Académie universitaire Wallonie-Europe
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Virology and viral diseases

VIROLOGICAL ASPECTS AND PATHOGENESIS OF NATURAL AND EXPERIMENTAL EQUID HERPESVIRUS 3 INFECTION IN HORSES

ASPECTS VIROLOGIQUES ET PATHOGÉNIE DE L'INFECTION NATURELLE ET EXPÉRIMENTALE DU CHEVAL PAR L´HERPÈSVIRUS ÉQUIN 3

Thesis presented to obtain the degree of Doctor in veterinary sciences

Academic year 2009-2010

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A:	adenine	**i.m:**	intramuscular
ATCC:	American Tissue Culture Collection	**i.v.:**	intravenous
bp:	base pairs	**Kb:**	kilobase
bwt:	total body weight	**kg:**	kilogram
°C:	Celsius degrees	**km:**	kilometer
C:	cytosine	**mg:**	milligram
CF:	complement-fixing	**min:**	minute
cm:	centimeters	**ml:**	milliliter
DNA:	deoxyribonucleic acid	**mm:**	millimeter
ECE:	Equine coital exanthema	**mo:**	month
E. Derm:	equine dermis	**NS:**	nasal swab
EDTA:	ethylenediaminetetraacetic acid	**ORF:**	open reading frame
EHV-3:	Equid herpesvirus 3	**PCR:**	polymerase chain reaction
EHV-1	Equid herpesvirus 1	PVS:	perineal/vaginal swabs
EHV-2:	Equid herpesvirus 2	**RE:**	restriction endonuclease
EHV-4:	Equid herpesvirus 4	**RK13:**	rabbit kidney cell line
EHV-5:	Equid herpesvirus 5	**T:**	thymine
G:	guanine	**TCID:**	Tissue Culture Infectious Dose
gG:	glycoprotein G	**UL:**	long unit
IgA:	immunoglobulin A	**US:**	small unit

Chapter 1

Introduction

Virological aspects and pathogenesis
of natural and experimental equid herpesvirus 3
infection in horses

Aspects virologiques et pathogénie
de l'infection naturelle et expérimentale
du cheval par l'herpèsvirus équin 3

Equine coital exanthema: a disease of concern for the equine industry

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Abstract

Equine coital exanthema (ECE), caused by equid herpesvirus 3 (EHV -3), is a contagious venereal disease characterised by the formation of painful papules, vesicles, pustules and ulcers on the external genitalia of both mares and stallions. EHV-3 is an alphaherpesvirus, distinct from the other equine herpesviruses, that is endemic in most horse breeding populations worldwide.The negative impacts of ECE on equine breeding enterprises are the forced, temporary disruption of mating activities of mares and stallions, additional care and supportive treatment in af fected horses, and the risk of virus spread by either fresh or frozen semen as well as by artificial insemination and embryo transfer practices. Because the diagnosis is made on the basis of typical clinical signs, its true prevalence and economic impact is difficult to assess and is probably underestimated.

The purpose of this review is to describe the advances in our understanding of EHV-3 infections and hypothesise about the ECE economic consequences in the current context of the equine industry.

Keywords: equid herpesvirus 3, equine coital exanthema, equine, reproduction, infectious diseases

1.1 Introduction

Equine coital exanthema (ECE), caused by equid herpesvirus 3 (EHV-3), is an acute, infectious, venereally transmitted disease resulting in the formation of papules, vesicles, pustules and ulcers on the penis and prepuce of stallions and on the vaginal and vestibular mucosa and perineal skin of mares. The virus is highly contagious but non-invasive, and the disease is relatively benign (Blanchard et al., 1992; Allen and Umphenour , 2004; Thiry, 2006; Tibary and Fite, 2007; Paccamonti and Pycock, 2009; Parkinson, 2009). The disease, under a variety of names such as genital horse pox, eruptive venereal disease, equine venereal vulvitis or balanitis and coital vesicular exanthema, was first described in Ireland in the early 1900s (Craig and Kehoe, 1921). In 1960, it was reported in Argentina (Monteverde et al., 1960) and Australia, where Osborne and Bain (1961) associated the disease with spirochetes seen in smears of semen and scrapings from early lesions. The causative agent of ECE, EHV -3, was first isolated in 1968 in the United States (Bryans, 1968), Canada (Girard et al., 1968) and Australia (Pascoe et al., 1968), concurrently. At present-days, EHV-3 has a worldwide distribution.

EHV-3 infection does not usually result in systemic illness. While some authors speculate that the direct effect of this infection on fertility remains debatable (Gleeson et al., 1976; Feilen et al., 1979; Tibary and Fite, 2007; Léon et al., 2008), there is currently sufficient evidence to suggest that infertility and abortion are not consequences of EHV-3 infection (Bryans, 1980; Pascoe, 1981; Rathor, 1989; van der Meulen et al., 2006; Léon et al., 2008; Barrandeguy et al., 2010a).

The negative impacts of ECE on the equine industry are the forced, temporary disruption of the mating activities of af fected stallions and mares, mainly in the thoroughbred breed (Allen and Umphenour, 2004; Barrandeguy et al., 2009a), the risk of iatrogenic EHV-3 dissemination and outbreaks of ECE in artificial insemination and embryo transfer facilities (Barrandeguy et al., 2010a).

The purpose of this review is to describe the advances in our understanding of EHV-3 infections and hypothesise about the economic consequences of ECE in the current context of the equine industry.

1.2 Horse industry and equine coital exanthema

Since the 1960s, there has been an unprecedented upsurge in the growth of the horse industry in many countries, both for commercial and recreational purposes, thus, the horse has joined the list of animal commodities that are traded globally (T imoney, 2007). The number of equine enterprises worldwide is huge; a total of 1 18,889 thoroughbred foals were registered in 2008, meaning that

approximately 237,000 mares were naturally mated by an estimated number of 14,000 stallions, worldwide. In 2008, Argentina was the fourth in production of thoroughbred foals, after the United States, Australia and Ireland (Anonymous, 2009), and first in producing polo horses (Buchanan, 2009).

Additionally, since the 1970s there have been tremendous changes in equine reproduction. Most breeds now allow the use of artificial insemination, embryo transfer, oocyte collection and transfer, and even cloning. Certainly, one of the main changes in the horse industry is the widespread acceptance of cooled / frozen transported semen, even internationally, and embryo transfer, which allows multiple foals to be registered from a given donor mare. According to the data reported by the International Embryo Transfer Society, a total of 24,875 fresh and 340 frozen equine embryos were transferred in 2007, of which Argentina performed 9,600; Brazil, 8,100; and the United States, 6,500 (International Embryo Transfer Society, 2008).

In contrast to the use of artificial insemination and embryo transfer, the thoroughbred industry allows only natural mating. The number of mares bred by thoroughbred stallions has increased from 50-60 to above 100 mares per year. In addition, many of these stallions, which are called "shuttle" stallions, fulfil a breeding season in both hemispheres in the same calendar year (Squires, 2009). In the 2009 breeding season, 25 shuttle stallions covered approximately 3000 mares in Argentina, as well as mares from Uruguay, Chile and Brazil, which all visited Argentina temporarily to be live covered by those high quality stallions (R. Soler. Argentine Thoroughbred Breeders Association, personal communication).

In the last decade, ECE has met the criterion to be considered a "re-emer ging disease" (Toma and Thiry, 2003); several outbreaks of ECE have been reported in diferent countries (Cochard et al., 2002; Aurich et al., 2003; Kleiboeker and Chapman, 2004; Seki et al., 2004; van der Meulen et al., 2006; Costa et al., 2009; Barrandeguy et al., 2009a; 2010b; 2010a). Additionally, ECE is considered an important equine venereal infectious disease that could spread both within and between countries with the movement and trade of equids. ECE could also be transmitted by infected artificial insemination/embryo transfer equipment or contaminated semen (Metcalf, 2001; Aurich, 2003; Seki et al., 2004; Samper and Tibary, 2006; van der Meulen et al., 2006; Timoney, 2007; Barrandeguy et al., 2009a; 2010a). Moreover, the Horserace Betting Levy Board has recently incorporated, for the first time, ECE in their "Codes of Practice 2010". This code updates recommendations concerning the prevention and control of equine infectious diseases that represent a potential major threat to equine breeding in Britain and worldwide (Horserace Betting Levy Board, 2010).

1.3 Economic impact

In intensively managed stud operations, which have heavily-scheduled breeding dates for thoroughbred stallions, breeding disruptions may translate into significant end-of-season decreases in the number of entries into the mare book of affected stallions. Also, delayed foaling dates or reduced pregnancy rates may occur in mares that miss breeding opportunities due to the disease (Allen and Umphenour, 2004, Barrandeguy et al., 2009a).

Similarly, in the face of an ECE outbreak in artificial insemination and embryo transfer centres, affected mares (both donor and recipient) show such discomfort that they are reluctant to be inspected, inseminated or transferred with the consequent loss of opportunity to be pregnant.Additional time and necessary precautions required to manage the donor and receptor mares due to the presence of the disease, also have a substantial negative impact (Barrandeguy et al., 2009a; 2010a).

1.4 Aetiology

The causal agent of ECE, equid herpesvirus 3 (EHV-3), is a member of the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus Varicellovirus (Allen and Randall, 1979; Pellet and Roizman, 2007, Roizman et al., 2007; Davidson et al., 2009).

EHV-3 is antigenically, genetically, and pathogenically distinct from equid herpesvirus 1 (EHV-1), EHV-2, EHV-4 and EHV-5 (Allen et al., 1977; Gutekunst et al., 1978; Staczek et al., 1983; Baumann et al., 1986; Hartley et al., 1999; Allen and Umphenour, 2004; Kleiboeker and Chapman, 2004).

The 149 Kb double-stranded DNA of EHV-3 is a type D genome comprised of a long and a small unit (UL and US). The US segment is flanked by inverted repeats (Atherton et al., 1982).The genomes of EHV-1 and EHV-4 have been fully sequenced (Telford et al., 1992; Telford et al., 1998), but much less is known about EHV-3; only the complete nucleotide sequence of the glycoprotein G (gG) precursor gene (Hartley et al., 1999) [GenBank Accession number AF081188], the partial sequence of EHV-3 DNA polymerase (Kleiboeker and Chapman, 2004) [GenBank Accession number AF514779], the EHV-3 putative DNA packaging protein gene (Kleiboeker and Chapman, 2004) [GenBank Accession number AF514778], and the EHV-3 DNA-dependent DNA polymerase gene [GenBank Accession number AF 141885] (Ehlers et al., 1999) have been determined.

The nucleotide sequence of EHV-3 gG, which comprises a 1344 bp ORF, is located in the US region of the genome, and encodes a class 1 membrane protein of 448 amino acids. EHV-3 gG contains both conserved and variable regions (amino acids 1-295 and 296-375, respectively), which are also found in EHV-1 and EHV-4. Similar to EHV-1 and EHV-4 gG, the C-terminal variable region of EHV-3 gG contains strong type-specific B cell epitopes in the natural host (Hartley et al., 1999).

