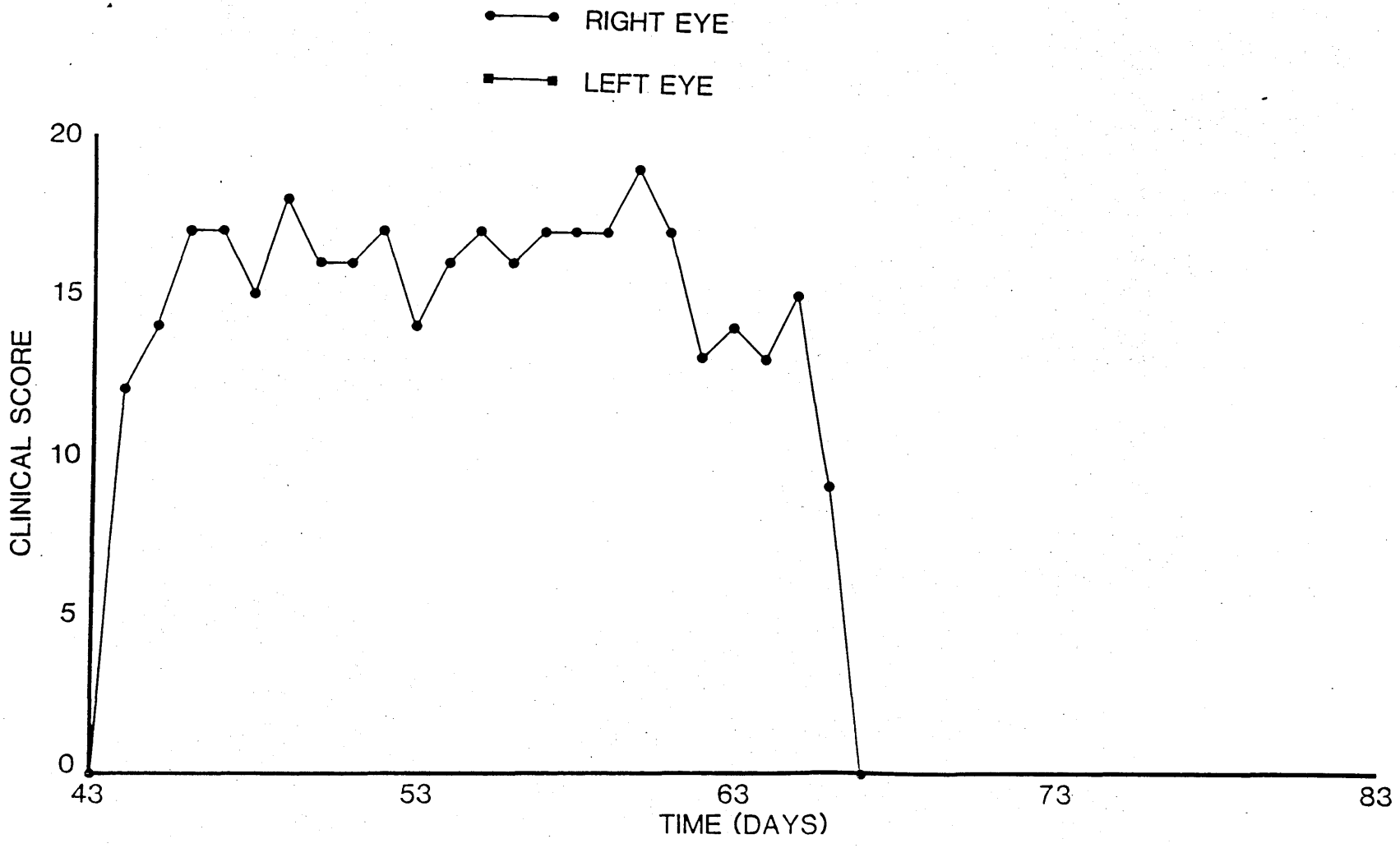
Vaccination experiment, calf 1. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 2

The clinical socres from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. The severity, duration and resolution of lesions in this eye were similar to those in the right eye of calf 1. The calf was free from clinical ocular lesions prior to inoculation on day 43. On day 44, epiphora, conjunctivitis, blepharospasm, increased blinking and iridospasm were present and a hazy corneal opacity was noted, surrounding 1.5mm diameter anterior-polar ulcer. The area covered by the ulcer increased, reached a maximum size of 8mm x 10mm on day 49 with a concurrent increase in opacity, the cornea becoming totally opaque by day 48. Vascularisation of the cornea was first noted on day 47, advanced across the cornea at a rate of 1mm per day, and reached the dorsal and ventral edges of the ulcer on days 53 and 54, respectively. Ridges of granulation tissue formed over the advancing capillary bed, reached a maximum width of 3mm on day 59 and met at the centre of the ulcer on day 63. By day 65, a single, central ridge of granulation tissue 6mm long, 4mm wide and projecting 4mm had formed and a decrease in corneal opacity was noted peripherally. Signs of ocular irritation were much reduced and were absent by day 66. Opacity decreased and the granulation tissue resolved slowly such that, by day 78, the cornea was clear peripherally with a central scar at the site of the ulcer. Granulation tissue was still present, forming a ridge 3mm x 4mm x 2mm high with a ring of capillaries at the base supplied by two dorsal and two ventral vessels, which had a slight overlying haze; this granulation tissue was still present, though slightly reduced, on day 83.

- Left eye. This eye was free from lesions throughout the experiment.



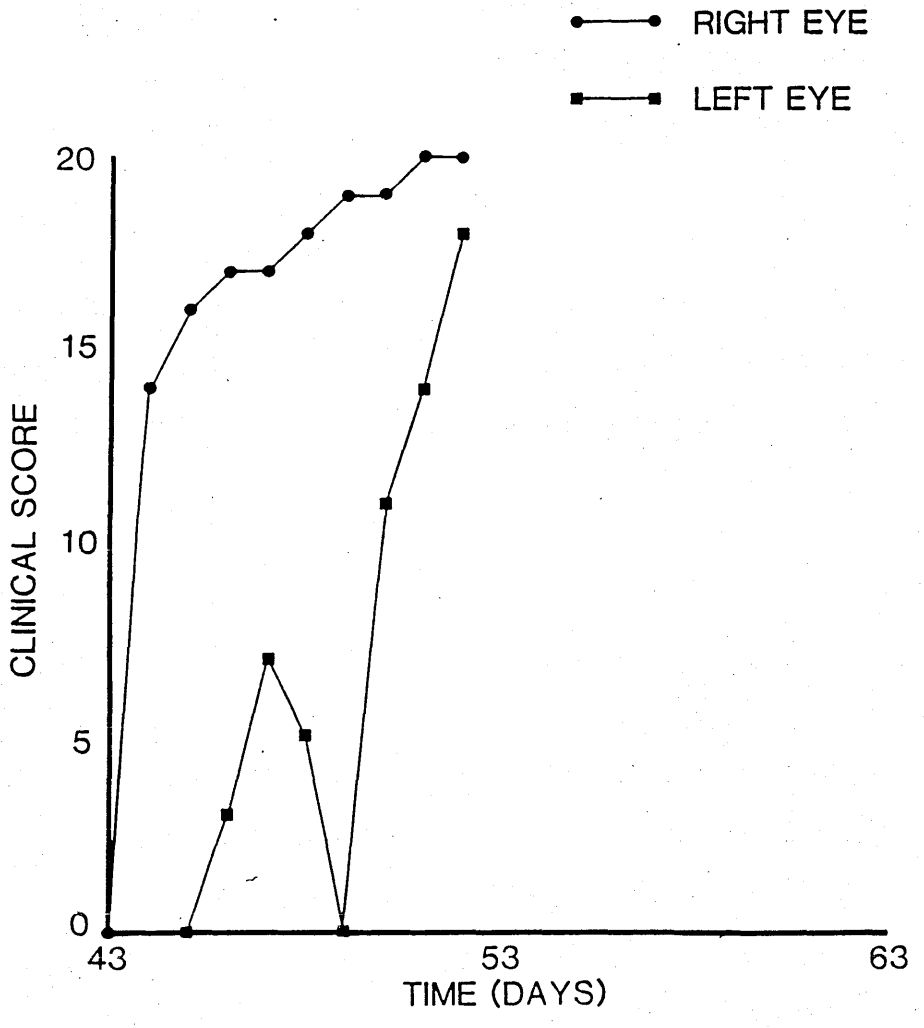
Vaccination experiment, calf 2. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 3

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eyes. On day 44, epiphora, conjunctivitis, blepharospasm, increased blinking, iridospasm, a moderate, diffuse corneal opacity and a 2mm, anterior-polar ulcer were noted. The ulcer increased in area and depth such that, by day 46, it had a diameter of 1cm and, by day 48, over 80% of the corneal area was affected with a step of over 1mm between the normal corneal epithelium and the ulcer floor. A severe purulent discharge was noted from day 49 onwards. The ulcer increased in depth centrally with a corresponding decrease in opacity centrally due to thinning of the cornea. On day 51, the rarefied centre of the cornea was ruptured, and the iris had fallen forward, prolapsing through the ulcer. The calf was slaughtered on day 52.

