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3 **Malignant catarrhal fever**

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Abstract

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of cattle and other ungulates caused by the ruminant γ-herpesviruses alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2). These viruses infect their reservoir hosts inapparently (wildebeest for AlHV-1 and sheep for OvHV-2) but cause fatal lymphoproliferative disease when they infect MCF-susceptible hosts that include cattle, deer, bison, water buffalo and pigs. MCF is an important disease wherever reservoir and MCF-susceptible species mix and is currently a particular problem in Bali cattle in Indonesia, bison in the USA and in pastoralist cattle herds in eastern and southern Africa.

MCF is characterised by the accumulation of lymphocytes (predominantly $CD8^+$ T lymphocytes) in a variety of organs, often associated with areas of tissue necrosis. Only a small proportion of these lymphocytes has been shown to contain virus, although more recent results with virus-gene-specific probes indicate that more infected cells may be present than previously thought. The tissue damage in MCF is hypothesised to be caused by the indiscriminate activity of MHC-unrestricted cytotoxic T/NK cells. The pathogenesis of MCF and the virus life cycle are poorly understood, there is also no effective disease control.

However, the recent sequencing of the OvHV-2 genome and construction of an AlHV-1 bacterial artificial chromosome (BAC) is facilitating studies to understand the pathogenesis of this extraordinary disease. Furthermore, new and improved methods of disease diagnosis have been developed and promising vaccine strategies are being tested. The next few years are likely to be exciting and productive for MCF research.

Keywords: Ovine herpesvirus 2; Alcelaphine herpesvirus 1; Large granular lymphocytes; Pathogenesis; Diagnostics

Introduction

Malignant catarrhal fever (MCF) is a dramatic, fatal disease of cattle and other ungulates including deer, bison and pigs (Reid and Buxton, 1984a; Loken et al., 1998; Schultheiss et al., 2000). MCF is characterised by fever, inappetence and often (in cattle in particular) ocular and nasal discharge. Death can occur within a few days or up to several weeks after the onset of clinical signs. MCF is characterized by tissue destruction in multiple organs occurring as a consequence of virus infection.

Two forms of the disease were originally described, with distinct geographical distributions. In Africa, MCF was first recorded as disease outbreaks in cattle caused by transmission of the infectious agent from wildebeest (*Connochaetes taurinus*; Plowright et al., 1960) that are inapparent carriers. Wildebeest-associated MCF (WA-MCF) is a particular problem with pastoralists in eastern and southern Africa where wildebeest are found (Cleaveland et al., 2001; Bedelian et al., 2007). As well as the economic and welfare implications of MCF, it has been suggested that driving cattle off the short grass plains into upland regions to avoid MCF brings them into contact with other serious pathogens that cause east coast fever (theileriosis), contagious bovine pleuropneumonia and trypanosomiasis (Cleaveland et al., 2001). WA-MCF has also been a problem in zoological collections that contain wildebeest (Meteyer et al., 1989; Whittaker et al., 2007).

The other common form of the disease is sheep-associated MCF (SA-MCF), which was initially observed in Europe, but is found worldwide, wherever sheep and cattle (or other MCF-susceptible species) are kept together. SA-MCF is currently an important economic and welfare problem in Bali cattle in Indonesia and in bison in the USA. SA-MCF has been reported worldwide, including North and South America (Reid and Robinson 1987; Berezowski et al., 2005; Rech et al., 2005), Europe (Collery and Foley, 1996; Frolich et al., 1998; Desmecht et al., 1999; Yus et al.,

66 1999), the Middle East (Brenner et al., 2002; Abu Elzein et al., 2003), Asia, Africa and Australasia
67 (Rossiter, 1981, Wilson, 2002; Dabak and Bulut, 2003).
68
69 As MCF surveillance has extended worldwide, so too has the recorded MCF-susceptible
70 host range. As well as being diagnosed in pigs (Albini et al., 2003; Syrjälä et al., 2006), MCF has
71 also been reported in captive moose (Clauss et al., 2002) and water buffalo (Martucciello et al.,
72 2006).
73
74 The best characterised causative agents of MCF are the two γ-herpesviruses alcelaphine
75 herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2). AlHV-1 is present in wildebeest and is
76 a known cause of WA-MCF. OvHV-2 is present in sheep and is a known cause of SA-MCF. An
77 interesting feature of MCF is that the natural reservoir species for the viruses causing MCF
78 (wildebeest and sheep) do not exhibit any clinical signs of infection, whereas the disease is dramatic
79 in MCF-susceptible species, some of which are closely phylogenetically related to the reservoir
80 hosts. There are excellent animal models of MCF. Rabbits and hamsters can be infected with
81 AlHV-1 or OvHV-2 to develop MCF that is very similar to that seen in the naturally MCF-
82 susceptible species (Buxton and Reid, 1980; Buxton et al., 1984, 1988; Jacoby et al., 1988; Reid et
83 al., 1989a).
84
85 A feature of MCF, with respect to cattle, is that many outbreaks are sporadic with single or
86 only a few individuals in a herd being affected. However, there are occasional more serious
87 outbreaks that can affect up to 40% of a herd. The reasons for this are not known. Certain breeds of
88 deer (e.g., Pere Davids; Reid et al., 1987), Bali cattle (Wiyono et al., 1994) and bison (Berezowski
89 et al., 2005) appear to be particularly susceptible to MCF in terms of rapid death following clinical
90 signs and the numbers of animals affected in closed herds. In cattle, recovery from MCF has been
91 recorded and is therefore part of the clinical spectrum of the disease (Milne and Reid, 1990;

92 O'Toole et al., 1997; Penny, 1998). The purpose of this review is to cover recent advances in our
93 understanding of MCF, with particular attention to the molecular virology, diagnosis and
94 pathogenesis of the disease.
95
96 **Transmission of MCF viruses**