No antigenic variants of EHV-3 have been described, and there has been no detailed analysis of the antigenic and immunogenic proteins/glycoproteins of EHV -3 (Studdert, 1996; Allen and Umphenour, 2004).

The partial sequence of the gG gene obtained from 25 field isolates and compared with those in GenBank has revealed that there are at least four genetically distinguishable EHV-3 strains in circulation. Three base substitutions in the gG gene have been found at positions 904, 1103 and 1264, which result in strains CAT (Australia), AAT (the United States and Brazil), CAG (Argentina) and ACT (Argentina). The substitution at position 904 is a silent mutation, whereas the others produce changes on the deduced gG protein amino acid sequence (D to A and S to A at the 368 and 422 amino acid (aa) residues, respectively). The antigenic implications of these changes have yet to be fully studied (Barrandeguy et al., 2009c).

When EHV-3 is digested with restriction endonuclease, the cleavage patterns are unique when compared to EHV-1, EHV-2, EHV-4, and EHV-5 (Sullivan et al., 1984; Kamada and Studdert, 1983; Jacob et al., 1985; Bouchey et al., 1987).

In cell culture, EHV-3 replicates only in equid-derived cell lines (Allen and Bryans, 1977). The optimal temperature for in vitro replication of EHV-3 is 34 °C; at 39 °C, the production of infectious virus is reduced to 10-6 of its maximum yield (Bouchey et al., 1987; Jacob and Steiner , 1988). Nevertheless, 37°C is a permissive temperature to isolate EHV-3 in E. Derm (ATCC number CCL-57) cells from the majority of clinically affected horses (M. Barrandeguy et al., unpublished data).

The virus is labile in the environment, and infectivity is quickly cleared by lipid solvents, detergents, heat, drying and the common disinfectants available for veterinary use (Dwyer, 2004).

1.5 Transmission

Infection by EHV-3 occurs via direct cutaneous contact, either during the act of coitus or by the transfer of virus-containing secretions from contaminated objects (Studdert, 1996; Allen and Umphenour, 2004). Non-genital, iatrogenic transmission of EHV -3 by the use of a contaminated endoscope likely occurred during an outbreak of unilateral rhinitis, an atypical manifestation of EHV-3 infection, in thoroughbred horses in training (Barrandeguy et al., 2010b). Similarly, iatrogenic virus spread by contaminated hands, gloves or ultrasound scanner occurred among mares during an outbreak of ECE at an embryo transfer centre in Argentina (Barrandeguy et al., 2010a). Mechanical transmission by stable flies has also been suggested (Gibbs et al., 1972). Non coital transmission has also been

associated with the genito-nasal contact by behavioural nuzzling/sniffing, with lesions on the lips and nostrils (Crandell and Davies, 1985).

EHV-3 can be potentially transmitted to the ejaculate through penile contact with an artificial vagina or sleeve and, consequently, by fresh or frozen semen (Metcalf, 2001).

1.6 Pathogenesis

Viral replication is limited to the stratified epithelium of epidermal tissue present within the skin or at muco-cutaneous margins. The destruction of epithelium caused by the lytic virus infection elicits a vigorous, localised inflammatory response that gives rise to the formation of the characteristic cutaneous lesions of ECE. Although systemic dissemination of EHV-3 does not occur, it is not clear whether this represents a genuine tissue tropism or is due to the temperature-sensitive nature of the virus (Allen and Umphenour, 2004). Anorectal lymphadenopathy was a consistent feature during an outbreak of ECE in mares at an embryo transfer centre in Argentina (Barrandeguy et al., 2010a). However, whether the enlargement of the anorectal lymph nodes was due to the EHV-3 infection, the outcome of the inflammatory process in the region or a secondary bacterial infection could not be elucidated and needs further studies. Thus, lymphadenopathy provides a new concern associated with ECE (Barrandeguy et al., 2010a).

Secondary bacterial infection with *Streptococcus zooepidemicus* is common and influences the nature, severity and duration of the epithelial lesions. Recovery from ECE is complete in a matter of two to three weeks and occurs without permanent sequelae (Studdert, 1996; Allen and Umphenour, 2004).

Immunity to EHV-3 has not been studied in detail; the horse responds to infection by EHV -3 with the production of serum complement-fixing (CF) and virus-neutralising (VN) antibodies that reach maximal levels 14 to 21 days after infection (Bryans and Allen, 1973). Allen and Umphenour (2004) suggested that the equine humoral immune response mounted against EHV-3 is lower than the response to systemically infecting herpesviruses. Nevertheless, in our experience, the virus-neutralising antibodies titres are similar to those against EHV -1 (M. Barrandeguy et al., unpublished data). There are controversial data regarding post-exposure immunity to reinfection with EHV -3. Development of ECE in consecutive breeding seasons has been observed (Uppal et al., 1989; Allen and Umphenour, 2004); however, results obtained after experimental infection revealed that the average score of typical ECE lesions were much higher in seronegative than seropositive mares. Also, differences were detected in the duration and intensity of virus shedding (Barrandeguy et al., 2009b).

Reactivation from latency can be associated with low (or decreasing) neutralising antibody titres, as seen for caprine herpesvirus 1, another genital alphaherpesvirus (Tempesta et al., 1998). Nevertheless, EHV-3 spontaneous reactivation and reexcretion in the presence of invariable levels of neutralising antibodies was detected in mares kept in isolation (Barrandeguy et al., 2010c).

1.7 Epidemiology

The horse is the only recognised host for EHV-3. Latency has been inferred based on epidemiological observations and serological studies (Allen and Umphenour , 2004) and recently demonstrated by reactivation and reexcretion of the virus after corticosteroid treatment as well as spontaneous virus shedding in mares kept in isolation for eleven months (Barrandeguy et al., 2008; 2010c).

Such episodes of virus reactivation and re-excretion, with subsequent recrudescence of clinical or subclinical infection, serve as the source for spread of EHV-3 to other animals (Allen and Umphenour, 2004; Seki et al., 2004; Thiry, 2006; Tibary and Fite, 2007). In a field study performed at the time of breeding, the prevalence of EHV-3 shedding in mares without clinical evidence of ECE was as high as 6% (Barrandeguy et al., 2010c).

The anatomical site that harbours latent EHV-3 is unknown, but it is hypothesised – by comparison

with human herpesvirus 2 and caprine herpesvirus 1 that the virus establishes a latent infection in sciatic and/or sacral ganglion cells (Studdert, 1996; Tempesta et al., 1999).

The seroprevalence of EHV-3 infection ranges between 18 to 53% of breeding age horses (Aurich et al., 2003; Allen and Umphenour, 2004; Barrandeguy et al., 2009a; 2010c).

Once recrudescence of EHV-3 infection has occurred, ECE is highly contagious; postcoital infection rates as high as 100% have been reported.

1.8 Clinical signs

Lesions initially appear as small (1 to 2 mm), raised and reddened papules, which often go unnoticed. Then, lesions progress sequentially to a vesicle, a pustule and, after epidermal sloughing of the necrotic dome of the pustule, a shallow, raw or encrusted erosion or ulcer (Figure 1 and Figure 2).

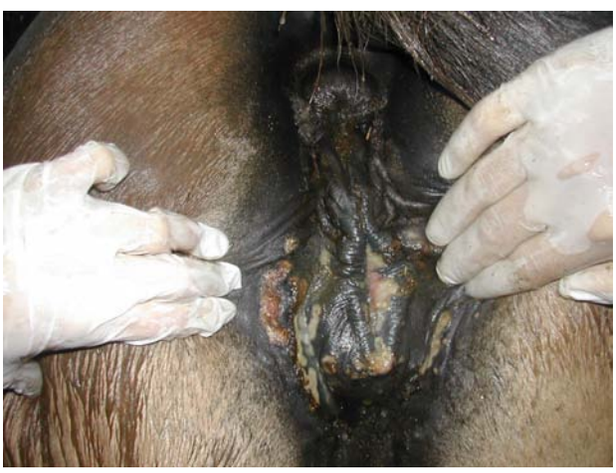

Figure 1: *Lesions of equine coital exanthema on the shaft of the penis of stallion.*

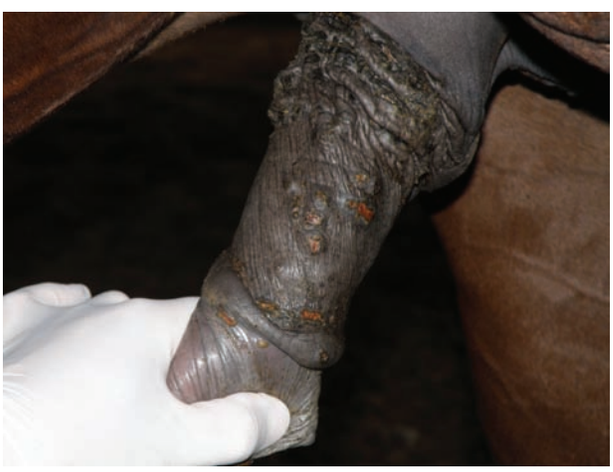

Figure 2: *Lesions of equine coital exanthema on the vulva and perineal skin of a mare.*

There is also localised inflammation reddening, congestion, and oedema. The lesions may be few or numerous and may occur at dif ferent clinical stages. In uncomplicated cases, healing ECE lesions is complete by 10 to 14 days, but lesions may be marked by depigmented, cutaneous scars that persist for many weeks (Blanchard et al., 1992; Studdert, 1996; Allen and Umphenour, 2004).

General signs of infection such as fever, anorexia or dullness are sometimes more severe in stallions than in mares, but vulvar discharge, tail switching, frequent urination or arching of the back have been reported in severely af fected mares (Allen and Umphenour , 2004). Anorectal lymphadenopathy, constipation, tenesmus and evacuation of firm, dry, mucus-covered faeces were associated with lesions around the anus during a severe ECE outbreak at an embryo transfer centre in Argentina (Barrandeguy et al., 2010a). Occasionally, stallions with extensive ECE lesions exhibit discomfort, loss of libido and refusal to mount and copulate with mares (Studdert, 1996;Allen and Umphenour, 2004; Barrandeguy et al., 2009a).

1.9 Diagnosis

ECE genital lesions in both mares and stallions are usually characteristic enough for a clinical diagnosis to be made with reasonable certainty. However, diagnosis of subclinical infections, which is critical to prevent outbreaks of the disease, is not routinely carried out. Moreover , a pen side EHV-3 detection assay, which could facilitate the diagnosis of subclinical infected mares, is still unavailable.