- Left eye. This eye was free from lesions until day 50 when signs of ocular irritation, a diffuse corneal opacity and a pinpoint, anterior-polar ulcer were noted. On day 51 extensive vascularisation was noted extending 10mm from the corneoscleral junction and the ulcer had increased to 4mm. Or day 52, the cornea was totally opaque, and the ulcer had increased to over 8mm.



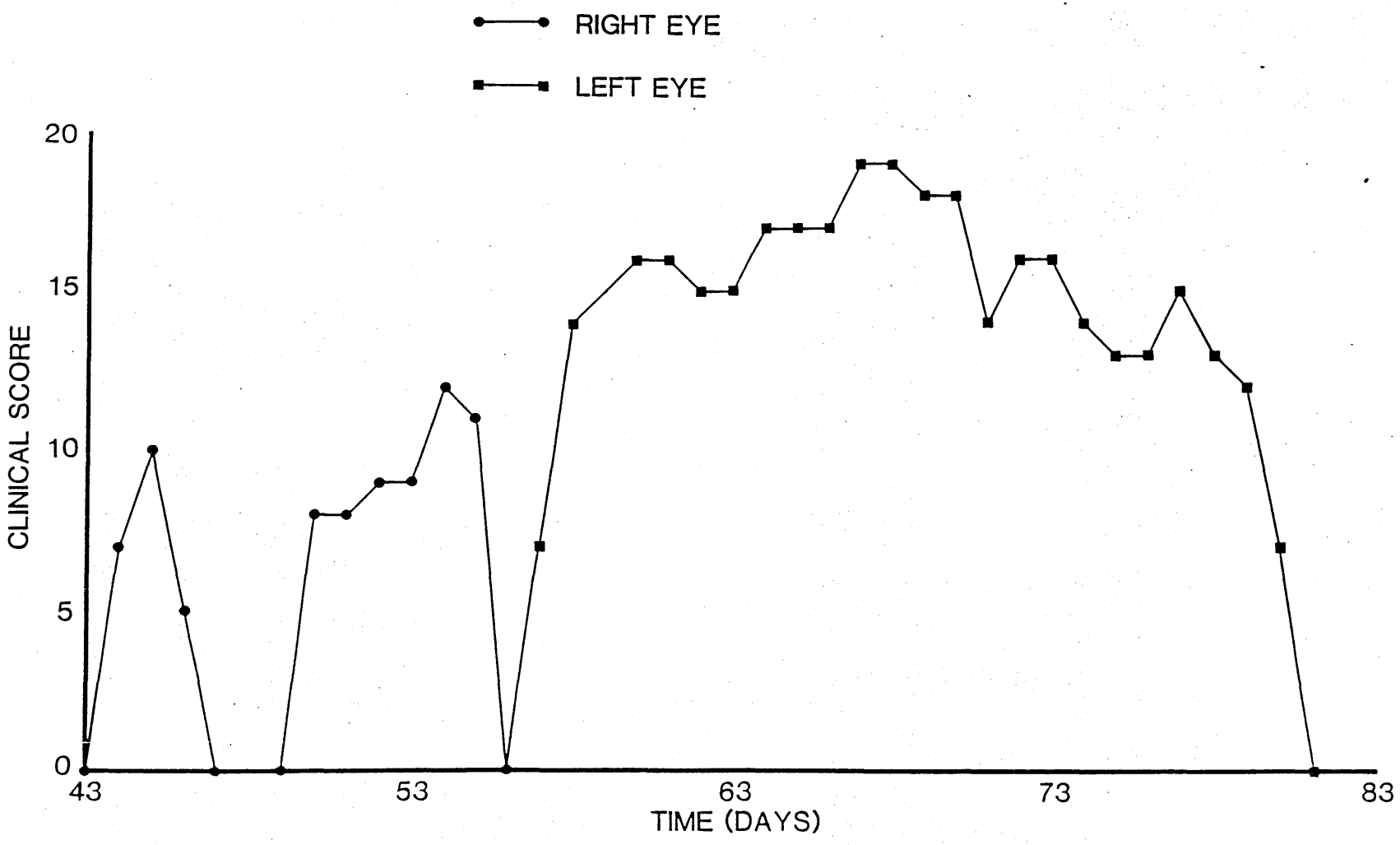
Vaccination experiment, calf 3. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 4

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. Signs of ocular irritation were first noted on day 44, although the cornea appeared to be normal. On day 45, a faint diffuse opacity and three pinpoint ulcers, lateral to the anterior pole, were noted. By day 47, only a residual corneal haze remained. Mild epiphora and conjunctivitis were noted on day 49 in conjunction with a faint increase in corneal opacity. On day 50, a 2mm, shallow ulcer was noted in the upper medial quadrant of the cornea. On day 54, a second, shallow, anterior-polar ulcer was noted. Mild signs of ocular irritation were present until day 55 after which they did not recur. The ulcers which developed remained small and healed without obvious vascularisation, leaving shallow depressions at the ulcer sites.

- Left eye. This eye remained healthy until day 57 when mild signs of ocular irritation were noted and a pinpoint ulcer and surrounding opacity were present medially. On day 58, the signs of ocular irritation were severe, the ulcer was 3mm in diameter and the cornea diffusely opaque. Vascularisation was first noted on day 50 and, by day 61, the ulcer had a diameter of 6mm. On day 62, there was a 10mm x 11mm centrally ulcerated, anterior-polar vesicle. By day 64, this had extended to 10mm x 15mm with vascularisation extending 5mm from the corneoscleral junction. On day 67, the cornea was deeply eroded and a descemetocoele 2mm in diameter was noted. By day 68 vascularisation extended to the entire rim of the ulcer and advanced centripetally across the floor at a rate of about 1mm/day. On day 71, an anterior synechia was noted. By day 73, a ridge of granulation tissue, 4mm wide, had formed round the circumference of the ulcer, the peripheral cornea decreased in opacity and a slight purulent discharge noted. A "C" shaped ridge of granulation tissue formed medially by day 77, surrounding the central transparent area which remained non-vascularised. Signs of ocular irritation were much reduced and were completely absent by day 81. By day 83, the periphery of the cornea was transparent, there was an opaque scar of 8mm diameter, a central vascularised zone 3-4mm in diameter, with a transparent centre of less than 0.5mm.