97 Both AlHV-1 and OvHV-2 are likely to be transmitted by contact or aerosol, mainly by
98 wildebeest calves (AlHV-1) and lambs (OvHV-2) under 1 year old (Mushi et al., 1981; Baxter et
99 al., 1997; Li et al., 1998). Incubation periods after experimental inoculation of cattle are between 2
100 and 12 weeks (Plowright et al., 1975; Buxton et al., 1984; Taus et al., 2006).
101
102 The causal viruses are passed between individuals of the reservoir hosts and from reservoir
103 to MCF-susceptible species by the horizontal route, although vertical transmission has been inferred
104 from the detection of anti-MCF virus antibodies in the serum of some gnotobiotic or specific-
105 pathogen-free lambs (Rossiter, 1981) and from recovery of AlHV-1 from a wildebeest foetus
106 (Plowright, 1965). The principal source of free virus is in the tears and nasal secretions of
107 wildebeest (Mushi et al., 1981), while OvHV-2 viral DNA was detected in samples from the
108 alimentary, respiratory and urogenital tracts (Hussy et al., 2002). This may account for some
109 infection of offspring occurring during or shortly after lambing/calving.
110
111 Experimental induction of MCF in cattle has been achieved using wildebeest nasal
112 secretions containing AlHV-1 (Plowright, 1964). For OvHV-2, the presence of the infectious virus
113 in ovine nasal secretions has recently been reported (Kim et al., 2003; Li et al., 2004). OvHV-2
114 appears to be difficult to isolate from this source because the period of virus shedding is short for
115 any given animal. OvHV-2 collected from ovine nasal secretions will infect naïve sheep (Taus et
116 al., 2005) and can also induce MCF in cattle and bison (Taus et al., 2006).
117

118 While sheep and bison could be infected by intranasal nebulisation with 10^3-10^5 genome
119 copies of OvHV-2, infection of cattle was not reliable, even at 1000-fold higher doses (Taus et al.,
120 2005; 2006). Interestingly, at very high doses, intranasal inoculation of OvHV-2 induced MCF-like
121 clinical signs in naïve sheep, confirming a previous report that this carrier species can develop a
122 mild form of MCF (Buxton et al. 1985, Li et al., 2005a).
123
124 The MCF-susceptible species are all thought to be dead-end hosts that do not pass the virus
125 to one another. This has the beneficial effect of limiting the spread of disease during outbreaks.
126 However, some transmission between infected deer has been reported (Reid et al., 1986) but such
127 cases appear to be unusual. The reason for lack of spread between MCF-susceptible animals is
128 likely to be that the virus replicates in a cell-associated manner in these species (cell-free virus is
129 not produced).
130
131 **Clinical course, pathology and histopathology of MCF**
132 Several overlapping but distinct clinical patterns have been described for MCF in cattle:
133 peracute; head and eye; alimentary; neurological; and cutaneous (OIE, 2004). The head and eye
134 form is the most common in cattle (Fig. 1a). Typical signs include fever, inappetence, ocular and
135 nasal discharge, lesions of the buccal cavity and muzzle and, in some cases, diarrhoea and
136 depression. The clinical signs depend to some extent on the species infected, the virus and how long
137 the animal survives after the onset of clinical signs. Many deer die within 48 h of the first clinical
138 signs, and affected bison generally die within 3 days (O' Toole et al. 2002). In contrast, cattle may
139 survive a week or more.
140
141 Gross findings at postmortem examination include petechial haemorrhages on the tongue,
142 buccal mucosa, in the gastrointestinal and respiratory tracts and urinary bladder (Fig. 1b-d).
143 Commonly, there are raised foci on the surfaces of the kidneys (Fig. 1e) and these may extend into

144 the cortex. There is also general enlargement of lymph nodes. Histologically, MCF is characterised
145 by the accumulation of lymphocytes in a range of tissues, some of these being associated with
146 vasculitis and necrotic lesions (Fig. 1f).
147
148 A characteristic of MCF is that, despite the profound pathological changes seen, there is
149 little evidence of virus antigen in affected organs, although viral DNA can be detected by *in situ*
150 hybridisation or PCR (Bridgen et al., 1992; Baxter et al., 1997). In rabbits, the disease is seen as a
151 progressive T cell hyperplasia involving local proliferation and infiltration of both lymphoid and
152 non-lymphoid organs associated with extensive vasculitis, followed by tissue destruction caused by
153 dysregulated cytotoxic lymphocytes (Buxton et al., 1984; Schock and Reid, 1996).
154
155 More recently, a detailed analysis of MCF in rabbits (Anderson et al., 2007) has confirmed
156 and extended earlier observations, showing specific differences between MCF caused by OvHV-2
157 and AlHV-1 (Table 1). OvHV-2-associated lesions were more apparent in 'central' lymphoid tissue
158 (e.g., mesenteric lymph nodes) than those resulting from infection with AlHV-1, which were found
159 more frequently in peripheral lymphoid tissue. In addition, OvHV-2 associated lesions contained
160 more areas of necrosis than those of AlHV-1, in which the lymphoid accumulation appeared to be
161 relatively more 'benign'.
162
163 However, with both viruses, lymphoid cell infiltrations consisted mainly of T cells (by pan-
164 T marker), of which $CD8^+$ T cells predominated, with very few $CD4^+$ T cells. However, a
165 proportion of the infiltrating T cells were neither $CD4^+$ nor $CD8^+$ and for which appropriate
166 antibody probes are not currently available in the rabbit (Anderson et al., 2007). Interestingly,
167 $CD8^-CD4^-$ large granular lymphocyte (LGL) lines (see below) infected with OvHV-2 have been
168 isolated from the MCF-affected tissues of rabbits, cattle and deer (Burrells and Reid, 1991; Swa et
169 al., 2001) in addition to $CD8^+$ LGL.

Small numbers of virus-infected cells have been detected in AlHV-1-infected animals by indirect immunofluorescence (Patel and Edington, 1981) or by in situ hybridisation (Bridgen et al., 1992), suggesting that the T cell hyperplasia may not always be due to the proliferation of infected cells. Indeed, treating infected rabbits with cyclosporin A suppressed lymphocyte proliferation, but did not prevent establishment of necrotic lesions and lethal MCF, showing that hyperplasia per se was unlikely to be critical to the development of MCF (Buxton et al., 1984).