A presumptive diagnosis can be confirmed in the laboratory by isolation of the virus, detection of EHV-3 DNA by PCR or demonstration of either seroconversion or a four-fold or greater rise in antibody titre in paired serum samples (Ehlers et al, 1999; Dynon et al., 2001; Allen and Umphenour, 2004; Seki et al., 2004; van der Meulen et al., 2006; Barrandeguy et al., 2008; Léon et al., 2008). Molecular diagnostic techniques were still useful to detect EHV-3 in severely desiccated skin samples from which the virus could not be isolated (Kleiboeker and Chapman, 2004). The utility of molecular diagnostic tests in detecting EHV-3 contamination in semen samples has not yet been explored.

Specimens to be submitted for laboratory confirmation of ECE should be clinical material collected from the edges of fresh, active lesions by firm swabbing or scraping (Figure 3).

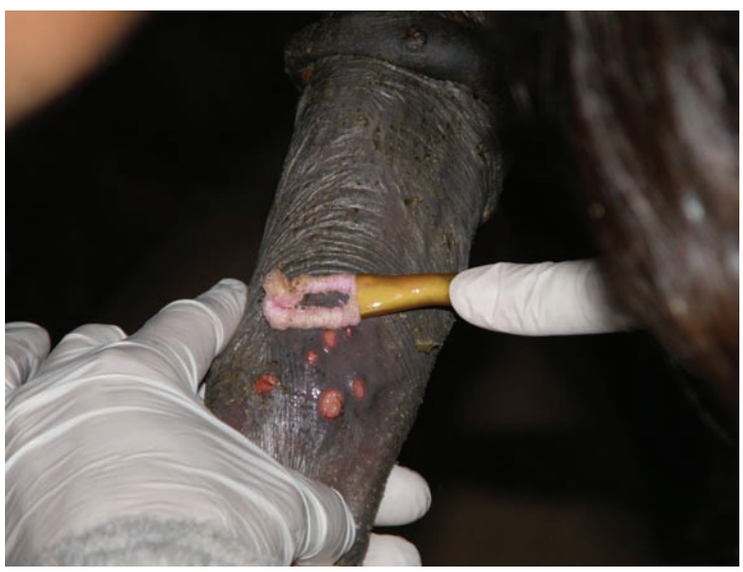

Figure 3: *Collection of samples for virological studies from fresh lesions of equine coital exanthema on the shaft of the penis of stallion.*

Two clotted blood samples, one taken during the acute illness and the other three to four weeks later, are also required. Specimens for virus isolation should be submitted to a competent laboratory in 2–3 ml of viral transport medium (containing antibiotics and antimycotics) on ice.

Isolation of EHV-3 requires inoculation of equine-derived cell cultures: foetal equine kidney, equine thyroid, equine testis or equine embryonic lung cells; or the unique cell line, E. Derm (NBL-6), available at the American Tissue Culture Collection (ATCC). Recently, a novel equine-derived cell line, named FHK-Tcl3, has proven useful to propagate EHV-3 (Maeda et al., 2007). Increased success of virus isolation is achieved if material collected from cutaneous erosions is inoculated directly (i.e. without prior homogenisation, centrifugation or filtration) onto monolayers of susceptible cells (Allen and Umphenour, 2004). Also, a 1:2 dilution in foetal calf serum has been shown to be useful in diminishing the unspecific toxicity of the inocula to the cells in culture (M. Barrandeguy, et al., unpublished data).

Definitive identification of the virus can be achieved by performing infectivity-neutralisation tests with EHV-3 reference antiserum, which is not commercially available, and PCR (Dynon et al., 2001; Kleiboeker and Chapman 2004; Seki et al., 2004; van der Meulen et al., 2006; Léon et al., 2008) or real time PCR (Barrandeguy et al., 2008).

Restriction endonuclease fingerprint analysis of viral DNAs has been used to establish possible epidemiological relatedness (Bouchey, et al., 1987; Barrandeguy, et al., 2009c).

A serological diagnosis of a recent infection with EHV-3 can sometimes be made, retrospectively, by demonstration of a significant rise in neutralising antibody levels between the samples obtained during the acute and convalescent stage of the disease. Detection of EHV -3 serum CF antibody is indicative of viral infection within the previous 60 days (Allen and Umphenour, 2004)

Currently, the diagnosis of EHV-3, mainly by PCR, could be made by several laboratories,

including some private, commercial laboratories (Zoologix, US) around the world. Knowing the causing agent of the disease is very useful, both in confirming a clinical diagnosis and aiding in early prevention of virus spread.

1.10 Treatment, prevention and control

Treatment of ECE is, generally, limited to sexual rest until the lesions have healed to prevent further spread of infection. Administration of broad-spectrum antimicrobials may be indicated when severe lesions are complicated by secondary bacterial infections. Treatment of breeding stallions and mares exhibiting ECE shortens the required period of suspended mating by promoting rapid and uncomplicated healing of genital lesions. This consists of daily cleansing of the genitalia with antiseptics/astringents, reducing inflammation with glucocorticosteroid anti-inflammatory agents and preventing secondary bacterial infection with antimicrobials (Allen and Umphenour, 2004). However, clinical inspection should be complemented by testing the horse for absence of virus excretion or re-excretion.

Topical use of the antiviral compound, acyclovir, for facilitating the healing of ECE lesions has not been fully explored. However, a 5% acyclovir topical cream formulation marketed for treatment of human herpetic skin lesions has been used in the treatment of coital exanthema in both stallions and mares (Cullinane et al., 1994; Barrandeguy, personal communication).

At the present time, vaccines are not commercially available, and vaccination to prevent ECE has not been investigated. Promising results have been shown for caprine herpesvirus 1 genital infection using mucosal vaccination; inactivated vaccines induce production of secretory IgA and provide significant clinical protection (Camero et al., 2007, Tempesta et al., 2007). This same approach could be developed for the prevention of ECE.

Prevention of coital exanthema in stallions requires examination of mares before breeding; nevertheless, as reactivation of latent virus is not preventable, and subclinical virus excretion is a not a rare event, the basis for controlling the impact of ECE outbreaks in breeding establishments is containment of the spread of infection. Cessation of breeding of clinically affected animals until the end of the excretion period, heightened vigilance by the personnel for early recognition of new clinical cases and strict adherence to breeding shed hygiene procedures designed to eliminate mechanical transmission of the virus are the primary ways to avoid the spread of infection if a case of ECE is observed during the breeding season (Allen and Umphenour, 2004).

Thoroughly rinsing each stallion's penis and prepuce with plain warm water after each mating should be carried out, with the aim of reducing the virus load of any potential inoculum of EHV -3 acquired from the covered mare. However, this practice should be investigated experimentally to determine its efficacy. Finally, all instruments, buckets and assist devices used during the breeding procedure should be washed and sterilised between uses or fitted with clean, disposable covers or liners (Allen and Umphenour, 2004).

Considering that EHV-3 infection does not affect the outcome of pregnancy, affected mares can be artificially inseminated during the symptomatic stage of the disease or wait for the complete healing of ECE-associated lesions and cessation of virus excretion before natural service (Paccamonti and Pycock, 2009).

The use of a pen-side test would assist practitioners in knowing the status of mares or stallions with regards to virus excretion or re-excretion after reactivation. This approach would be more reliable than using only visual inspection of the mucosa to search for lesions.

Even though the use of stallion with active disease for artificial insemination is not contraindicated, it must be taken into account that the virus can potentially be transmitted in the ejaculate through penile contact with the artificial vagina or sleeve (Metcalf, 2001).Alternatively, using an open-ended artificial vagina for semen collection would reduce the risk of virus transmission to the mares being (Youngquist and Threlfall, 2009). Checking the semen for the presence of EHV -3 by molecular

techniques or pen-side diagnostic tests would provide definitive security.

The control of outbreaks of ECE in reproductive centres, as well as in artificial insemination and embryo transfer centres, must be based on strict adherence to hygiene procedures designed to prevent mechanical, "iatrogenic", transmission of the virus. The personnel who have direct contact with mares and stallions should wear long, disposable examination sleeves and short latex gloves which should be changed between each inspection. The ultrasound scanner should be covered with a disposable glove or carefully disinfected prior to the inspection of each mare. Finally, all instruments and other devices used during the inspection procedure, artificial insemination and embryo collection must be either disposable or washed and sterilised between each use (Barrandeguy et al., 2010a).

1.11 Conclusions

Although the true incidence and economic impact of ECE is hard to assess, EHV-3 infections and ECE outbreaks can have profound economic impact under the current horse reproductive practices because they disrupt breeding activities, cause economic loss to mare and stallion owners, compromise welfare and are costly to deal with.

Preventing transmission of the virus from subclinically infected animals remains a challenging area of research. Efforts should be focused on prevention of virus shedding using topical vaccines, local "neutralisation" of the virus by antibody enrichment ointments or local treatment with effective antiviral drugs. Another strategy worth further study is the detection of EHV-3 shedding mares to segregate them from the breeding service; thus, the development of field tests for rapid screening, or pen-side tests such as lateral flow, should be a priority. Latency, reactivation and re-excretion and virus shedding by clinically healthy mares are critical features of EHV-3 epidemiology, prevention and control.

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Chapter 2

Objectives

Virological aspects and pathogenesis
of natural and experimental equid herpesvirus 3
infection in horses

Aspects virologiques et pathogénie
de l'infection naturelle et expérimentale
du cheval par l'herpèsvirus équin 3

1. General objectives

EHV-3 is an alphaherpesvirus, distinct from the other equine herpesviruses and endemic in most horse breeding populations worldwide. EHV-3 is primarily transmitted through coitus, although there is also evidence supporting the possibility of non-coital spreading through infected fomites and contacts other than coitus. The infection does not usually result in systemic illness. Previous epidemiological observations and serological monitoring suggest the existence of latently infected animals from which EHV-3 is periodically reactivated and transmitted to cohorts but latency of EHV-3 has not been formerly demonstrated.

The negative impacts of ECE on equine breeding enterprises are the forced, temporary disruption of mating activities of mares and stallions, additional care and supportive treatment in affected horses, and the risk of virus spread by either fresh or frozen semen as well as by artificial insemination and embryo transfer practices. In intensively managed stud operations, which have heavily-scheduled breeding dates for thoroughbred stallions, breeding disruptions may translate into significant end-of-season decreases in the number of entries into the mare book of affected stallions. Similarly, in the face of an ECE outbreak in artificial insemination and embryo transfer centres, afected mares (both donors and recipients) show such discomfort that they are reluctant to be inspected, inseminated or transferred with the consequent loss of opportunity to become pregnant. The additional time and necessary precautions required to manage the donor and receptor mares due to the presence of the disease also have a substantial negative impact because delayed foaling dates and/or reduced pregnancy rates may occur in mares that miss breeding opportunities due to the disease.

Because ECE is a not a notifiable disease and the diagnosis is made on the basis of typical clinical signs, most cases and outbreaks of ECE remain unnoticed and its true prevalence and economic impact is difficult to assess and is probably underestimated.