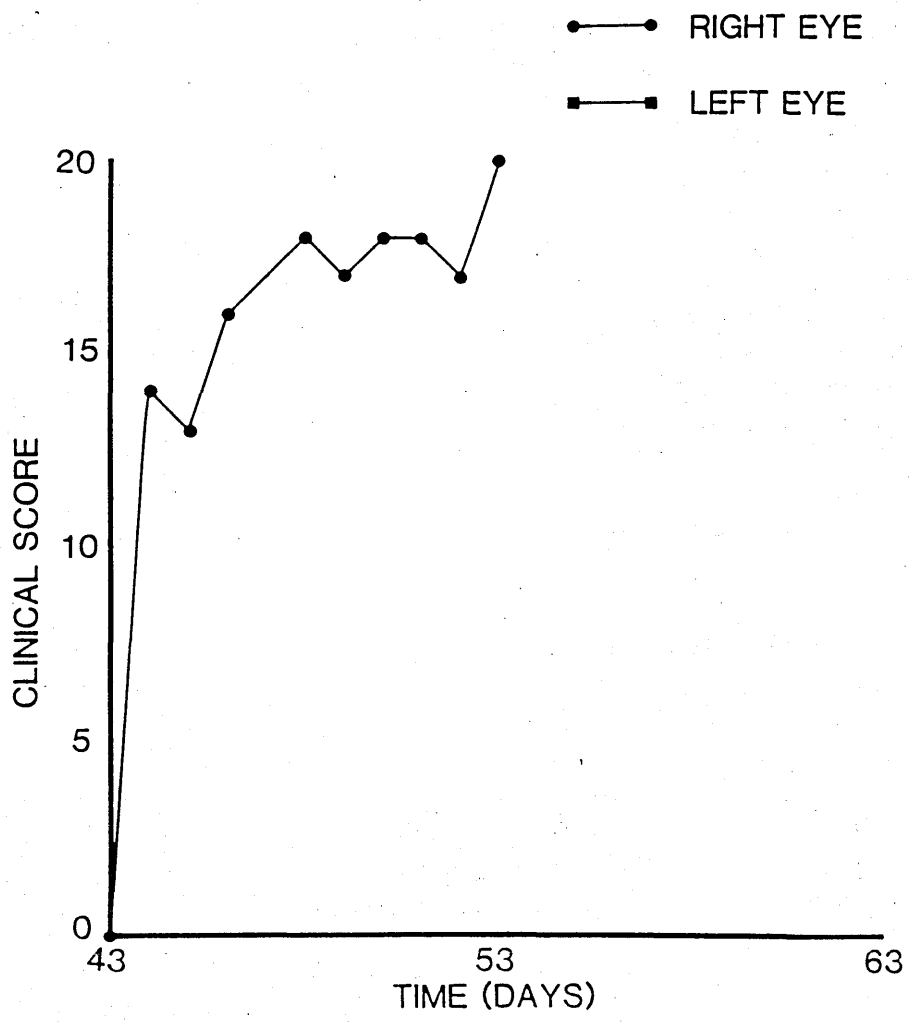
Vaccination experiment, calf 4. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 5

The clinical scores from this calf for the period from days 43 to 53 are illustrated overleaf.

- Right eye. Severe signs of ocular irritation were noted on day 44 accompanied by a vesicle of 3mm diameter in the medioventral quadrant of the cornea and a diffuse corneal haze. The vesicle extended to 6mm by day 46 and, on day 47, had ruptured, leaving a 7mm ulcer, and vascularisation was first noted. On day 52, a central area of the ulcer, 4mm in diameter, was noted to be transparent but the cornea was still intact. The cornea had perforated by day 53, there was a severe purulent ocular discharge and the calf was slaughtered.

- Left eye. The left eye of this calf was injured during the initial observation period resulting in corneal ulceration on the medial corneoscleral junction. On day 43, vascularisation extended 3mm into the corneal ulcer and there were no signs of ocular irritation present. A slight opacity was still present at this site on day 53.



Vaccination experiment, calf 5. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 7

- Right eye. This eye remained healthy throughout this trial apart from the development of a pinpoint ulcer at the lateral corneoscleral junction on day 65, accompanied by signs of mild ocular irritation. On day 66, there was a pinpoint opacity at the ulcer site but no signs of irritation were present. The eye was completely normal on day 67.

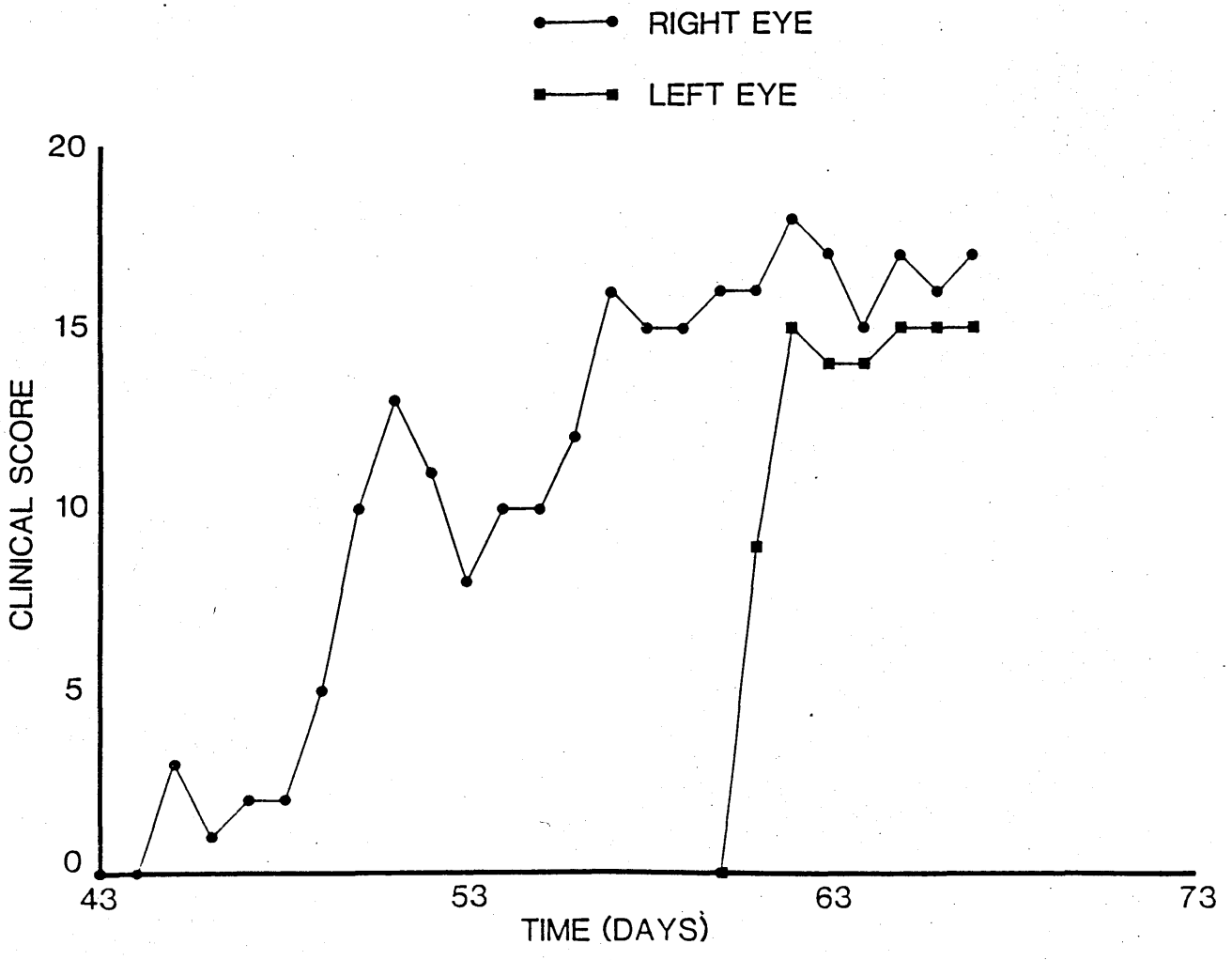
- Left eye. There were no signs of irritation in this eye at any time during this trial although a linear opacity, 1mm x 0.5mm, was present in the upper medial quadrant of the cornea from day 48 onwards.

APPENDIX IV: Calf 8

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. Epiphora and mild conjunctivitis were first noted on day 45 and continued as the only signs present until day 49 when a slight opacity was noted. On day 50, a 2mm ulcer, lateral to the anterior pole, blepharospasm and blinking were noted. Iridospasm was first noted on day 51. On day 53, the corneal changes were noted to be resolving although epiphora and slight blepharospasm persisted. On day 55, two fresh ulcers, both 2mm diameter, one in the lateral lower quadrant and one on the anterior pole, epiphora, mild conjunctivitis, blepharospasm and blinking were noted. By day 57, the anterior-polar ulcer had expanded to 4mm and signs of ocular irritation were more severe. Vascularisation was first noted on day 58. Or day 62, a 6mm x 8mm vesicle was noted, ulcerated centrally. The ulcer attained its maximum size of 8mm x 10mm on day 64. The animal was judged to be bilaterally blind on day 67 and was slaughtered.

- Left eye. Epiphora, blepharospasm, increased blinking, conjunctivitis and iridospasm were noted, on day 62, in the absence of corneal changes. On day 63, a 2mm, anterior-polar ulcer was noted with a generalised diffuse corneal opacity. The ulcer attained its maximum size of 4mm diameter on day 65 when vascularisation was first noted as veil like vessels extending from the corneoscleral junction, denser at the dorsal and ventral aspects. On day 67 the animal was judged to be bilaterally blind and was slaughtered.