The small numbers of infected lymphocytes observed in lesions characterised by lymphocyte accumulation have been interpreted to suggest that MCF has an autoimmune-like pathology, caused by the cytotoxic action of uninfected cells under the regulatory influence of a small number of infected cells (Buxton et al., 1984; Reid et al., 1984b; Schock and Reid, 1996).

However, more recent work is challenging this view. In situ polymerase chain reaction (PCR) has shown that vascular lesions in the brains of MCF-affected cattle and bison contained $CD8^+$, OvHV-2-infected lymphocytes in larger numbers than has been previously recorded at any MCF-affected tissue site (Simon et al., 2003). Furthermore, preliminary data from the Moredun group (Haig et al., unpublished data) indicates that several viral gene sequences or gene products can be detected by in situ hyridisation in many more lymphocytes accumulating at various tissue sites in OvHV-2-infected rabbits than has previously been seen.

This raises the possibility that the pathogenesis of MCF is due to the direct action of virus-infected, dysregulated cytotoxic T cells at lesional sites and that the frequency of virus positive cells in vivo has been underestimated. It also indicates that LGLs obtained in culture from MCF-affected tissues are likely to be biologically relevant effector cells that are derived from and representative of the infected cells in vivo.

196

197 **Large granular lymphocytes of MCF**

198 The presence of MCF viruses can be detected by PCR of viral DNA prepared from blood or
199 infected tissue samples or inferred from the ability to culture infected LGLs from the tissues of
200 MCF-affected animals. These LGLs have cytotoxic activity and appear to have T cell or natural
201 killer (NK) cell phenotypes (Cook and Splitter, 1988; Reid et al., 1989b; Burrells and Reid, 1991;
202 Wilkinson et al., 1992). They can be developed in culture from various tissues of MCF-affected
203 animals infected with OvHV-2 or AlHV-1 (Reid et al., 1983).

204

205 In contrast to the apparent paucity of cells containing virus in MCF-affected tissues in vivo,
206 >90% of LGL cells are infected with virus, as detected by immunocytochemistry or in situ
207 hybridisation detection of viral DNA or mRNA. Viral particles detectable by electron microscopy
208 (EM) can be seen in the cytoplasm of AlHV-1 LGLs, but are rare in OvHV-2 LGLs (Cook and
209 Splitter, 1988; Rosbottom et al., 2002).

210

211 In spite of this latter observation, some OvHV-2$^+$ LGL lines of bovine or cervine origin can
212 induce MCF when adoptively transferred to rabbits (Reid et al., 1989b). These LGLs are
213 indiscriminately cytotoxic, killing various target tissue cells in MHC-unrestricted fashion; they do
214 not exhibit concanavalin A (ConA)-stimulated proliferation, whereas control uninfected T cells
215 proliferate. In the case of OvHV-2$^+$ LGLs, cytokines tumour necrosis factor-α, interferon-γ,
216 interleukin (IL)-4 and IL-10 are constitutively expressed (Schock et al., 1998).

217

218 The activated, constitutively-cytotoxic phenotype and ConA unresponsiveness of the LGL
219 cell lines could be the consequence of constitutive activation of the T cell signalling molecules Lck
220 and Fyn kinases, along with activation of downstream p42 and p44 mitogen activated protein
221 (MAP) kinases. In uninfected T cells, these are only transiently activated after stimulation of

222 antigen and co-receptors on the T cell surface (Swa et al., 2001). The viral mechanism inducing this
223 change in the LGLs is currently unknown.
224
225 **MCF-associated viruses**
226 The two viruses known to cause MCF have been genome-sequenced and are well
227 characterised (Ensser et al., 1997; Coulter et al 2001; Hart et al., 2007; Fig. 2). A feature of AlHV-1
228 is that it is predominantly cell-associated on primary isolation in culture and probably also in cells
229 of the MCF-susceptible species. OvHV-2 has never been propagated in monolayer culture, however
230 an incompletely enveloped virus has been detected by EM from a rabbit LGL lysate (Rosbottom et
231 al., 2002). The absence of a permissive cell culture system has limited the study of OvHV-2 and the
232 search for such a system continues to be a priority.
233
234 In contrast, AlHV-1 replicates as cell-free, as well as cell-associated virus, that is virulent
235 (in terms of inducing disease in rabbits or cattle) for up to 5 passages in culture in bovine turbinate
236 cells seeded with free virus or infected lymphoid cells from MCF-affected rabbits. After this, there
237 is a period during which the viral genome undergoes rearrangements leading to attenuation. These
238 rearrangements involve gene deletions and translocations from the central region of the genome to
239 the terminal repeat region (Wright et al., 2003). Later in culture (>20 passages), AlHV-1 becomes
240 cell-free and is attenuated with respect to disease induction in animals. Thus, cell-free virulent and
241 attenuated AlHV-1 virus can be produced for vaccine and pathogenesis studies.
242
243 The receptors used by the virus to enter cells (epithelial or lymphocyte) to establish latent or
244 lytic infection are not known, but this is currently the focus of a collaborative study by the authors.
245 The cellular site of virus production in wildebeest or sheep that allows cell-free virus to be shed in
246 nasal and ocular secretions is not known. Although OvHV-2 transcripts and DNA can be detected in
247 ovine blood leukocytes, the particular cellular site of latency is not known.

248

249 Less frequently, MCF associated with other viruses has also been described in deer and
250 experimental animals. Hippotragine herpesvirus-1, recovered in culture from cells of a roan
251 antelope, was able to induce MCF on inoculation into rabbits (Reid and Bridgen, 1991). Another γ-
252 herpesvirus was detected in white-tailed deer showing clinical signs of MCF and anti-MCF
253 antibodies, but no detectable AlHV-1 or OvHV-2 DNA (Li et al. 2000). This MCF virus of white-
254 tailed deer has been characterised by limited sequencing, but its natural reservoir has not been
255 identified. Caprine herpesvirus-2 appears to be endemic to at least some goat populations (Li et al
256 2001a) and has been associated with MCF-like lesions, characterised by lymphocytic infiltration, in
257 a range of cervid species (Crawford et al 2002; Vikoren et al., 2007).