Therefore, the general objective of this doctoral study was to increase the knowledge about the biology of EHV-3 infection with the aim to improve its control and reduce the economic consequences to the horse industry.

2. Specific objectives

Several questions need to be addressed in order to better understand the biology of EHV-3 infection in horses with the aim to improve the control of the infection.

First, the natural infection of horses was investigated in several situations: the occurrence of EHV-3 natural infection in horses in Argentina was studied with the aim of illustrate the impact of ECE on the horse industry; the non-venereal transmission of the EHV-3 infection was also investigated.

Secondly, although a large genetic diversity is not expected for an alphaherpesvirus, it was important to explore the variation between EHV-3 field isolates in order to find tools to follow the virus circulation in horse populations.

Thirdly, the main biological property of an alphaherpesvirus, latency , was indirectly studied in experimental conditions, by applying a corticosteroid treatment on seropositive mares to cause viral reactivation and re-excretion.

Fourth, an experimental model of reproduction of the infection and disease was set up in order to find out a tool to study the pathogenesis of EHV-3 infection and to evaluate the efficacy of vaccines or antiviral treatments.

Finally, as the silent carriage of EHV-3 is probably underestimated, the subclinical infection was studied under both natural and experimental conditions.

Chapter 3

Natural infections of horses with equid herpesvirus 3

Virological aspects and pathogenesis
of natural and experimental equid herpesvirus 3
infection in horses

Aspects virologiques et pathogénie
de l'infection naturelle et expérimentale
du cheval par l'herpèsvirus équin 3

3.1 Occurrence of Equine Coital Exanthema in mares from an embryo transfer center

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——————— Journal of Equine Veterinary Science, 2010, **30**, 145-149.

Summary

An outbreak of ECE, characterized by typical lesions around the anus and on the perineal skin, discomfort and anorectal lymphadenopathy, occurred in a large number of donor and recipient mares of an embryo transfer center of Argentina. We found that the horses' movement pattern, the environmental conditions, and the management procedures could have favoured the introduction and/or reactivation from latency and spread of EHV-3. Although this is the first report of an outbreak of ECE in an embryo transfer facility, it reinforces the need to introduce additional hygienic and preventive measures to avoid EHV-3 spread and future ECE outbreaks.

SHORT COMMUNICATION

Occurrence of Equine Coital Exanthema in Mares from an Embryo Transfer Center

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ABSTRACT

Equine coital exanthema (ECE) is an acute, venereal disease caused by equid herpesvirus 3 (EHV-3), characterized by the formation of papules, vesicles, pustules, and ulcers on the vaginal and vestibular mucosa as well as on the skin of the penis, prepuce, and perineal region of mares and stallions. The present work describes an outbreak of ECE characterized by typical lesions around the anus and on the perineal skin, discomfort, and anorectal lymphadenopathy, which occurred in a large number of donor and recipient mares at an embryo transfer center in Argentina. We found that the horses' movement pattern, the environmental conditions, and the management procedures could have favored the introduction and/or reactivation from latency and spread of EHV-3. Although this is the first report of an outbreak of ECE in an embryo transfer facility, it reinforces the need to introduce additional hygienic and preventive measures to avoid EHV-3 spread and future ECE outbreaks.

Keywords: Equid herpesvirus 3; Equine coital exanthema; Virus spread; Embryo transfer center

INTRODUCTION

Equine coital exanthema (ECE) is an acute, venereal disease caused by equid herpesvirus 3 (EHV-3), characterized by the formation of papules, vesicles, pustules, and ulcers on the vaginal and vestibular mucosa as well as on the skin of the penis, prepuce, and perineal region of mares and stallions. In contrast with infections caused by EHV-1 and EHV-4, EHV-3 appears to be self-limiting and does not usually affect internal organs of the host. EHV-3 is primarily transmitted through coitus, although there is also evidence supporting the possibility of noncoital spread through infected fomites.[1,2]

The introduction of artificial insemination as a reproductive practice in almost all breeds of horses has minimized the impact of ECE outbreaks during the last decades, although ECE is still a sanitary problem mainly for thoroughbred enterprises.

Embryo transfer has been expanded in the last 10 years in Argentina, where about 5000 recipient mares become pregnant by embryo transfer every year. There are 10 large facilities and an average of four embryos per donor mare, per year, are obtained[3] (Buchanan G. Argentine Association of Polo Breeders, personal communication). In the previous years, extensive outbreaks of ECE, which impeded the routine procedures, have occurred in equine embryo transfer and reproductive facilities (personal communication). This article describes the epidemiological, clinical, and virological findings during an outbreak of ECE in donor and recipient mares in an embryo transfer center of Argentina.

MATERIALS AND METHODS

Embryo Transfer Facilities, Horse Population, and Horse Movement

La Irenita is a full-service embryo recovery and transfer facility for polo horses located in Daireaux, Buenos Aires province, Argentina. This center manages the entire breeding and transfer process and its main goal is to obtain viable pregnancies and healthy foals from donor mares. The 2008–2009 period was the 11th embryo transfer season of this center, where approximately 9000 foals have been born from embryo transfer.

La Irenita breeding season starts in September and ends in April each year. The center has an intensive annual horse movement: an average of 400 donors (belonging to approximately 50 different owners) and 2000 recipient mares.

Around December 15th, the peak of the season, 80–90 mares are evaluated by transrectal ultrasonography and palpation to determine ovulation, 30 are inseminated and 10 are flushed at the donor area, daily. At the same time, 200–300 mares are evaluated in order to select an

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appropriate recipient for the embryo transfer. A total of 25 mares are selected, and 10 embryos are transferred in the receptor area also daily. Each mare enters the stocks to be assessed by a veterinarian an average of five times (days).

The donor mares are inseminated with fresh semen of selected stallions on the day before ovulation.

The recipient mares leave La Irenita once pregnancies are confirmed at 60 days. The age of the donor and receptor mares ranges between 3 and 20 years. Donor mares are managed separately from the recipient mares, that is, different facilities (4 km apart), paddocks, veterinary staff, and auxiliary personnel; the only connection is a special container where the embryos are transported from the donor to the recipient area, once a day. In the donor area, beside the barn where the mares are inspected, there are also laboratories for fresh semen evaluation and preparation, embryo recovery, identification, washing and conditioning for transfer, and a cleaning room to disinfect, wash, prepare, and sterilize all the materials, equipment, and solutions used in this area. The recipient mares' area is simpler; it consists of only a large barn near the paddock where the mares live on grass.

The donor and recipient mares do not have contact with stallions, teaser stallions, foals, yearlings, or any other category of horses during their stay in La Irenita.

The stallions reside in individual small paddocks close to the donor area. The semen is obtained from clinically healthy stallions, certified free of equine infectious anemia and equine viral arteritis.

All the procedures are supervised by eight trained veterinarians, and mares showing clinical signs of systemic diseases, depression, or fever, are excluded as donors or recipients.

All the instruments, equipments, and solutions (gloves, pipettes, syringes, artificial vaginas, filters, etc) used during the entire process, inspection of mares, collection of semen, artificial insemination, and collection and transfer of embryos are either new and disposable or carefully disinfected and/or sterilized before use. The only two pieces of equipment that were shared between different mares inside the same group (donor or recipient) before the outbreak of disease reported in this work was the pair of gloves worn by the veterinarian to examine the mares and the ultrasonography scanner.

Mares

In the outbreak reported here, 110 donor and 500 recipient mares living 4 km apart, in La Irenita embryo transfer center were involved.

Clinical and Reproductive Records

The mares in both, the donor and recipient areas, were daily inspected clinically and by transrectal palpation and ultrasonography.

Clinical Samples

Swabs were obtained from the anal and perineal region from 12 affected mares and placed in viral transport media. Samples of unclotted blood for serum were obtained from 26 convalescent mares.

Virological Studies

Virus isolation on EDerm tissue culture cells, DNA extraction, and polymerase chain reaction (PCR) were performed on anal and perineal swabs, and EHV-3 antibody titers on serum samples were determined by seroneutralization test as described previously.[4]

RESULTS

Clinical and Virological Findings

From October 30 until December 15, 2008, approximately 32% (n = 35) of the donor mares and 25% (n = 125) of the recipient mares showed lesions resembling those observed in mares suffering from ECE. The lesions consisted of raised papules, round erosions, pustules, and ulcers on the mucocutaneous border of the anal ring (Fig. 1) and in the skin around the anus as well as on the adjacent perineal region (Fig. 2). Some mares showed only small (5–10 mm in diameter) individual erosions, whereas others revealed coalescent raw epidermal ulcers. Fissures (cracks) in the skin surrounding the anus, associated with anal pain, tenesmus, and constipation were commonly observed in severely affected mares.

In addition, in most of the mares showing these lesions, a firm, tender, and swollen structure could be palpated on the rectal wall immediately after the introduction of the hand, 10 cm cranial, through the anus. The structure presented a variable size, ranging between 1 and 7 cm in diameter, and elicited a pain response on finger pressure. The anatomical site was coincident with the location of anorectal lymph nodes, retroperitoneally on the dorsolateral surface of the rectum and canalis analis, for which the finding was interpreted as anorectal lymphadenopathy. Also, in some of these mares, the rectal mucosa become thickened and friable, giving blood-tinged mucus on the rectal sleeve, and in a few cases many focal intramural small nodules the size of rice were also palpated in the rectal wall, thus suggesting an inflammatory condition. Constipation, tenesmus, and evacuation of firm, dry feces covered by mucus (indicative of prolonged stasis) were also reported in affected mares.

The lymphadenopathy and extreme pain and discomfort associated with the lesions at the anal ring discouraged the mares from any type of examination procedures.

No systemic signs, fever, anorexia, or dullness were observed. The disease apparently does not cause any long-term impairment of fertility because the rate of pregnancy

M Barrandeguy et al • Vol 30, No 3 (2010)

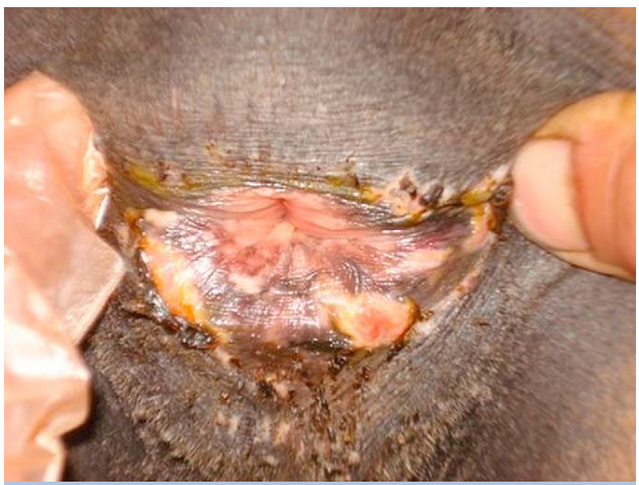

Figure 1. ECE lesions observed on the mucocutaneous border of the anal ring.

in donor and recipient mares was not affected and remained between the averages of rates of previous years.