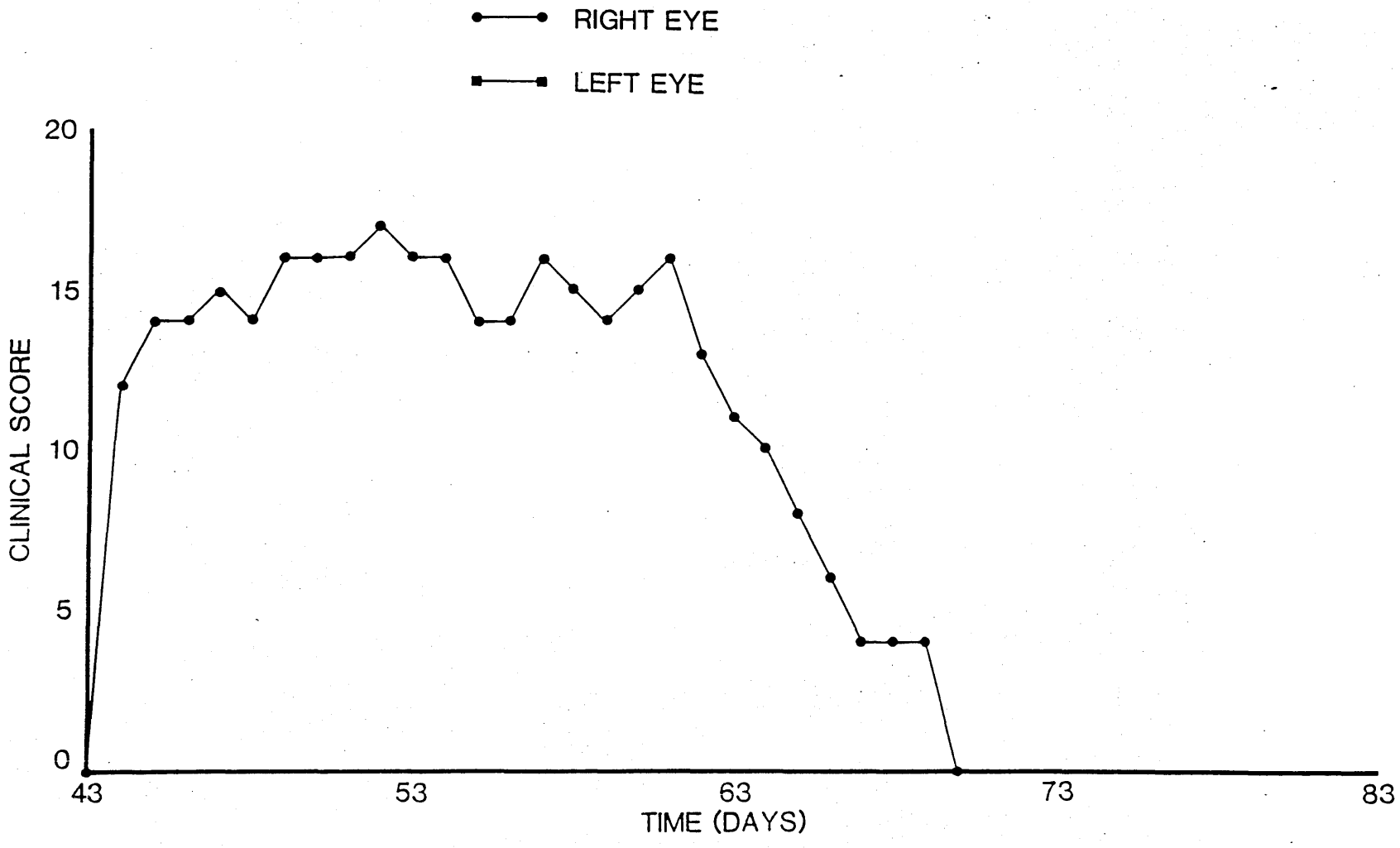
Vaccination experiment, calf 8. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 9

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. On day 44, epiphora, conjunctivitis, blepharospasm, increased blinking, iridospasm, a diffuse corneal opacity and three ulcers, located close together lateral to the anterior pole, were noted. On day 45, these ulcers had coalesced to form a single, 4mm diameter ulcer and a fresh 4mm diameter ulcer had formed medially. The maximum size for the ulcers was attained on day 46, the medial ulcer being 5mm in diameter and the lateral 7mm. Vascularisation was first noted circumferentially on day 47, advanced at a rate of 1mm per day, and reached the lateral edge of the lateral ulcer on day 53. On subsequent examinations vascularisation was noted to be most pronounced laterally. On day 58, only faint vascularisation was present in the medial quadrant and only the lateral ulcer was vascularised, with the formation of a lateral ridge of granulation tissue. Signs of ocular irritation decreased from day 60 and, apart from slight conjunctivitis, were absent by day 66. The advancing capillary had reached the medial ulcer on day 63, growing across the base of the ulcer without the production of granulation tissue. The lateral ulcer on this examination was noted to be covered by a raised area of granulation tissue, 4mm in diameter. Vascularisation continued to fade as blood supply to the lateral ulcers became more organised. By day 73, only two dorsal and two ventral blood vessels remained visible laterally, supplying a pale area of granulation tissue 1.5mm diameter and raised by 1mm, the site of the medial ulcer was marked by an area of opacity 3mm in diameter. On day 83, there was only faint evidence of the medial ulcer and, at the site of the lateral ulcer, there was a slight thickening due to resolving granulation tissue. Supply vessels were still faintly visible surrounded by slight opacities.

- Left eye. This eye remained clinically normal at all times.



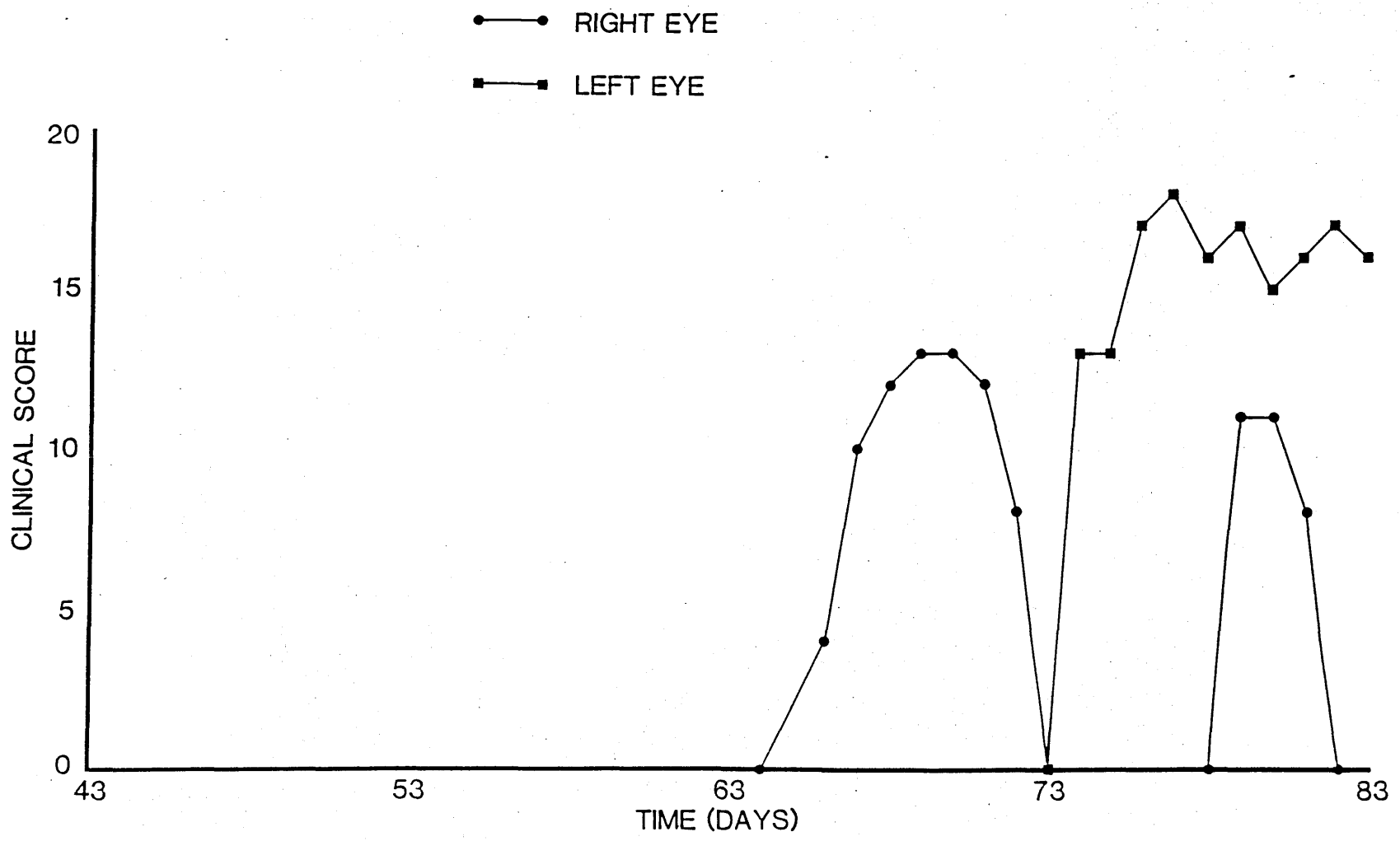
Vaccination experiment, calf 9. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 10