258

259 AlHV-1 and OvHV-2 belong to the *Rhadinovirus* genus of the *Gammaherpesvirinae*
260 (McGeoch et al., 2005). They have genomes with unique segments of about 130 kbp, bounded by
261 terminal repeats of 1.1 kbp (AlHV-1; Ensser et al., 1997) or 4.2kbp (OvHV-2; Hart et al., 2007).
262 The recent sequencing of OvHV-2 from a LGL cell line derived from an infected cow (BJ1035) has
263 revealed that its genome is highly similar to AlHV-1 and is co-linear with the other known
264 rhadinoviruses (Fig. 2).

265

266 There are 73 predicted open reading frames (ORFs) in the OvHV-2 sequence, compared
267 with 71 in AlHV-1. Of the 10 unique genes described in AlHV-1 (A1 through A10; Ensser et al.,
268 1997), eight have clear homologues in OvHV-2: there are no equivalents of A1 or A4 (Fig. 2; Table
269 2). OvHV-2 encodes additional unique genes, named Ov2.5, Ov3.5, Ov4.5 and Ov8.5 to indicate
270 their positions relative to neighbouring unique genes (Table 2). Thus, Ov2.5 lies between Ov2 and
271 Ov3 (homologues of A2 and A3) and encodes a spliced IL-10 homologue. This gene reproduces the
272 host IL-10 splice sites exactly but has greatly reduced intron sizes. The expressed recombinant
273 Ov2.5 protein has typical cellular IL-10 functions (Stewart et al., unpublished data).

Ov3.5 occupies a position analogous to A4, but has no obvious sequence similarity. Both genes encode small proteins with predicted signal sequences and so may perform similar functions.

Ov4.5 lies between ORFs 3 and 6 of OvHV-2 and AlHV-1 carries an homologous gene (A4.5), which was not annotated originally (Ensser et al., 1997), but was added recently (Mills et al., 2003; Fig. 2). The Ov4.5 and A4.5 predicted protein sequences have similarity to the Epstein-Barr virus BALF1 gene product and the Bcl-2 family of apoptosis-related proteins and may therefore be involved in the regulation of cell death.

Ov8.5 lies between ORF69 and ORF73 in OvHV-2 and encodes a proline-rich protein of unknown function. Expression of these unique genes of OvHV-2 has been demonstrated recently in LGL lines derived from MCF-affected rabbits and cattle, suggesting that they are genuine (Thonur et al., 2006).

In respect of lytic (productive) and latent virus life cycles, LGLs from OvHV-2-infected rabbit and cattle tissues have distinct features (Rosbottom et al., 2002). The cattle LGL line had viral genomes that were mainly circular, suggestive of latency, while the rabbit LGLs contained a large proportion of linear DNA, suggestive of productive replication. The rabbit cell line also expressed RNA corresponding to a lytic cycle gene (ORF75) and viral capsids could be detected after concentration of cell lysates (Rosbottom et al., 2002).

These results were confirmed and extended by the use of OvHV-2 sequence data to produce probes for the OvHV-2 unique genes (Thonur et al., 2006). This showed that, in sheep peripheral blood mononuclear cells, OvHV-2 viral genomes were mainly circular and mRNA for only Ov3.5 of the unique genes was detected. In contrast, rabbit LGL cultures contained mainly linear viral

300 DNA and expressed most of the unique genes, while cattle LGLs contained mainly circular viral
301 genomes and also expressed most of the unique genes. Interestingly, no evidence of transcription of
302 the latency-associated gene, ORF73, or the productive cycle regulator, ORF50, could be found in
303 the cattle LGL. Treatment of the cells with the topoisomerase inhibitor doxorubicin induced unique
304 and productive cycle gene expression and linear genome production, while treatment with 5-
305 azacytidine inhibited unique gene expression and induced circular genome accumulation in both
306 LGL lines (Thonur et al., 2006).
307
308 Thus, it appears that LGL cell lines derived from MCF-affected animals may have features
309 of both latent and productive life cycles, suggesting that the normal programme of viral gene
310 expression is defective in these hosts (Thonur et al., 2006). The ability to manipulate viral gene
311 expression and DNA replication in these cultures should be a useful tool for future research, with
312 potential for generating a cell culture system for the propagation of OvHV-2.
313
314 In parallel with the sequencing of OvHV-2 derived in the UK from a clinically affected cow
315 (Hart et al., 2007), a second OvHV-2 sequence has recently been determined from OvHV-2 virus
316 particles isolated in the USA from the nasal secretions of 13 sheep (Taus et al., 2007). The two
317 sequences are highly similar, with amino acid identities of 94-100% between corresponding ORFs,
318 except for ORF73. Much of the ORF73 gene is taken up by three tandem repeat elements and the
319 sequence could not be determined clearly from pooled viral DNA. ORF73 cloned from three
320 individual sheep were 94-98% identical, differing mainly by insertions/deletions in one of the repeat
321 regions. The UK isolate, in contrast, differed by multiple insertions/deletions within repeat areas
322 and by over 20 missense changes, concentrated in the N-terminal part of the protein sequence. The
323 C-terminal 130 residue segment of ORF73 was highly conserved in all isolates, with only one
324 missense difference recorded in the UK isolate. This gene therefore appears to be highly variable,
325 both within and between geographical isolates of OvHV-2, and may be a useful tool for