No specific treatment was implemented; the anal and adjacent perineal regions were carefully washed with commercial iodine soap solution both before and after transrectal examinations.

Among the severely affected mares, only a few (n = 13) at the donor area were treated with anti-inflammatory drugs, and oral mineral oil was used to minimize the discomfort and constipation. Mares were not examined for reproduction purposes and placed in the paddock for 5 to 7 days.

The skin lesions dried out and within weeks evolved to depigmented spots. In contrast, the lymphadenopathy demanded a longer time (2 months) to be reduced with the larger ones taking the longest time to return to normal.

The disease was most commonly observed and more severe in new incoming mares.

No ECE lesions were observed in the stallions from which semen was obtained daily to inseminate the donor mares.

Because the lesions on the skin were initially attributed to irritation or allergic reaction as a consequence to the lubricant used for transrectal palpation and ultrasonography (carboxymethylcellulose 4% aqueous solution), the lubricant was changed to mineral oil plus 10% of commercial iodine soap. Because no significant improvement was noted on the course of the outbreak, an infectious disease, particularly equine coital exanthema, was suspected, and thus a laboratory test for EHV-3 detection was requested.

EHV-3 was detected in seven (58%) of the affected mares sampled, five of which were positive by both virus isolation and PCR, and two of which were positive only by PCR. EHV-3 specific antibodies were detected in 23 (88%) of the convalescent mares sampled and the antibody titer ranged between 32 and 1024 (data not shown).

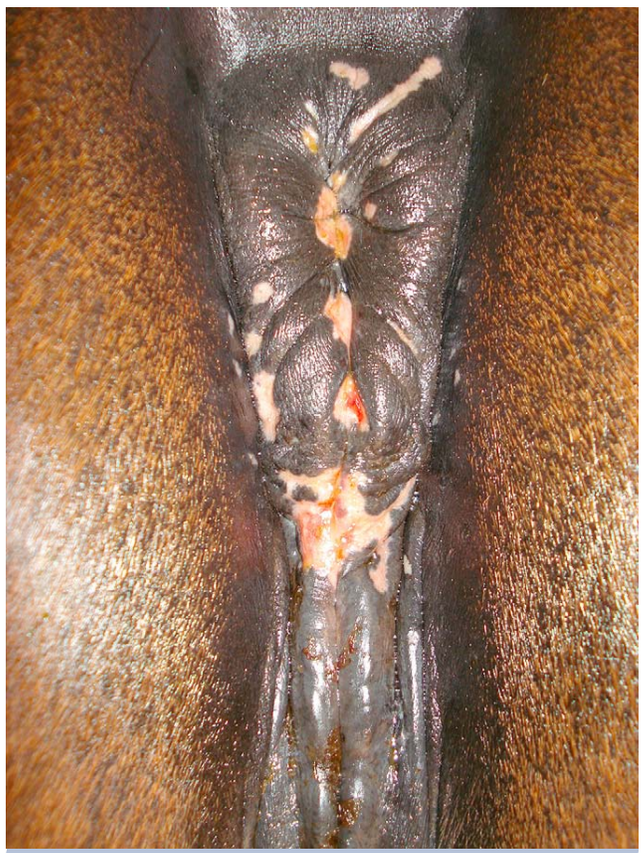

Figure 2. ECE lesions observed in the skin around the anus and on the adjacent perineal region.

A relationship between the ECE lesions and the enlarged and painful anorectal lymph nodes was assumed on the basis of the clinical observations, physical examination, and laboratory findings.

Once the diagnosis of EHV-3 infection was confirmed, some additional preventive and hygienic measures were routinely adopted: the veterinarian performing the examination now changes the disposable plastic gloves between subsequent inspections; the ultrasonography scanner was covered with a different glove for each mare and the person assisting the veterinarian wore disposable surgical gloves to wash the perineal region with disinfectant (commercial iodine soap solution 10% in plain water).

Only two new cases with skin lesion and anorectal lymphadenopathy were recorded after the adoption of these preventive and hygienic measures.

DISCUSSION

The clinical signs and lesions, except for the lymphadenopathy, found in affected mares during the outbreak here reported were consistent with ECE and were confirmed by

virological and serological tests. It could be hypothesized that the virus spread among mares in both donor and recipient areas was a consequence of contamination by means of the gloves or ultrasonography scanner used and that a subclinically infected mare, without visible noticeable lesions, was the primary source of the EHV-3 infection. The index case could not be established because the first cases either proceeded unnoticed or were attributed to irritation due to the lubricant used for transrectal examinations. Likewise, how the infection moved from the donor to the recipient area or vice versa remains unknown. In agreement with previous reports,[1,2,5,6] a possible explanation is that iatrogenic transmission of infection by virus-contaminated objects took place.

The embryo as a source of EHV-3 infection could be ruled out for the following reason: the absence of viremia prevents the intrauterine infections of the embryo; during the collection procedure, the embryo is not exposed to the vagina, vulvar mucosa, or skin; the embryo is not in contact with the hands of the veterinarian or potentially contaminated equipment. Therefore, the transmission of EHV-3 from donor to recipient mares via EHV-3–infected embryos was not likely during this outbreak.

Thus far, it is known that EHV-3 replication is limited to the stratified epithelium of the epidermal tissue present within the skin or at mucocutaneous margins. Viral invasion of tissue within blood vessels, stroma of the dermis underlying the epithelial lesions, or systemic dissemination of EHV-3 does not occur.[1]

Normal anorectal lymph nodes are not noticed by transrectal palpation. Anorectal lymphadenopathy, a consistent feature of this ECE case, has not been previously reported. Considering that afferent vessels reach the anorectal lymph node from the rectum, anus, muscles, and skin of the tail, uterus, vagina, vulva, and clitoris,[7] the enlargement of these lymph nodes would be the outcome of an inflammatory process in the region. ECE lesions and anorectal lymphadenopathy appeared to be highly associated during this outbreak and could have been a consequence of a secondary bacterial infection. Infections with *Streptococcus equi zooepidemicus* have been reported to commonly influence the features, severity, and duration of the epithelial lesions of ECE.[1] The lymphadenopathy observed could be a lesion specifically associated with the EHV-3 infection in the embryo transfer practice due to the particular reproductive procedures. Several factors such as EHV-3 and ECE lesions, a secondary bacterial infection, repeated transrectal palpation in donor and recipient mares, and mechanical (transrectal palpation) and chemical irritation (lubricants) of the area could contribute to this. The lymphadenopathy recorded in La Irenita had the most relevant clinical consequence of the ECE outbreak because it caused constipation, pain, and reluctance of the mares to be inspected. Lymphadenopathy provides a new concern associated with ECE. Whether the enlargement of the anorectal lymph nodes was due to the EHV-3 infection or to a secondary bacterial infection could not be elucidated and needs further studies.

The index case remains to be identified. Considering that EHV-3 induces a latent infection,[1,4] spontaneous reactivation and re-excretion related to stress could have occurred in one of the mares and the infection could then have been transmitted by the examination gloves or the ultrasonography scanner. There may also be a relationship between the EHV-3 spread and the "iatrogenic" transmission because when the disposable gloves worn by the veterinarian and the ultrasonography scanner protection gloves were changed between subsequent inspections, the number of new ECE cases was abruptly reduced to only two. Also, the use of iodine compounds as disinfectants in the perineal area could have helped to reduce the load of this enveloped virus, easily inactivated by disinfectants in the absence of organic matter.[8] In agreement with that previously reported, the present work highly suggests that the virus can be transmitted not only by mating but also mechanically from one mare to another.[1,2,9,10]

Because of the existence of latently infected carrier animals in most horse populations, occasional reactivations of the latent virus are not preventable. Thus, the control of outbreaks of ECE in reproductive centers like La Irenita must be based on strict adherence to hygiene procedures designed to prevent both the direct and indirect transmission of the virus. The personnel who have direct contact with mares should wear long, disposable examination sleeves, and short latex gloves which should be changed between subsequent inspections. The ultrasonography scanner should be covered with a disposable glove or be carefully disinfected previously to the inspection of each mare. Finally, all instruments and other devices used during the inspection procedure, artificial insemination, and embryo collection must be either disposable or washed and sterilized between one use and the next.

Even though neither the life of mares nor their fertility are compromised by ECE, this virus has an important negative impact on embryo transfer enterprises due to the additional time and precautions required to manage the donor and recipient mares because of the presence of the disease.

ACKNOWLEDGMENTS

We thank La Irenita staff for sharing the information and the valuable details related to the outbreak. We also gratefully acknowledge Dr Julien Thiry for his generous corrections on the manuscript.

M Barrandeguy et al • Vol 30, No 3 (2010)

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3.2 Outbreak of rhinitis caused by equid herpesvirus type 3

Barrandeguy M., Ulloa N., Bok K., Fernandez F.

Veterinary Record, 2010, 6, 178-179.

Summary

The primary method of EHV-3 transmission is through direct skin-to-skin contact with an acutely infected, virus-shedding horse, usually during coitus. This section describes an outbreak of unilateral rhinitis associated with EHV-3, most likely spread by the use of a contaminated endoscope, in horses.

Short Communications

Outbreak of rhinitis caused by equine herpesvirus type 3

M. Barrandeguy, N. Ulloa, K. Bok, F. Fernández

EQUINE herpesvirus type 3 (EHV-3) is the causal agent of equine coital exanthema (ECE), an acute venereal mucocutaneous disease of horses characterised by the development of papules, vesicles, pustules and ulcers on the vaginal and vestibular mucosa, as well as on the skin of the penis, prepuce and perineal region (Allen and Umphenour 2004). The primary method of virus transmission is through direct skin-to-skin contact with an acutely infected, virus-shedding horse, usually during coitus; non-venereal and iatrogenic transmission of the infection has also been reported sporadically (Crandell and Davis 1985, Allen and Umphenour 2004). Careful observations, frequent serological monitoring of a closed breeding herd and experimental reactivation of the virus from seropositive horses has demonstrated the existence of latently infected carrier animals in which EHV-3 is periodically reactivated, and from which it can be transmitted to horses in close contact (Allen and Umphenour 2004, Barrandeguy and others 2008).

This short communication describes an outbreak of unilateral rhinitis associated with EHV-3, most likely spread by the use of a contaminated endoscope, in horses.

The cases were observed within a population of approximately 2000 adult thoroughbred horses, stabled at the Palermo, San Isidro and Cardales training centres in Argentina. Forty horses showed exudative lesions of the transitional epithelium at the mucocutaneous junctions of only the right nostril, and one horse showed these lesions in both nostrils. The lesions were characterised by epidermal sloughing of the necrotic domes of pustules, giving rise to shallow, raw or encrusted erosions and ulcers. No pyrexia, general distress or lesions on the genital mucosa were observed. Affected horses recovered in approximately one week and no permanent sequelae were observed. No specific treatment other than rinsing the affected area with saline solution twice a day was administered.