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. Epiphora and a small localised opacity on the anterior-pole were first noted on day 66. On day 67, mild conjunctivitis, blepharospasm, iridospasm, a more diffuse opacity and a 2mm ulcer were noted. On day 68, the ulcer was 3mm in diameter and did not increase in size thereafter, vascularisation was noted medially and had extended to the entire corneoscleral junction on day 69. Blood vessels extended into the cornea at a rate of 1mm/day and, by day 72, formed a thin veil 3mm wide. Conjunctivitis, blepharospasm, iridospasm and blinking were not present on day 72. On day 73 epiphora had stopped, the capillary bed had faded and there was a slight depression at the ulcer site. The lesion flared up on day 79 with epiphora, conjunctivitis, blepharospasm, blinking and a generalised increase in opacity. Signs of irritation were completely resolved on day 82 and faint vascularisation was noted, extending 4mm from the ventral corneoscleral junction.

- Left eye. Signs of ocular disease were first noted on day 74, when epiphora, conjunctivitis, blepharospasm, increased blinking, iridospasm and a moderate, diffuse opacity were noted. In addition, the animal demonstrated a rearward jerk of the head coincident with blinking. The cornea became underrun and, by day 80, a vesicle 10mm x 12mm had formed. Sloughing of the loose flap of cornea left an ulcer, of similar size, on day 81. Faint vascularisation was first noted on day 75 as a 2mm fringe at the corneoscleral junction, a solid band of vascular tissue 2mm wide was first noted on day 77, which progressed at a rate of 1mm/day and which reached the ulcer's edge on day 83.



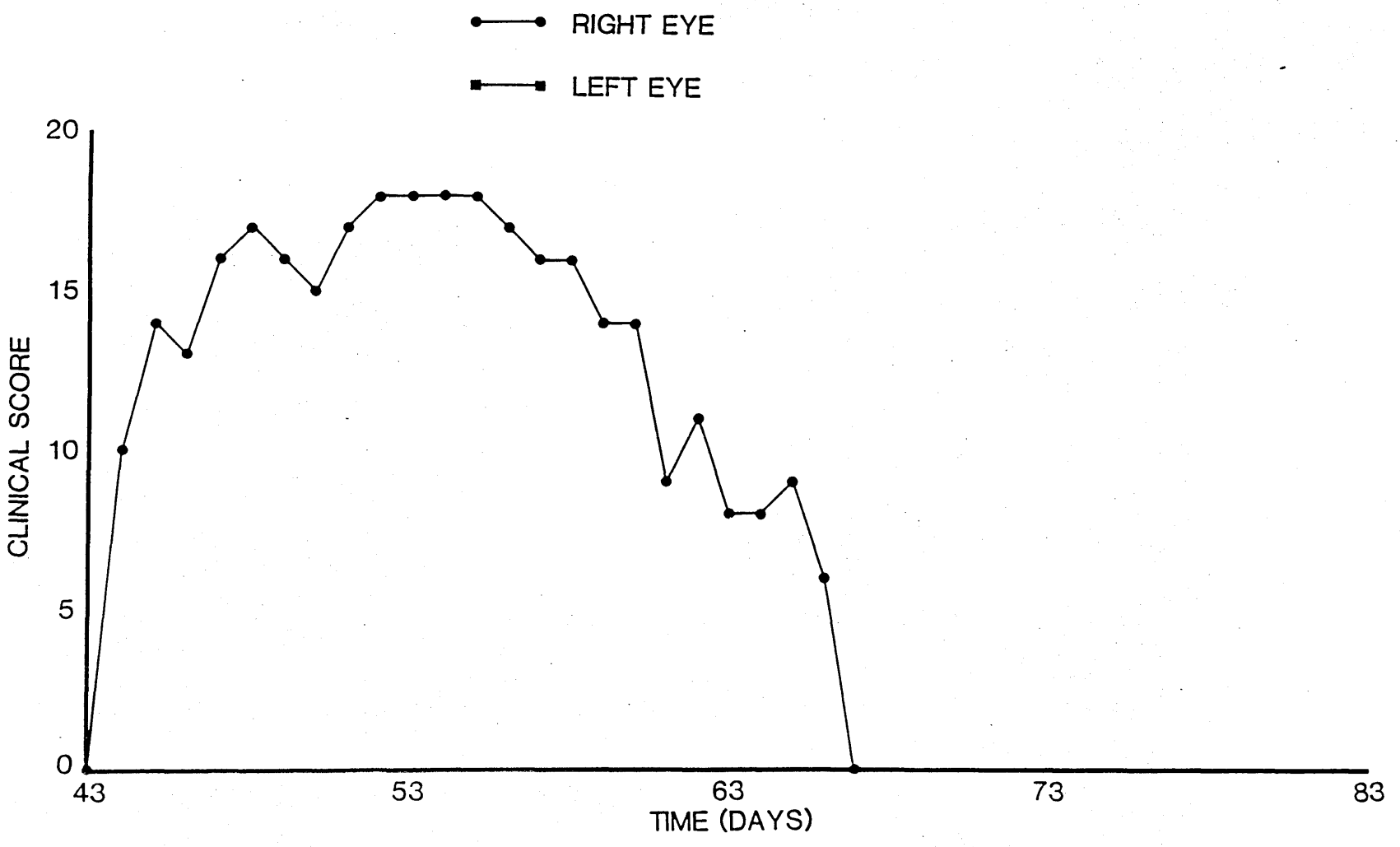
Vaccination experiment, calf 10. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 11

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. On day 44, epiphora, conjunctivitis, blepharospasm, increased blinking, iridospasm and a 1mm diameter, anterior-polar ulcer surrounded by a zone of opacity were noted. The ulcer expanded, attaining a maximum diameter of 7mm by day 46, and corneal opacity increased, the cornea being totally opaque on day 48. Vascularisation was first noted on day 47 as a 1mm rim along the entire corneoscleral junction, grew at a rate of just under 1mm/day, with poorer capillary development in the lateral quadrant, and reached the ulcer edge on day 55. Corneal opacity decreased peripherally from day 57 as smaller capillaries atrophied and signs of ocular irritation decreased from days 59 to 67. Ridges of granulation tissue were initially noted on the dorsal and ventral edges of the ulcer on day 58, grew towards each other and formed a single central ridge, 5mm wide by 8mm long and projecting 3mm above the corneal surface, on day 62. By day 65, the granulation tissue had decreased in area to 2mm x 4mm overlying a dense white scar 8mm in diameter, a slight depression was present lateral to the granulation tissue. On day 59, the corneal surface was level with a central, pale pink scar 8mm in diameter. Vessels supplying the scar were still visible with a slight haze surrounding them. On final examination on day 83, a single supply vessel remained visible ventrally, with capillaries fanning out over the white, 8mm diameter, scar.

- Left eye. The left eye remained clinically normal throughout this trial.