326 epidemiological studies of OvHV-2 variation. The ORF73 protein is known to be antigenic (see
327 below) and it is possible that its variation reflects relatively (to other genes) rapid evolution in the
328 presence of immunological pressure from the host.
329
330 The expansion of herpesvirus sequence availability and the use of PCR as a diagnostic tool
331 have facilitated phylogenetic analysis both within the rhadinoviruses and across all herpesvirus
332 groups (Li et al., 2005b; McGeoch et al., 2005, 2006). Analysis of herpesvirus phylogeny based on
333 multiple genes found across all herpesviruses (McGeoch et al., 2006) or all γ-herpesviruses
334 (McGeoch et al., 2005) has shown that AlHV-1 and OvHV-2 are most closely related to porcine
335 lymphotropic herpesvirus-1. These analyses have led to the suggestion that the γ-herpesviruses
336 should be split into four genera rather than two, with the current *Rhadinovirus* genus being divided
337 to form two additional genera, *Macavirus* (including AlHV-1 and presumably OvHV-2) and
338 *Percavirus* (including EHV-2), based on likelihood of co-evolutionary origin (McGeoch et al.,
339 2006).
340
341 An analysis of over 20 ruminant rhadinoviruses based on the amino acid sequence of a
342 fragment of the DNA polymerase gene suggested they could be divided into two major subgroups,
343 one of which contained MCF viruses that express the 15A epitope, used previously to identify MCF
344 virus infection, and a second group of lymphotropic herpesviruses that do not express the 15A
345 epitope (Li et al., 2005b). Both of these clades are likely to lie within the *Macavirus* genus
346 described above. Bovine herpesvirus 4 was not grouped with either of these clades by sequence
347 analysis, in agreement with other analyses that suggested that BoHV-4 had diverged further from
348 other artiodactyl γ-herpesviruses and was retained in the *Rhadinovirus* genus (McGeoch et al.,
349 2005; 2006).
350

351 The study of MCF pathogenesis has been facilitated by the recent production of a bacterial
352 artificial chromosome (BAC) clone carrying the entire pathogenic AlHV-1 genome (Dewals et al.,
353 2006). This clone propagated infectious AlHV-1 virus in permissive cells and produced MCF in
354 rabbits that was indistinguishable from the disease caused by non-recombinant virus. The AlHV-1
355 BAC is currently proving a useful tool for analysing the contribution of individual genes to the
356 pathogenesis and host range of MCF and will move our understanding of MCF pathogenesis to a
357 new level.

358

359 **MCF diagnosis**

360 Diagnosis of MCF depends on a combination of clinical signs, histopathology and detection
361 of virus-specific antibodies or DNA in blood cells or tissue samples. Diagnosis in MCF-susceptible
362 species has benefited from recent developments in molecular virology. Sequencing of the genome
363 of AlHV-1 (and genome fragments from other γ-herpesviruses) has allowed the development of
364 both generic and specific reagents for the PCR amplification of diagnostic fragments of both AlHV-
365 1 and OvHV-2 genomes (Bridgen and Reid, 1991; Katz et al., 1991; Baxter et al., 1993; Flach et al.,
366 2002).

367

368 The use of PCR allows sensitive confirmation of the presence of MCF viruses in infected
369 animals and may also be useful for phylogenetic and epidemiological studies in both the natural and
370 MCF-susceptible hosts. Indeed, serological or PCR-based testing of apparently healthy cattle and
371 free-living bison and caribou demonstrated infection in the absence of clinical signs (Zarnke et al.,
372 2002; Powers et al., 2005). This suggests that inapparent infection with OvHV-2 or recovery from
373 MCF may be more frequent than was previously thought.

374

375 The highly cell-associated nature of MCF viruses has made it difficult to study the aetiology
376 of MCF and, to date, AlHV-1 is the only MCF virus that can be propagated cell-free in culture and

377 is consequently the best studied. The mode of infection has, however, long been inferred to be via
378 contact, aerosol transmission or ingestion of infected material.
379
380 The similarity of the clinical signs to other enteric or vesicular diseases, the lack of unique
381 disease-specific clinical diagnostic features and the variability in the presentation of the disease
382 make laboratory confirmation of a clinical diagnosis of MCF important (Holliman, 2005).
383 Histopathological analysis of postmortem samples should give a clear diagnosis. OIE recognises
384 histopathology as the definitive diagnostic test, but laboratories have adopted other approaches,
385 such as indirect immunofluorescence to detect antibodies specific for MCF virus antigens and PCR
386 assays that detect MCF virus DNA sequences (OIE, 2004).
387
388 Using a monoclonal antibody (15A) specific for a conserved MCF virus antigen, a
389 competitive inhibition (CI)-ELISA test has been developed and refined (Li et al., 1994, 2001b). A
390 direct ELISA has been developed recently (Fraser et al., 2006), which offers a simple and
391 inexpensive alternative to other serological tests. Both conventional and real-time (quantitative)
392 PCR assays have also been developed for the detection of OvHV-2 and AlHV-1 viral DNA (Katz et
393 al., 1991; Baxter et al., 1993; Hussy et al., 2001; Flach et al., 2002; Traul et al., 2005).
394
395 The conventional assays employ a nested PCR approach, which makes them about 10-fold
396 more sensitive than quantitative PCR. In combination with an appropriate host gene assay, however,
397 real-time PCR assays have the potential to define viral loads in a range of tissues from both natural
398 and MCF-susceptible hosts. The higher sensitivity of the nested PCR assays may make them an
399 attractive alternative where viral load is low or in difficult samples, such as paraffin-embedded
400 sections. Detection of MCF-virus specific antibodies or DNA in an animal with clinical signs will
401 support a diagnosis of MCF.
402

Several studies have used both serological and PCR-based diagnostic tests to analyse MCF infection of both reservoir and MCF-susceptible hosts. In sheep, nested PCR and CI-ELISA showed that 99% and 94% of animals tested were OvHV-2 positive, respectively, suggesting that PCR might be slightly more sensitive (Li et al., 1995a). A similar study of healthy or clinically suspect cattle concluded that PCR was more sensitive than CI-ELISA, possibly due to the rapid onset of MCF in some cases leading to death before seroconversion (Muller-Doblies et al., 1998).

A longitudinal study of 48 apparently healthy dairy cattle showed evidence of OvHV-2 DNA in blood or milk samples from 17 cattle, of which 8 were also serologically positive (Powers et al., 2005). Three cattle were CI-ELISA positive but PCR negative. Detection of DNA was generally sporadic, with 1-3 positive tests within the 20 month study, while serological results were generally consistent over several months. MCF was not seen in any of the animals tested during the course of the study, suggesting that sub-clinical or latent infection with OvHV-2 can occur in cattle.