Swab specimens were collected from the edges of fresh, acute lesions in four horses. The swabs were placed in viral transport medium and immediately sent to the virology laboratory. Virus isolation was performed by inoculating the swab samples onto a cell line derived from equine dermis (EDerm) and onto the rabbit kidney cell line RK13.

A cytopathic virus was isolated on EDerm cells from the samples taken from three of the four horses. Virus identification was carried out using a PCR amplifying part of the glycoprotein G (gG) gene of EHV-3, as described by Dynon and others (2001). Extracts from the nasal swabs were also tested directly by PCR, after DNA extraction using a commercial kit. PCR products 520 bp in size, the same size as for the EHV-3 reference strain, were obtained from DNA extracted directly from the clinical samples and from the supernatant of inoculated EDerm cells showing a cytopathic effect.

The horses' records showed that they had been examined endoscopically by a veterinarian, using the same flexible endoscope, to evaluate their respiratory tract function in the week before the onset of the lesions; this was consistent with the observation that lesions were observed only in the right nostril in all but one of the affected horses.

EHV-3 was isolated and associated with lesions observed in three of four thoroughbred horses showing unilateral rhinitis; on this basis the authors consider it likely that EHV-3 was the aetiological agent responsible for the clinical signs in the 40 affected horses. The horses' history suggested that the endoscope used for respiratory tract examination in the week before the onset of the disease may have been responsible for spreading the infection. The index case in the outbreak was not identified. Typical lesions of ECE in the genital region were not recorded in horses living at the affected premises at the time of the outbreak of rhinitis or in the preceding few days. Genital lesions caused by EHV-3 are not expected to be observed in horse training centres, since the horses living in these facilities are used for non-reproductive purposes and almost all of them are maiden mares and unmated stallions.

The development of lesions in the mouth and nostrils of mares and foals naturally infected with EHV-3 has been reported before (Crandell and Davis 1985), and the onset of rhinitis in one stallion and one mare approximately three weeks after the onset of ECE was described by Gibbs and others (1970). In the present outbreak, a spontaneous and subclinical reactivation of EHV-3 from a naturally infected horse, leading to excretion of the virus in nasal secretions, may have been the initial source of contamination of the endoscope. Transmission of EHV-3 by the transfer of virus-containing secretions from contaminated objects, such as hands, gloves, instruments, palpation sleeves and sponges, has been previously described (Allen and Umphenour 2004). Non-coital transmission of infection via the genitonasal contact associated with behavioural nuzzling/sniffing has been proposed, since lesions on the lips and nostrils have been reported (Crandell and Davis 1985). Horizontal transmission by direct nose-to-nose contact could theoretically also have played a role in this outbreak, but does not explain the unilateral occurrence of the lesions in all cases but one.

Soaking in a diluted solution of chlorhexidine followed by thorough rinsing is a recommended procedure for disinfecting endoscopes between use in different horses. Like other herpesviruses, EHV-3 can be inactivated by common disinfectants, especially lipid solvents (Dwyer 2004).

The authors hypothesise that, in this case, non-genital iatrogenic transmission of EHV-3 occurred. It is necessary to reinforce the importance of strict application of hygienic measures to limit the risk of spread of infectious diseases. The importance of disease associated with EHV-3 in the horse industry is difficult to assess; nevertheless, EHV-3 should be considered as a potential aetiological agent in the event of an outbreak of rhinitis.

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3.3 Characterisation of Equid herpesvirus 3 Argentinean field isolates

Barrandeguy M., Vissani A., Miño S., Olguin C., Becerra L., Tordoya S., Thiry E.

Manuscript in preparation.

Summary

Twenty-five EHV-3 strains isolated from field outbreaks of ECE, subclinically infected animals and atypical presentations of EHV-3 infection, like rhinitis, were characterised by means of restriction endonuclease (RE) fragment patterns, plaque size and glycoprotein G (gG) gene partial nucleotide sequencing, and compared to the reference EHV-3 strain. Two different RE patterns were found with *Bam*HI (one of them identical to that of the reference strain), two with *Hind*III (both different from that of the reference strain) and one with *Eco*RI (different from that of the reference strain). The plaque size was homogeneous between the isolates, but was 1.64 and 2.88 times larger than that of the reference strain. Three base substitutions, at positions 904, 1103 and 1264 in the gG gene were detected, allowing a differentiation between two EHV-3 clusters, CAG and ACT, among the Argentinean field strains, distinguishable from the CAT strains from Australia and the AAT strains from the United States and Brazil. The RE patterns and the partial gG gene nucleotide sequence obtained revealed that there is a genomic heterogeneity between the EHV-3 strains isolated, thus showing that there are genetically distinguishable EHV-3 strains in circulation in Argentina. Not only the RE patterns, as previously described, but also the nucleotide sequence of the gG gene could be useful tools for further epidemiological studies.

Characterisation of Equid herpesvirus 3 Argentinean field isolates

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Abstract

Equid herpesvirus 3 (EHV-3) is the causal agent of equine coital exanthema (ECE), an acute venereal mucocutaneous disease of horses. EHV -3 has considerable economic impact on the horse industry worldwide. Twenty-five EHV-3 strains isolated from field outbreaks of ECE, subclinically infected animals and atypical presentations of EHV-3 infection, like rhinitis, were characterised by means of restriction endonuclease (RE) fragment patterns, plaque size and glycoprotein G (gG) gene partial nucleotide sequencing, and compared to the reference EHV-3 strain. Two different RE patterns were found with *Bam*HI (one of them identical to that of the reference strain), two with *Hind*III (both different from that of the reference strain) and one with *Eco*RI (different from that of the reference strain). The plaque size was homogeneous between the isolates, but was 1.64 and 2.88 times larger than that of the reference strain. Three base substitutions, at positions 904, 1103 and 1264 in the gG gene were detected, allowing a differentiation between two EHV -3 clusters, CAG and ACT, among the Argentinean field strains, distinguishable from the CAT strains from Australia and the AAT strains from the United States and Brazil. The RE patterns and the partial gG gene nucleotide sequence obtained revealed that there is a genomic heterogeneity between the EHV-3 strains isolated, thus showing that there are genetically distinguishable EHV-3 strains in circulation in Argentina. Not only the RE patterns, as previously described, but also the nucleotide sequence of the gG gene could be useful tools for further epidemiological studies.

Keywords: Equid herpesvirus 3; equine coital exanthema; field strains; virus characterisation

Introduction

Equid herpesvirus 3 (EHV -3), which is a major cause of venereally transmitted genital disease (equine coital exanthema), has been recognized in many countries. EHV 3 is an alphaherpesvirus antigenically and genetically distinct from the other equine herpesviruses (Allen and Umphenour, 2004). Although there has been no report of antigenic variation within EHV-3 strains, large and small plaque variants have been recognized and DNA restriction endonuclease analysis has been used to demonstrate the genetic individuality of EHV-3 isolates (Allen and Umphenour, 2004; Bouchey et al., 1987; Kamada and Studert, 1983). Of the numerous enzymes used, *Bam*HI, *Hind*III, and *Eco*RI have been found to be the most suitable to investigate the EHV -3 viral genome, yielding 23, 18 and 7 major fragments respectively (Sullivan et al., 1984; Atherton et al., 1982).

The nucleotide sequence of the EHV -3 gG gene, which comprises a 1344 bp ORF [GenBank Accesion number AF081188], is located in the US region of the genome, and encodes a class 1 membrane protein of 448 amino acids (Hartley et al., 1999). EHV -3 gG contains both conserved and variable regions (amino acids 1-295 and 296-375, respectively; the C-terminal variable region of EHV-3 gG contains strong type-specific B cell epitopes in the natural host (Hartley et al., 1999).

The phylogenetic relationship of EHV-3 isolates has not been previously examined and only parts of the genome have been sequenced (Barrandeguy and Thiry, in press).

Although it is not expected for an alphaherpesvirus to have a large genetic diversity, the aim of this work was to explore the diversity between EHV -3 field isolates in order to obtain a tool allowing epidemiological follow-up of the virus.

Material and methods

Viruses and cell culture

Twenty-five EHV-3 field isolates were propagated on confluent equine dermis (EDerm) cells monolayers. When the cytopathic effect reached 90% (approximately 48 hours post infection), the medium was removed

and clarified by centrifugation. The supernatants were aliquoted and frozen at -70°C pending laboratory studies.

The designation, origins, horse breed, gender , clinical patterns and references of the field isolates included in this work are listed in Table 1.

Strains	Farm	Breed**	Gender***	Clinical manifestation****
110 (1st Exc)[1]	INTA	P	M	ECE MILD
110 (2nd Exc)[5]	INTA	P	M	Subclinical
110 (3rd Exc)[5]	INTA	P	M	Subclinical
55 (1st Exc)[5]	INTA	P	M	Subclinical
E/1164/01[4]	San Isidro Racetrack	TB	M	Rhinitis
E/9283/07 (3)[2]	Four Fingers Embriones	P	M	ECE
E/976/07 (154)[5]	La Mission	TB	M	Subclinical
E/976/07 (169)[5]	La Mission	TB	M	Subclinical
E/1331/07 (5)[2]	Vacación	TB	S	ECE
E/1333/07 (1)[2]	La Quebrada	TB	S	ECE
E/1333/07 (2)[2]	La Quebrada	TB	M	ECE
E/3473/08 (1)[2]	La Mission*	TB	M	ECE
E/3426/08[2]	Leticia Marco	TB	M	ECE
E/3427/08 (1)[2]	La Mission*	TB	M	ECE
E/3427/08 (4)[2]	La Mission*	TB	M	ECE
E/3427/08 (5)[2]	La Mission*	TB	M	ECE
E/3535/08 (5)[2]	La Mission*	TB	M	ECE
E/3767/08 (11)[3]	La Irenita	P	M	ECE
E/3767/08 (12)[3]	La Irenita	P	M	ECE
E/3767/08 (14)[3]	La Irenita	P	M	ECE
E/3767/08 (17)[3]	La Irenita	P	M	ECE
E/3795/08 (4)[2]	La Mission*	TB	M	ECE
E/3900/08 (1)[2]	La Mission*	TB	M	ECE
E/3900/08 (2)[2]	La Mission*	TB	M	ECE
E/4564/09[2]	La Irenita	P	M	ECE
EHV-3 reference[6]	Cornell University			

*La Mission is a stallion station facility were thousands of mares come in every year season to be covered by shuttle stallions. All mares booked are clinically evaluated before mating. The EHV-3 strains included here were obtained from mares "suspicious" of ECE and rejected to be covered.