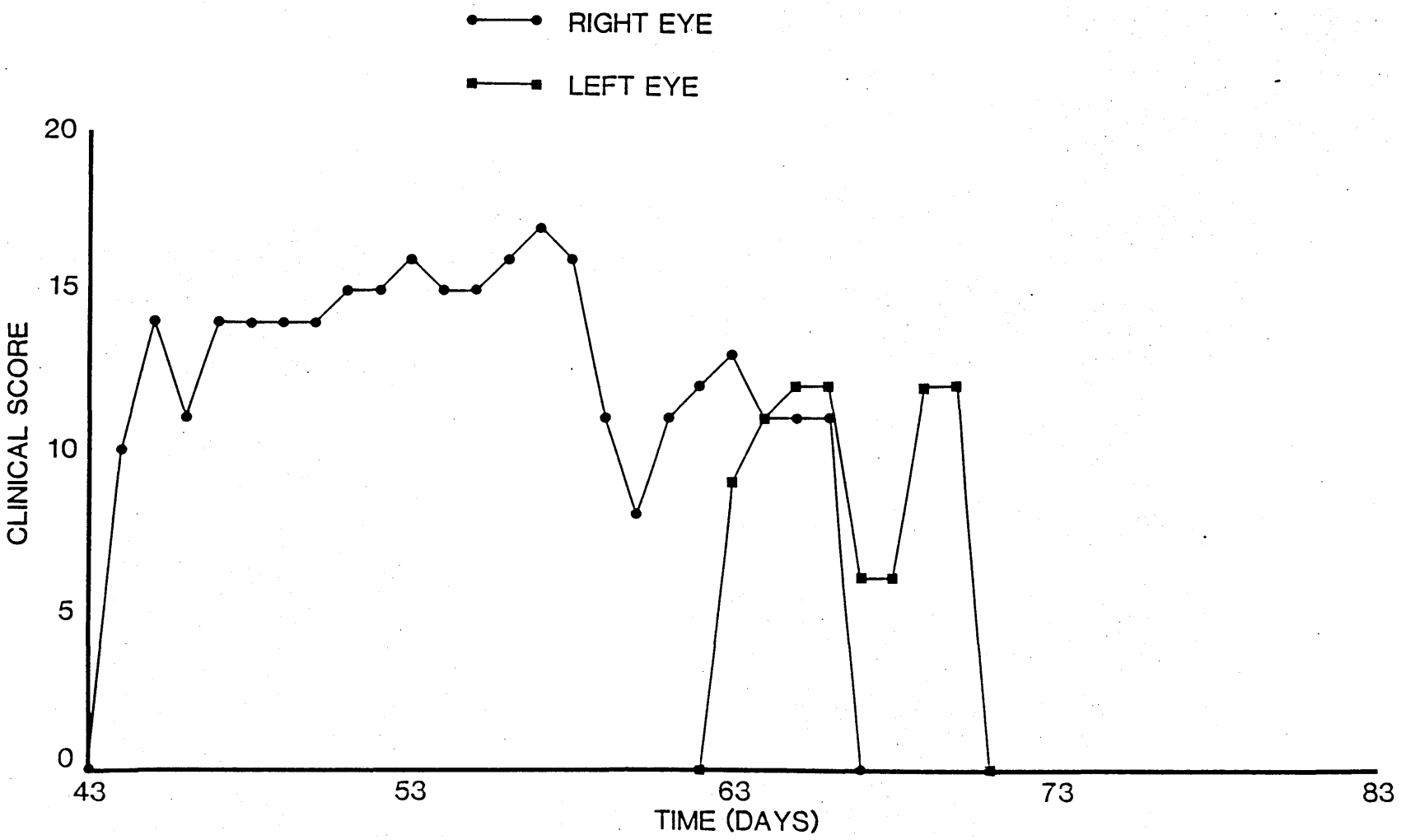
Vaccination experiment, calf 11. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 12

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. On day 44, epiphora, mild conjunctivitis, blepharospasm, increased blinking, iridospasm and a 1.5mm anterior-polar ulcer surrounded by a slight corneal haze were noted. By day 4t there was a 5mm diameter vesicle and vascularisation of the cornea was first noted along the dorsal and ventral corneoscleral junctions only on days 47 and 48, respectively. The dorsal band of vascular tissue reached the ulcer edge on day 57 at which time there was little change in the eye apart from a slight and gradual increase in corneal opacity. On day 59, a vascular ridge was noted on the dorsal aspect of the cornea, epiphora, conjunctivitis and blepharospasm were much reduced and blinking and iridospasm were absent. On day 60, only faint vascularisation was visible and mild blepharospasm was the only sign of ocular irritation present. A greater degree of ocular irritation recurred from days 61 to 66, although no further corneal changes were noted during this period. On day 70, the site of the ulcer was marked by a dense white scar, 6mm in diameter, with very faint vascularisation present. This gradually faded such that, by day 82, the scar was translucent with no blood vessels visible. However on day 83 signs of ocular irritation had recurred with a moderate diffuse corneal opacity present. When examined on day 88, these had resolved and faint vascularisation had recurred.

- Left eye. On day 63, epiphora, mild conjunctivitis, severe blepharospasm, increased blinking and iridospasm were noted although the cornea appeared normal. By day 64, a 2mm, dorsolateral ulcer, 3mm from the corneoscleral junction, and a moderate corneal opacity were noted. Vascularisation was first noted at the area of corneoscleral junction closest to the ulcer on day 65 and had reached the ulcer by day 67. Signs of ocular irritation were absent from day 70 onwards, the ulcer healed without formation of granulation tissue and, by day 80, there was no evidence of a corneal lesion visible.



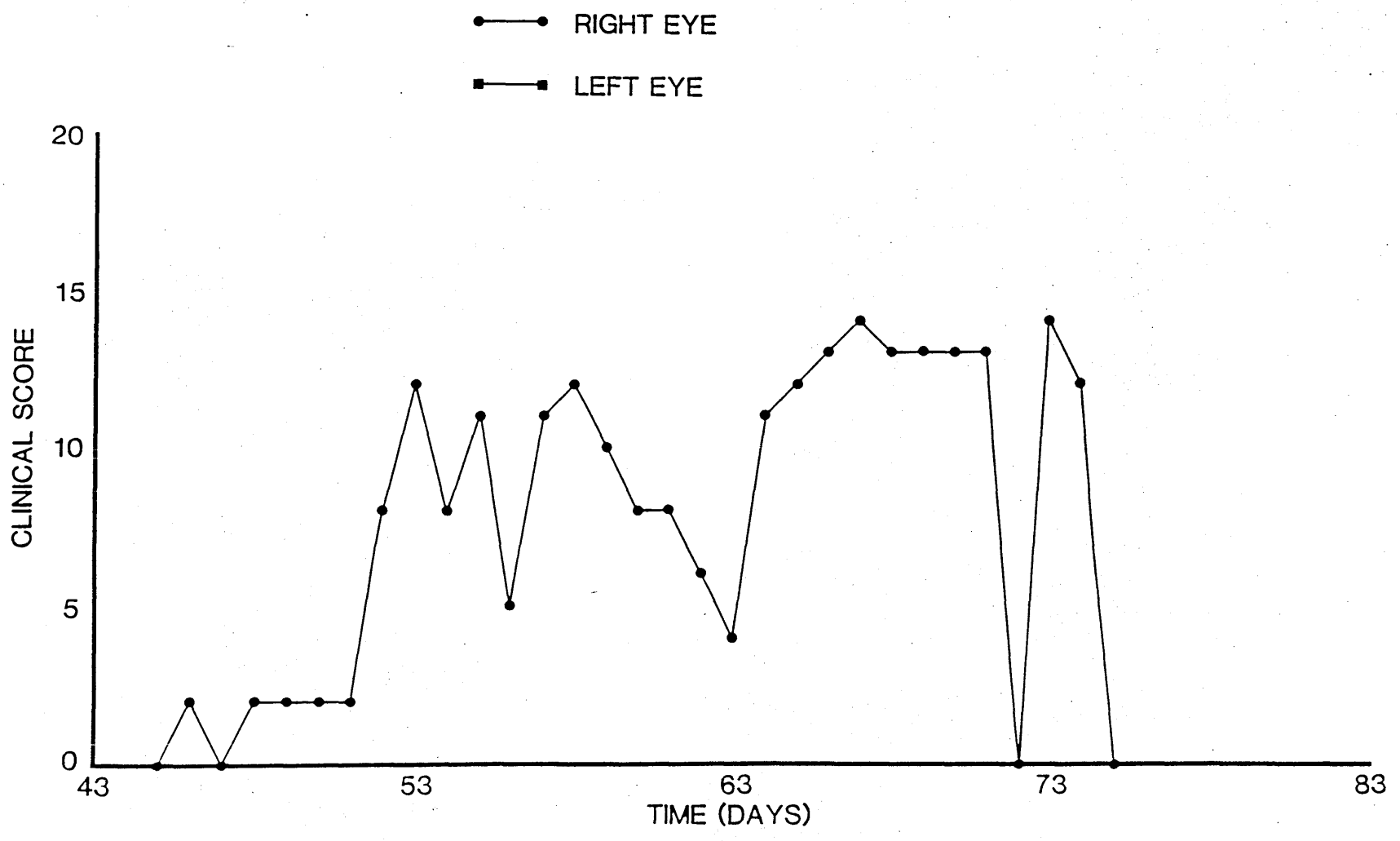
Vaccination experiment, calf 12. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 13