Serological studies of a range of species that may either harbour MCF viruses (MCFV) or be susceptible to MCF show interesting trends. Some species, including sheep, goats, wildebeest and musk oxen, have a high frequency of seropositivity (>90%; Plowright, 1967; Rossiter, 1981; Li et al., 1995a, 1996; Zarnke et al., 2002), indicating their status as inapparent carriers of MCFV. In contrast, the degree of seropositivity in MCF-susceptible species including cattle, bison, deer, caribou, elk and moose, ranges from a few percent to 50% seropositive (Li et al., 1996; Frolich et al., 1998; Zarnke et al., 2002; Powers et al., 2005). This variation in the frequency of MCF seroconversion suggests that infection of MCF-susceptible hosts with MCF viruses can have different outcomes that may depend on genetic variation in the host or infecting virus or on differences in the magnitude or route of challenge..

MCF antigens and vaccine development

429 To date, no effective treatment or vaccine for MCF has been described. Reservoir hosts and
430 MCF-susceptible species can develop antibody responses to MCF viruses and, as discussed above,
431 serological testing remains an important diagnostic and epidemiological tool.
432
433 Antibodies that recognise AlHV-1 antigens have been demonstrated in sera from carrier
434 sheep and MCF-affected cattle, showing that the agent responsible for sheep-associated MCF was
435 likely to be related to AlHV-1 (Rossiter, 1981; 1983). Further studies, using immunoprecipitation
436 and western blotting, showed that both wildebeest and sheep sera recognised similar polypeptide
437 profiles in either infected cell lysates or in purified AlHV-1 virus preparations (Herring et al., 1989;
438 Adams and Hutt-Fletcher, 1990; Li et al., 1995b). These studies identified the major antigens
439 recognised by the natural host species. Fewer antigens were detected by sera from cattle reacting
440 with MCF (Herring et al., 1989) suggesting a more restricted pattern of gene expression in these
441 hosts.
442
443 MCF virus-specific sera have also been used in attempts to identify important diagnostic or
444 protective antigens. Antibody screening of cDNA expression libraries has led to the identification of
445 candidate antigens from both AlHV-1 (Lahijani et al., 1995) and OvHV-2 (Coulter et al., 2002).
446 Interestingly, both studies identified cDNA clones encoding the C-terminal region of ORF73 as
447 being antigenic in OvHV-2 positive sheep and in AlHV-1 infected rabbits and wildebeest. These
448 ORFs encode the MCF virus homologues of the latency-associated nuclear antigen (LANA), a
449 multifunctional protein involved in the maintenance of latency and the association of virus genomes
450 with the host cell chromosomes. While LANA may be antigenic in latently-infected sheep or
451 wildebeest, it is unclear how useful it would be in MCF-affected animals in either a diagnostic or
452 protective role.
453

454 The development of monoclonal antibodies (mAbs) against AlHV-1 antigens (Adams and
455 Hutt-Fletcher, 1990; Li et al., 1995b) has facilitated a better understanding of the nature of the
456 antigens recognised and allowed the development of a serological diagnostic test (Li et al., 1994).
457 Both studies described mAbs that could immunoprecipitate a glycoprotein complex with
458 components approximately 115/110/105/78/45 kDa (mAb 12B5, Adams and Hutt-Fletcher, 1990;
459 mAb 15-A, Li et al., 1995b). However, western blotting showed that mAb 12B5 recognised the
460 115-78 kDa components, while mAb 15-A recognised only the 45 kDa band. Pulse-chase
461 experiments also suggested that the 78 and 45 kDa components were derived from larger bands by
462 proteolytic cleavage (Adams and Hutt-Fletcher, 1990). The complex was also shown to be
463 accessible to extrinsic labelling of AlHV-1 virions by ^{125}I, demonstrating that it was a mature virion
464 glycoprotein complex (Adams and Hutt-Fletcher, 1990).
465
466 Recent proteomic analysis of AlHV-1 virions has shown that glycoprotein B is found in the
467 mature virion as a complex of 2 furin-cleaved polypeptides of about 80 kDa (N-terminal fragment)
468 and 50 kDa (C-terminal fragment) (Dry et al., unpublished data). This suggests that both 12B5 and
469 15-A mAbs recognise gB, that the 115/110/105/78/45 kDa complex contains the various post-
470 translationally modified and cleaved forms of gB and that mAbs 12b5 and 15-A recognise distinct
471 epitopes in the N-terminal and C-terminal parts of the molecule, respectively. The widespread
472 conservation of the mAb 15A epitope in MCF viruses from both domestic and wild ungulates
473 demonstrates the importance of this glycoprotein complex in these viruses (Li et al., 2005b),
474 making it a good target for diagnostic or prophylactic use.
475
476 Early attempts to immunise cattle using live or inactivated formulations of the attenuated
477 WC11 strain of AlHV-1 were unsuccessful, providing no clear protection against either parenteral
478 or natural challenge, despite the development of virus neutralising antibodies in the serum (Piercy,
479 1954; Plowright 1968; Plowright et al., 1975). Later work in rabbits suggested that inactivated cell-

480 free virulent AlHV-1 C500 strain could protect against a cell-free virus challenge, but not against a
481 cell-associated virus challenge (Edington and Plowright, 1980). However, this work was not
482 transferred to cattle. Indeed, observations on the small numbers of immunised cattle that survived
483 an initial challenge suggested that their immunity was short-lived (Piercy, 1954). In contrast, cattle
484 surviving natural infection remained immune, despite having lower titres of serum neutralising
485 antibody than immunised animals (Plowright 1968; Plowright et al., 1975).
486
487 These observations suggested that serum neutralising antibody was not a critical component
488 of a protective immune response in cattle and raised the question of what the protective response
489 might be. Work on cellular immunity to MCF virus has been hampered by the lack of a good
490 experimental system in which animals can be immunised and challenged, and by the severe T cell
491 hyperplasia induced by MCF virus, which is a central part of disease pathology.
492
493 At MRI, we have developed an intranasal virus challenge system intended to mimic the
494 presumed natural route of challenge for MCF-susceptible species. Using cell-free preparations of
495 virulent AlHV-1, experimental infection of 100% of cattle can be achieved with an incubation
496 period of between 20 and 50 days (Haig et al., unpublished data). Furthermore, cell-free high
497 passage (in tissue culture) attenuated C500 AlHV-1 can be easily harvested and used as a vaccine
498 candidate.
499
500 This system has been used to test the proposition that a mucosal barrier of neutralising
501 antibody could protect against disease following intranasal challenge with AlHV-1. Initial
502 experiments have demonstrated that such an approach works, with immunised cattle protected
503 against disease (Haig et al., unpublished data). These studies may allow the development of a
504 protective vaccine for wildebeest-associated MCF and provide an experimental system to study the