**Breed: Polo (P); Thoroughbreed (TB).
***Gender: Mare (M); Stallion (S).
****Clinical Manifestation: Equine coital exanthema (ECE)

[1]Barrandeguy et al., 2008.
[2]Barrandeguy et al., 2009.
[3]Barrandeguy et al., 2010a
[4]Barrandeguy et al., 2010c
[5]Barrandeguy et al., 2010d
[6]Kindly provided by Dr. Ed Duvobi

Table 1: *EHV-3 isolates analysed by plaque size, restriction endonuclease and partial gG gene sequencing.*

Viral DNA extraction

Viruses propagated in large quantities on EDerm cells (ATCC) were clarified by centrifugation at 1,500 x g for 20 min. Supernatants were removed and further ultracentrifuged at 100,000 x g for 60 min to pellet virions. Pellets were resuspended in TE buffer (10mM TrisHCl pH 7.8, 1 mM EDTA) with 0.1% sucrose cushion. Pellets were resuspended in TE buffer with 0.5% sodium dodecyl sulfate (SDS) and digested with proteinase K (700 ug/ml) for 2 h at 56°C. Proteins were precipitated by the addition of half volume ammonium acetate (1.5M, pH 7.5) and DNA was precipitated by addition of 2 volumes of ethanol. Viral DNA was extracted as previously described (Lemaire et al., 1999).

Restriction endonuclease analysis

DNA was extracted from virus-infected EDerm cells and digested to completion (2 h) with *Bam*HI, *Hind*III and *Eco*RI. The digestion products were electrophoresed in a 0.7% Tris Acetate EDTA agarose submersion gel for 18 h at 40V/cm and 500 mA, stained with ethidium bromide, and photographed.

Plaque size determination

Field strain isolates and the EHV-3 reference strain were inoculated onto an EDerm-cell monolayer using E-MEM supplemented with 1.5 % of methylcellulose as post infection medium. Cells were stained with 0.1 % of crystal violet 72 h after inoculation. Twenty plaques from each strain were measured using IMAGE J software (W right Cell Imaging Facility , Toronto Western Research Institute, www.uhnresearch.ca/wcif - http://rsb.info.nih.gov/ij). The results obtained are expressed as the average of the area in pixels.

PCR analysis and DNA sequencing

DNA was extracted from the original clinical sample and from the EHV-3 reference strain, using the QIAamp DNA mini kit3 according to the manufacturer 's instructions. A primer pair capable of amplifying the partial-length glycoprotein G (gG) gene of EHV-3 was used as described by Dynon et al. (2001).

The unpurified PCR products were submitted to Macrogen Inc., Korea, (http://www.macrogen.com) for sequencing purposes.

Phylogenetic analysis

The phylogenetic data sets were constructed with the following previously published gG gene sequences: the EHV-3 334/74 strain [GenBank Accession number AF081188] (Hartley et al., 1999), an isolate from Brazil [GenBank Accession number GQ 336877] (Costa et al., 2009), and the 26 gG gene partial sequences (25 field isolates and the reference strain) obtained in this study.

The nucleotide sequence data were assembled and edited using the BioEdit Sequence Alignment Editor V7.0.9.0 software (Hall 1999). Sequences were aligned using ClustalX version 1.81 with the default setting (Thompson et al., 1997).

The distance analysis was performed by the Neighbour -Joining (NJ) method with Kimura-2 parameters as evolutional model (T amura et al., 2007). Branch support was estimated using Bootstrapping analysis with 1000 replicates.

Results

Genomic characterization

All the field isolates included in this work were differentiated from the reference strain by *Hind*III and *Eco*RI RE patterns. Most of the field strains yielded*Bam*HI RE patterns identical to the reference strain.

Two *Bam*HI RE patterns were found among the field strains; the *Bam*HI-A fragments of strains E/1331/07-5, E/1333/07-1, E/1333/07-2 and E/1 164/01 co-migrated with the *Bam* HI-B fragment (Figure 1a).

Polymorphism was observed in the *Hind*III K band; two *Hind*III RE patterns, different from the reference strain, were found among the Argentinean isolates (Figure 1b).

A homogeneous *Eco*RI RE pattern, which differed from that of the reference strain at the *Eco*RI-E fragment, was detected among the Argentinean field strains studied (Figure 1c).

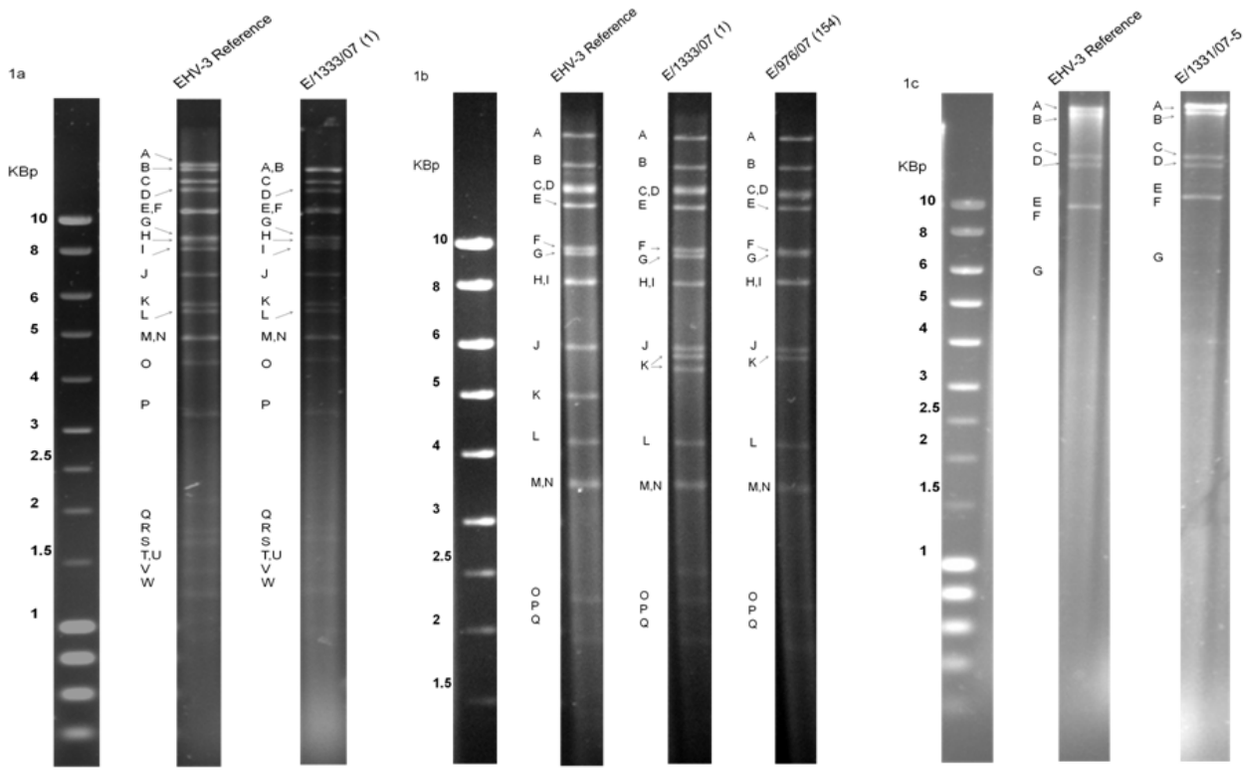

Figure 1: *BamHI (a), HindIII (b) and EcoRI (c) restriction endonuclease profiles of the Argentinean field EHV-3 isolates.*

Plaque size

The plaque size was homogeneous among the Argentinean EHV-3 isolates but was 1.64 and 2.88 times larger than that of the reference strain (Figure 2).

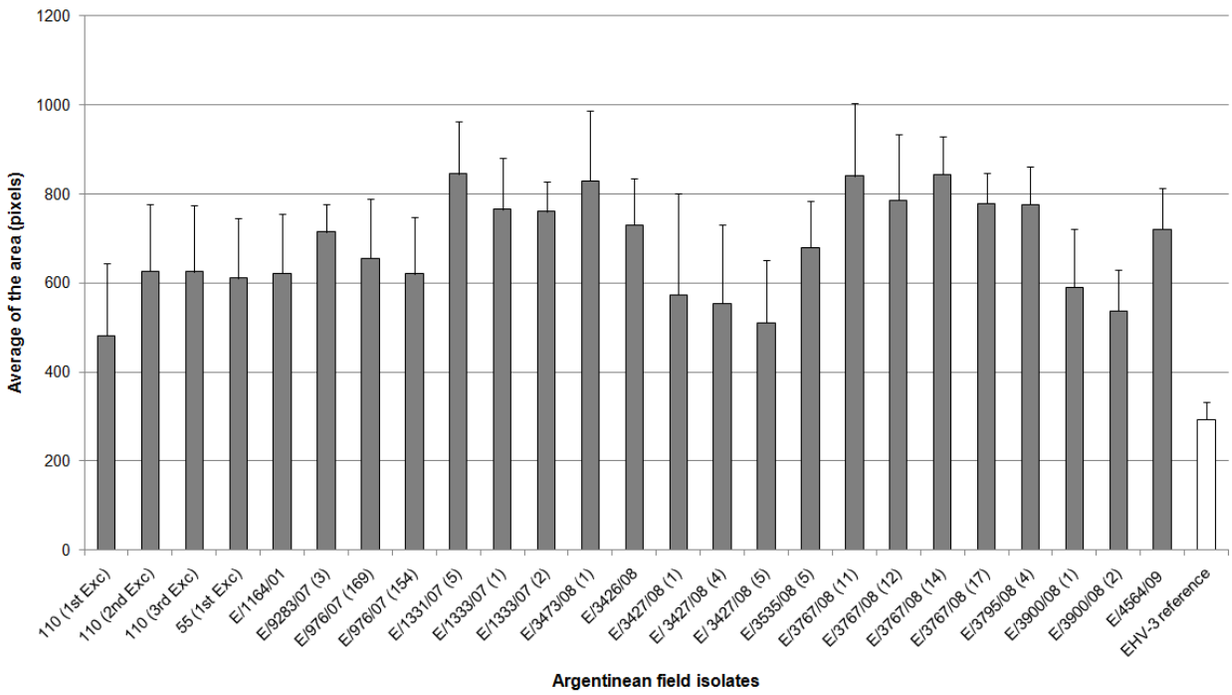

Figure 2: *Plaque sizes of EHV-3 field isolates 72 hours post inoculation (mean of 20 isolate plaques). Data are compared with the plaque size obtained for the reference strain.*

Phylogenetic relationship

The partial length of the gG gene sequence obtained was 496 bp, from nucleotides 802 to 1298. By analyzing multiple nucleotide sequence alignments, the Argentinean strains were grouped in two genotypes which differed from each other by three out of 496 bases.The three-base substitutions in the gG gene were found at positions 904, 1103 and 1264, which resulted in strains CAG and ACT. By comparison with the gG gene partial nucleotide sequences deposited at geneBank, the Argentinean strains differed also in three bases, being CAT (Australia) and AAT (the United States and Brazil). The substitution at position 904 is a silent mutation, whereas the others cause changes in the deduced gG amino acid sequence: D (aspartic acid) to A (alanine) and S (serine) to A (alanine) at the 368 and 422 amino acid residues, respectively.