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. On day 52, epiphora, conjunctivitis, blepharospasm, increased blinking and a diffuse corneal opacity were noted. On day 53, two small ulcers were noted in the lateral quadrant and these did not progress. Clinical signs abated and, on day 56, the ulcers appeared to have healed although a corneal opacity and mild epiphora were still present. Further small ulcers developed and healed from days 57 to 63 with varying degrees of ocular irritation. On day 65, a 3mm vesicle had formed on the anterior pole and faint vascularisation was noted extending 3mm from the corneoscleral junction. A second 3mm ulcer was noted ventral to the vesicle on day 66. Vascularisation progressed at a rate of just under 1mm per day such that, by day 71, vascularisation extended 9mm dorsally and 5mm ventrally. A third small ulcer was noted in the lateral quadrant on day 73, however, signs of ocular irritation abated and were absent on day 75 at which time the corneal vascularisation appeared to be fading. On day 83, the eye was healthy apart from slight opacities at the site of the ulcers.

- Left eye. The left eye remained clinically normal throughout this experiment.



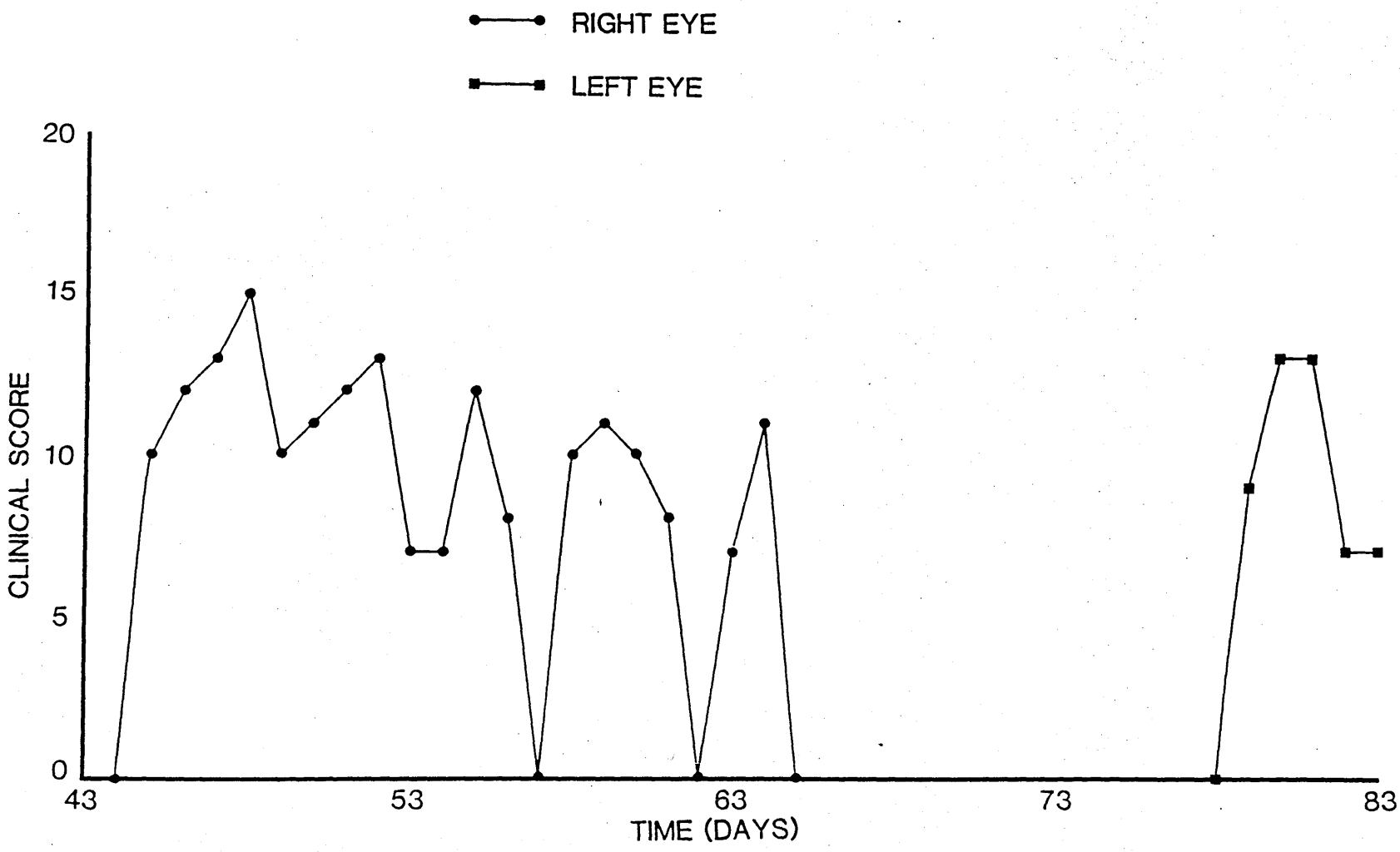
Vaccination experiment, calf 13. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 14

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. On day 45, epiphora, conjunctivitis, blepharospasm, increased blinking, iridospasm, a 1mm diameter, anterior-polar ulcer and a faint corneal opacity were noted. On day 46, the ulcer was 3mm in diameter but did not increase in size or depth after this. Vascularisation was not apparent until day 51 when tenuous strands were noted extending 3mm into the cornea. Mild signs of ocular irritation continued with varying intensity until day 64, vascularisation reaching the dorsal and ventral edges of the ulcer on day 66. Healing of the ulcer occurred without the formation of granulation tissue, vascular tissue in the cornea becoming too faint to be visible by day 75, leaving a milky white scar by day 83.

- Left eye. This eye remained normal until day 66 when mild signs of ocular irritation were noted. A slight opacity was noted on the lateral corneoscleral junction on day 67, resolving rapidly without the development of an ulcer such that the eye was free from signs on day 70. Signs recurred on day 79 when a pinpoint ulcer, epiphora, conjunctivitis, blepharospasm, increased blinking and iridospasm were noted. Corneal opacity was first noted on day 80, at which time, the ulcer was 2mm in diameter. The disease did not progress and signs had almost resolved on day 83.