505 immune response to MCF virus. Furthermore, the protective antigens in AlHV-1 can be identified
506 and the equivalents in OvHV-2 isolated to attempt vaccination control of sheep-associated MCF.
507

508 **Conclusions**

509 Malignant catarrhal Fever is an important and fascinating disease with many unanswered
510 questions concerning transmission, the sporadic occurrence of the disease and pathogenesis. One
511 outstanding question is, why do closely related species, such as sheep and cattle, exhibit such
512 different responses to infection with OvHV-2? The possible autoimmune pathology of MCF may
513 provide clues to its pathogenesis and help in the identification of therapeutic treatments. The recent
514 availability of the sequence of OvHV-2 and the development of a recombinant BAC carrying the
515 AlHV-1 genome will greatly facilitate and accelerate our understanding of virus-host interactions.
516 An OvHV-2 BAC clone is actively being sought.
517

518 The importance of MCF as a pathogen of farmed deer and bison, as well as cattle, is driving
519 research for improved diagnostic tools and development of effective vaccines. The recent
520 sequencing of the OvHV-2 genome, the production of recombinant AlHV-1 viruses and
521 developments in the use of intranasal challenges, for both OvHV-2 and AlHV-1, constitute
522 important steps forward in the development of vaccine strategies to protect against MCF.
523

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529

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802 Table 1

803 Comparison of histopathology of MCF induced by AlHV-1 and OvHV-2 in rabbits (based on

804 Anderson et al., 2007)

805

Observation[a]	AlHV-1	OvHV-2	Comments
Haemorrhagic foci in appendix	–	+	
Necrosis in lymphoid follicles of appendix	–	+	
Pan-T positive cells in appendix	+++	++	Mainly in inter-follicular areas
CD4 T cells in appendix	++	+	Mainly in inter-follicular areas
CD8 T cells in appendix	++	+	Throughout lymphoid areas
B cells in appendix	++	+	Lymphoid areas
Necrosis in MLN	–	+	
CD8 T cells in MLN	++	+	Cortex and medulla
MHC class II positive cells in MLN	+	++	Margins of follicles
CD8 T cells in spleen	++	+	Mainly in periarteriolar lymphoid sheath
Liver periportal lymphoid cell accumulations	+++	++	Large and numerous, with little debris or apoptotic cells, in AlHV-1 infection; Moderate size and frequency, with cellular debris and apoptotic cells in OvHV-2 infection
Lymphoid cells in liver	++	+	In parenchyma
MHC-positive cells in liver	++	+	In clusters or in periportal accumulations
Pan-T positive cells in lung lymphoid cell accumulations	+++	++	
Kidney lymphoid cell accumulation	+++	–	Perivascular location
$CD8^+$ in kidney	+++	+	Cortical accumulations and scattered through medulla
$CD4^+$ cells in kidney	++	+	In lymphoid cell accumulations
MHC^+ cells in kidney	++	+	In some lymphoid cell accumulations

806 [a] Each observation, where differences were found, is recorded as absent (–), present (+), frequent

807 (++), or very frequent (+++), for AlHV-1 and OvHV-2 MCF, respectively

808 MLN: Mesenteric lymph nodes; MHC: Major histocompatibility complex

809 Table 2

810 Unique MCF virus genes

811

OvHV-2 gene	AlHV-1 gene	Possible Function[a]
	A1	Unknown
Ov2	A2	Leucine zipper protein; Transcription regulation
Ov2.5		Viral IL-10
Ov3	A3	Semaphorin homologue; Intracellular signalling
Ov3.5		Unknown, signal peptide
	A4	Unknown, signal peptide
Ov4.5	A4.5	Bcl-2 homologue; cell death regulators
Ov5	A5	GPCR (G-protein coupled receptor); Intracellular signalling
Ov6	A6	Similar to Epstein-Barr virus BZLF1; Viral transactivator
Ov7	A7	Virus Glycoprotein
Ov8	A8	Virus Glycoprotein
Ov8.5		Unknown, proline-rich
Ov9	A9	Bcl-2 homologue; Cell death regulators
Ov10	A10	Nuclear localisation signal; Transcriptional regulation?

812 [a] Provisional assignment of function is based on analysis of the predicted amino acid sequences and