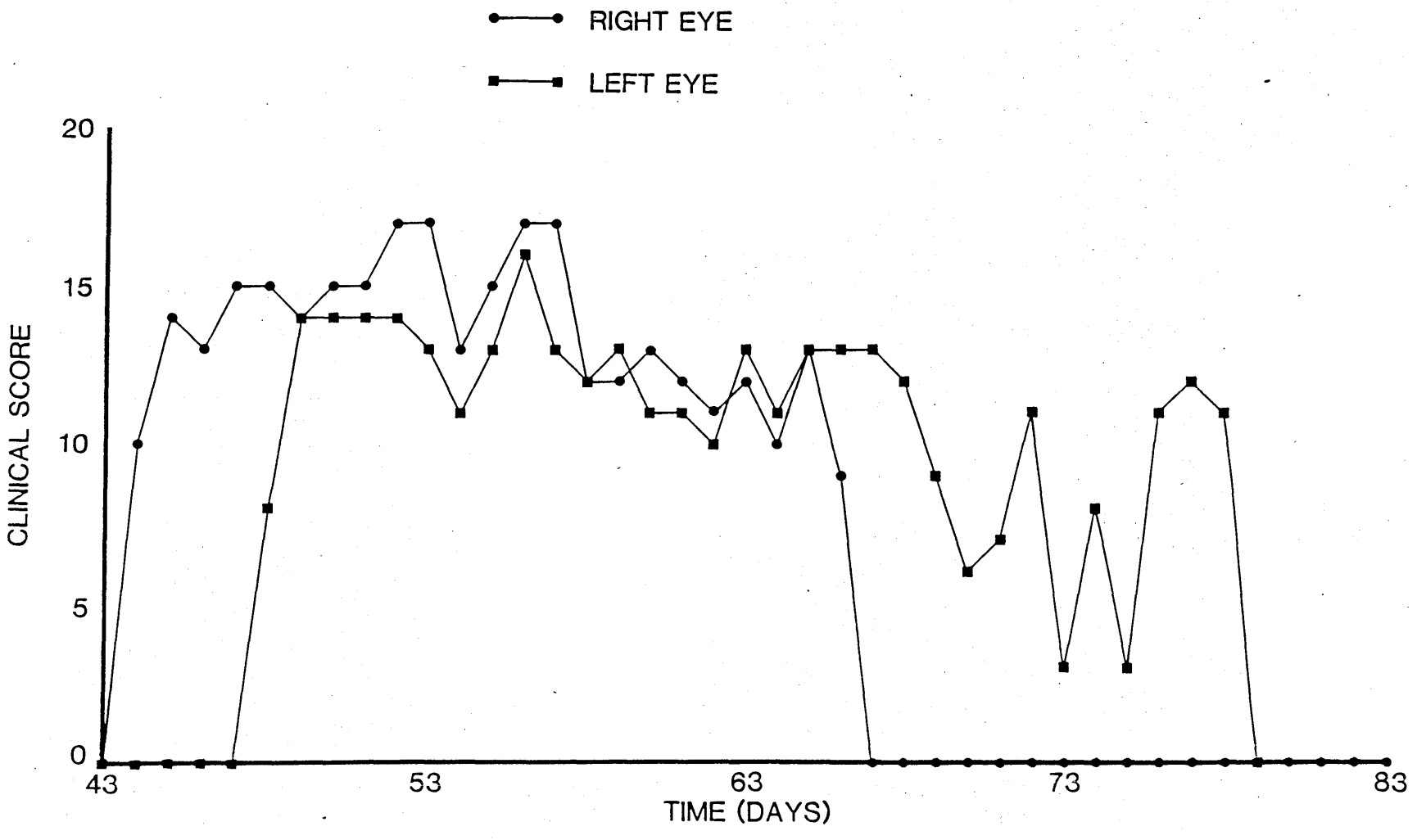
Vaccination experiment, calf 14. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 15

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. On day 44, mild epiphora, conjunctivitis, blepharospasm, increased blinking, iridospasm and a 2mm diameter corneal vesicle in the medial quadrant surrounded by an area of opacity, were noted. The vesicle expanded over a period of four days, reaching a maximum diameter of 5mm, accompanied by increasingly severe signs of ocular irritation. Vascularisation was first noted on day 47 as a tenuous band, 2mm deep, at the medial corneoscleral junction. This consolidated and grew across the cornea, reaching the medial edge of the ulcer on day 52. Although the capillary bed infiltrated the floor of the ulcer, obvious granulation tissue was not seen. Signs of ocular irritation decreased from day 62 and were absent on day 67, at which time, the level of the ulcer floor was confluent with the surrounding normal corneal tissue. On day 69, there was no corneal vascularisation visible although a tenuous horizontal scar, 1mm long by 3mm wide, remained at the ulcer site until day 83.

- Left eye. On day 48, mild signs of ocular irritation and a pinpoint ulcer in the medial quadrant were noted. Corneal opacity was first noted on day 49 and the ulcer reached its maximum diameter of 5mm by day 50, on which occasion, tenuous strands of vascularisation were present medially. The vascular tissue reached the medial edges of the ulcer on day 57 and had completely covered the floor by day 64. On day 65, a new anterior-polar ulcer, 3mm in diameter, was noted accompanied by more severe signs of ocular irritation. On day 66, areas of corneal vascular tissue immediately dorsal and ventral to the new ulcer, were injected. These grew under the base of the ulcer and met on day 69 without the formation of granulation tissue. This ulcer healed rapidly and signs of ocular irritation had resolved by day 73, leaving two scars with a very faint vascular supply. A new 2mm, lateral ulcer was noted on day 76, accompanied by extensive injection of corneal blood vessels and mild signs of ocular irritation which lasted until day 79.



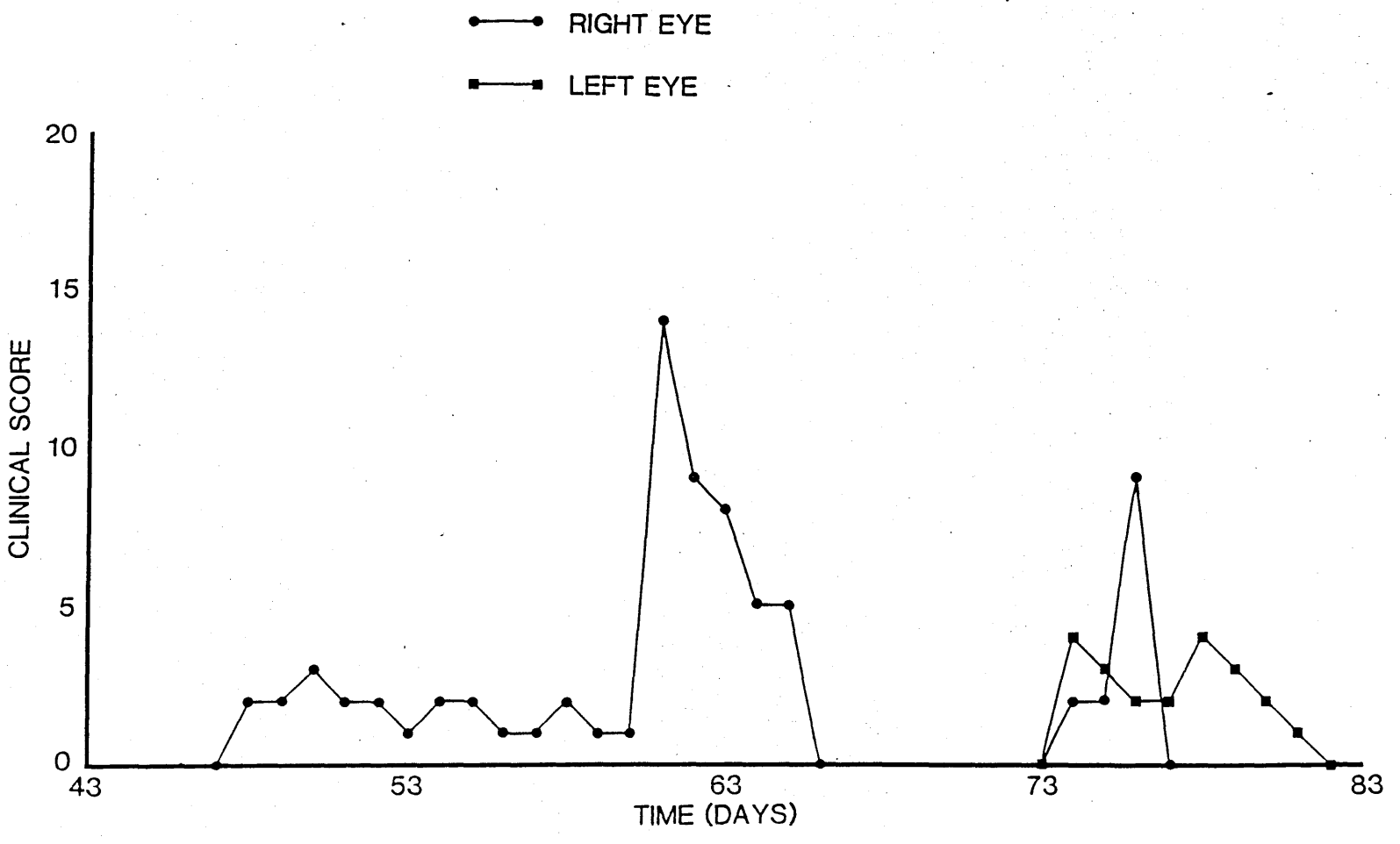
Vaccination experiment, calf 15. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

APPENDIX IV: Calf 16

The clinical scores from this calf for the period from days 43 to 83 are illustrated overleaf.

- Right eye. Mild signs of ocular irritation and a 2mm ulcer, lateral to the anterior pole, were noted on day 61. The lesions did not progress and had resolved without vascularisation by day 66, leaving a slight facet and opacity at the ulcer site. A second pinpoint ulcer was noted at the anterior pole on day 76 surrounded by a slight corneal haze. This ulcer had healed by day 78.

- Left eye. The left eye remained normal apart from a small anterior-polar opacity which was noted on day 74 and which was present until day 79.



Vaccination experiment, calf 16. Clinical score following instillation of M.bovis (GS) into the right conjunctival sac on day 43.

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