813 similarity to proteins of known function

814 **Figure Legends**

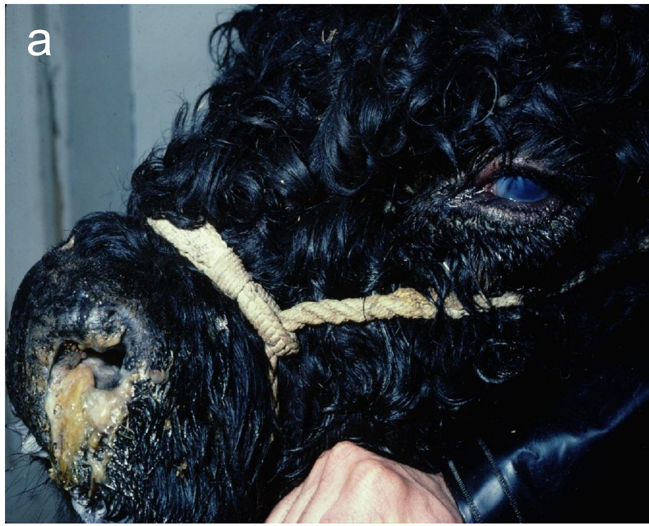
815

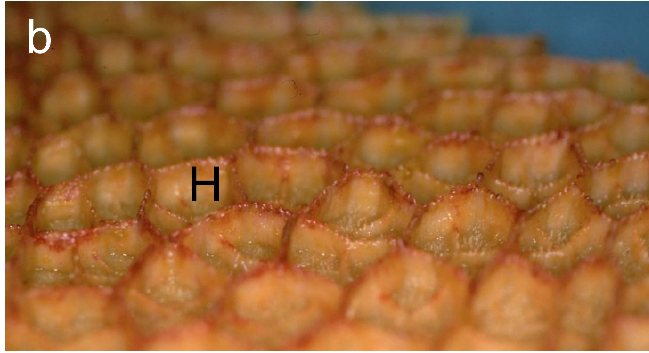
816 Fig. 1. Gross and histological signs of MCF. a. Dexter bull showing classic head-and-eye signs of
817 MCF with corneal opacity and mucopurulent nasal discharge. b-e. MCF lesions on reticulum,
818 urinary bladder, kidney and buccal papillae, respectively. H indicates areas of haemorrhage and L
819 indicates pale focal lesions on the kidney. f; Haematoxylin and eosin-stained section of buccal
820 papillae with early MCF lesions. In addition to areas of haemorrhage (H), the section shows the
821 beginning of epidermal erosion (E) and areas of early lymphocyte infiltration (I).

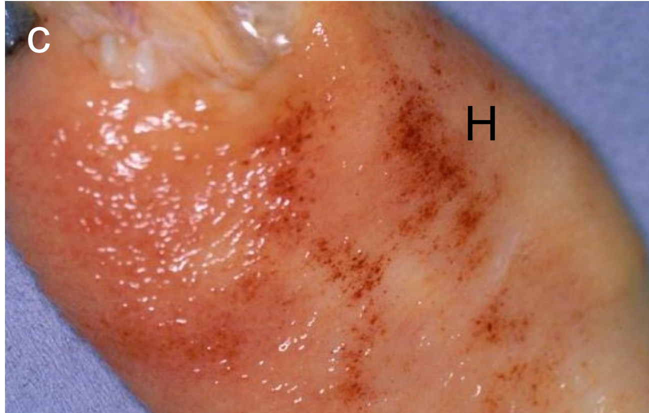
822

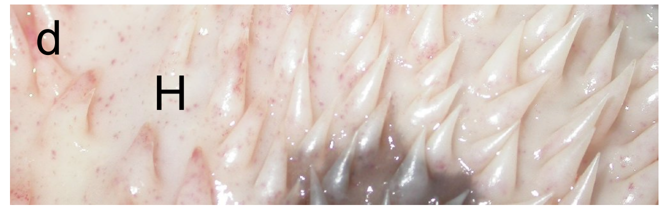
823 Fig. 2. Genome organisation of AlHV-1 and OvHV-2. Schematic maps show the relative
824 organisation of genes in the OvHV-2 and AlHV-1 genomes. Genes are shown to scale as block
825 arrows indicating the position and orientation of open reading frames. Gene designations are given
826 beneath, following the numbering scheme for *Herpesvirus saimiri*. Conserved γ-herpesvirus genes
827 are shown as open arrows, while genes found only in the MCF virus genomes are shaded grey and
828 named beneath each map with an A prefix for AlHV-1-specific genes and Ov prefix for OvHV-2.
829 The terminal repeat (TR) sequences are shown to scale and are shaded pale grey..

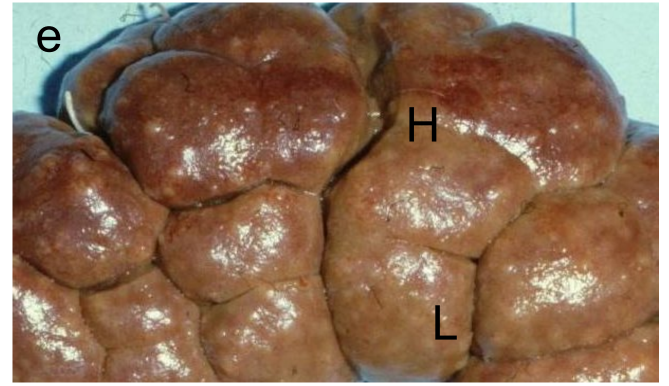
Figure 1











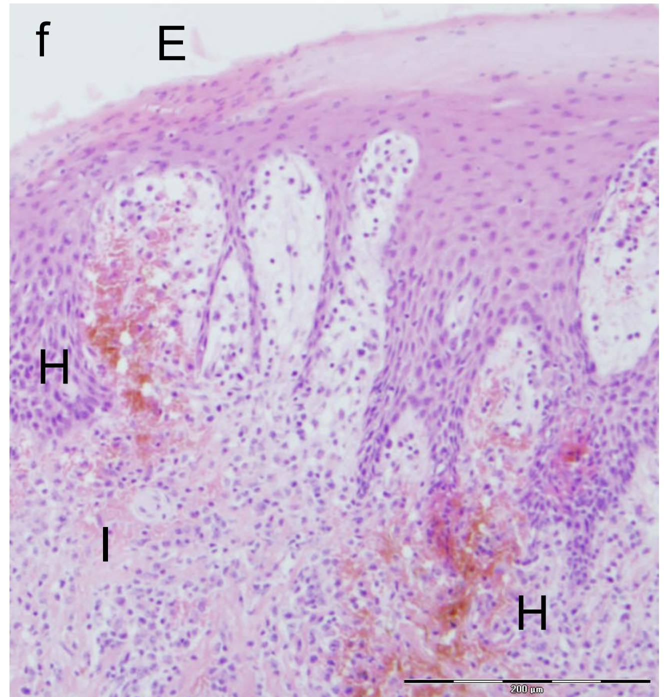


Figure 2