Phylogenetic analysis was also performed to assess the relationship between the regional isolates and also with those isolated in other geographical regions; two major clusters were detected (Figure 3).

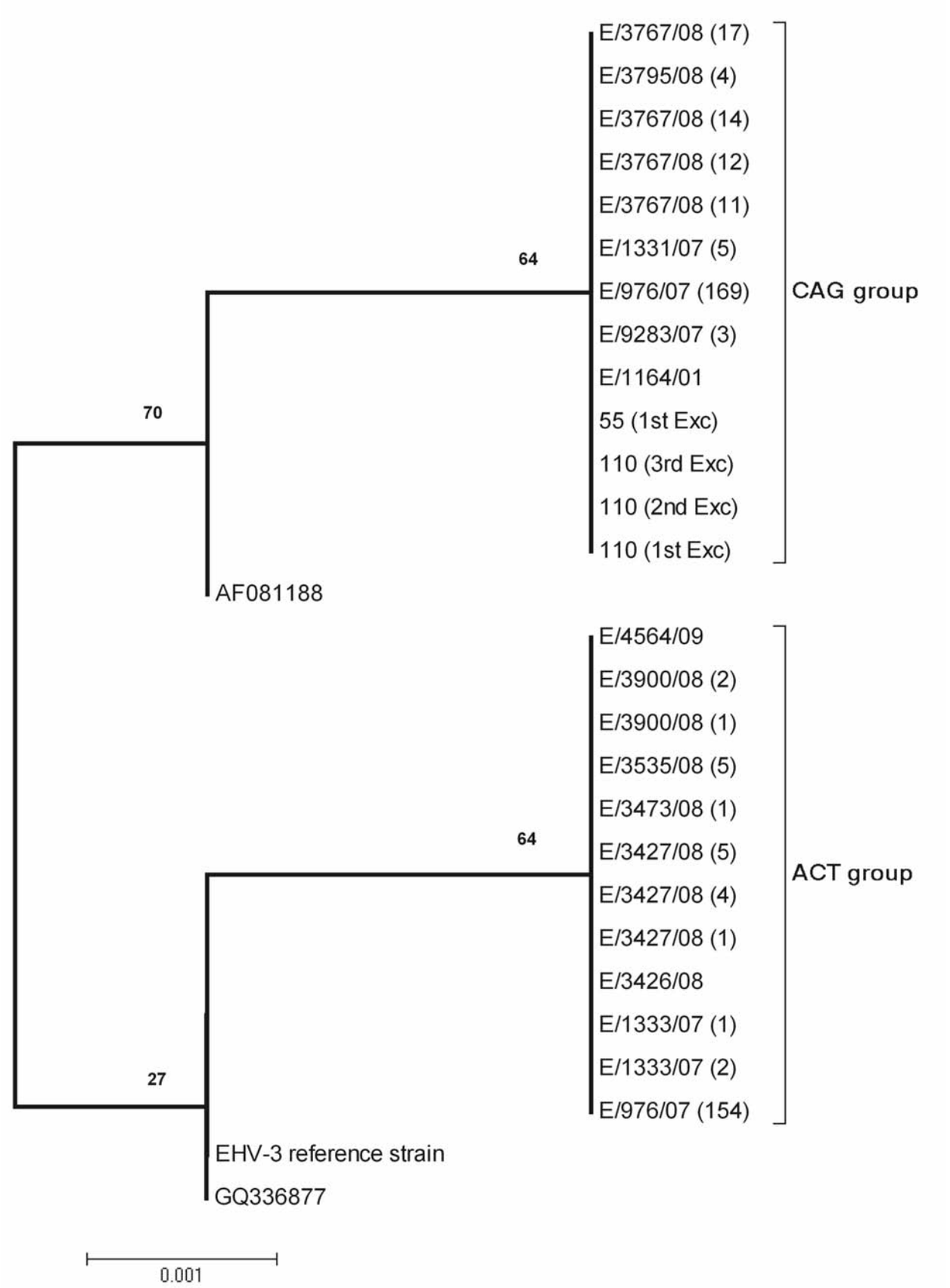

Figure 3: *Phylogenetic r elationship between EHV -3 Argentinean field isolates and EHV -3 isolated in other geographical regions. Two major clusters are shown. The Neighbour-Joining tree was generated by using partial 497 bp fragment of ORF gG gene. Bootstrap values are shown per 1000 replicates. Bar, 0.001 substitutions per nucleotide.*

Discussion

Despite the intense international movement of horses, Argentinean EHV-3 field isolates were distinguished from others obtained in geographically distant locations by means of RE patterns, plaque size and gG gene sequencing. Plaque size revealed the Argentinean isolates as a homogeneous group that differs from the reference strain. Although as small-plaque variants of EHV-3 may arise during passage in cell culture (Allen and Umphenour , 2004), this is not likely to happen in our working conditions, because only low-passaged isolates, except for the reference strain, were included.

In agreement with previous reports (Allen and Umphenour , 2004; Bouchey et al., 1987; Kamada and Studert, 1983), RE analysis allowed distinguishing different patterns among EHV-3 isolates: two with *Bam*HI, two with *Hind*III and one with *Eco*RI; in addition, *Hind*III and *Eco*RI showed that the Argentinean strains are different from the reference strain.

Moreover, the gG gene sequence revealed that at least two clusters of EHV -3 are currently in circulation in Argentina.

Although differences were detected, neither the RE pattern nor the "genotype" could be associated with any particular clinical manifestation; when more than one isolate was obtained from a particular ECE outbreak, e.g. E/3767/08, homogeneity between those isolates was found. In the present study, we did not find co-circulation of different genotypes of EHV-3, e.g. three isolates (E/3767/08 12, 14 and 17) from an outbreak of ECE at an embryo transfer facility (Barrandeguy et al., 2010a) showed the same genotype. The two isolates from subclinically infected mares (Barrandeguy et al., 2010d) were different at the gG gene level, but the genotype could not be associated with the "subclinical character" of the infection.

The strain E/9283/07, an isolate from ECE lesions occurred in a polo mare in February 2007, was identical by plaque size, RE patterns and gG gene sequencing to the isolate 1 10 first excretion (July 2007) obtained after corticosteroid treatment of the same polo mare (Barrandeguy et al., 2008) and also to the 110 second excretion (October 2007) and 110 third excretion (February 2008) occurred spontaneously in the same animal (Barrandeguy et al., 2010). Then, these data provide additional evidence related to latency and reactivation of EHV-3. As expected, no changes were detected in EHV-3 after reactivation and re-excretion.

The genome of EHV-3 has not been fully sequenced and only a few partial sequences are available (Barrandeguy and Thiry, 2010b). In the present study , the nucleotide substitution at position 904 is a silent mutation, whereas the others cause changes in the deduced gG amino acid sequence, but the implications of these changes are still unknown. Considering that the C-terminal variable region of EHV-3 gG has been found to be strongly immunogenic in the natural host (Hartley et al., 1999), the observed amino acid substitutions situated in the corresponding gene region could be relevant in the pathogenesis of EHV-3 infections.

The Argentinean isolates were also distinguishable by gG gene sequencing from the ones from Australia [GenBank Accession number AF081188] (Hartley et al. 1999), and Brazil [GenBank Accession number GQ 336877] (Costa et al., 2009).

In conclusion, the RE patterns and the nucleotide sequence of the gG gene obtained revealed that there are genetically distinguishable EHV-3 strains in circulation in Argentina. The biological implications of these changes are still unknown. Not only RE patterns, as previously described, but also the nucleotide sequence of the gG gene could be useful tools to follow the virus circulation in horse populations.

Acknowledgments

We wish to thank Dominique Ziant for his excellent technical assistance with RE analysis. This work was partially supported by INTA HARAS agreement.

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Chapter 4

Pathogenesis of equid herpesvirus 3 infections

Virological aspects and pathogenesis
of natural and experimental equid herpesvirus 3
infection in horses

Aspects virologiques et pathogénie
de l'infection naturelle et expérimentale
du cheval par l'herpèsvirus équin 3

4.1 Experimental infection with Equid herpesvirus 3 in seronegative and seropositive mares

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——————————————————— Manuscript in preparation.

Summary

In order to set up an experimental EHV -3 infection protocol, seronegative and seropositive mares were topically inoculated in the perineal region with 4 x 106 TCID50/ml of EHV-3. Clinical signs were evaluated by means of a designed scoring system and body temperature was recorded daily. Virological, serological and haematological studies were also performed. Typical ECE lesions were observed in seronegative animals; the clinical scores of these animals were 172 and 90 for the mares included in the first experiment and 160 and 92 in a second experiment. Only mild ECE lesions were observed in seropositive mares, being the clinical score 53 and 41. Both groups of mares shed the virus but the duration was shorter and the intensity was lower in seropositive mares than in seronegative ones. EHV-3 antibody response was detected in both seronegative and seropositive mares after experimental infection and re-infection, being more moderate in seropositive ones.

In conclusion, EHV-3 infection of mares was experimentally achieved in a reproducible manner and thus provides an infection model for further research. The typical lesions of ECE were observed after topical EHV -3 infection in seronegative mares, in association with virus excretion and neutralising antibody kinetics.

Experimental infection with Equid herpesvirus 3 in seronegative and seropositive mares

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Abstract

Equine coital exanthema (ECE) caused by equid herpesvirus 3 (EHV-3) has been recognized as an economically significant venereal disease for years, but, surprisingly, no infection models on the natural host have been established. In order to set up an experimental infection protocol, seronegative and seropositive mares were topically inoculated in the perineal region with 4 x 106 TCID50/ml of EHV-3. Clinical signs were evaluated by means of a designed scoring system and body temperature was recorded daily. Virological, serological and haematological studies were also performed. Typical ECE lesions were observed in seronegative animals; the clinical scores of these animals were 172 and 90 for the mares included in the first experiment and 160 and 92 in a second experiment. Only mild ECE lesions were observed in seropositive mares, being the clinical score 53 and 41. Both groups of mares shed the virus but the duration was shorter and the intensity was lower in seropositive mares than in seronegative ones. EHV-3 antibody response was detected in both seronegative and seropositive mares after experimental infection and re-infection, being more moderate in seropositive ones.

In conclusion, EHV-3 infection of mares was experimentally achieved in a reproducible manner and thus provides an infection model for further research. The typical lesions of ECE were observed after topical EHV-3 infection in seronegative mares, in association with virus excretion and neutralising antibody kinetics.
Keywords: Equine coital exanthema, equid herpesvirus 3, equine, experimental infection

1. Introduction

Equine coital exanthema (ECE) caused by equid herpesvirus 3 (EHV-3) is an acute, venereal disease characterized by the formation of papules, vesicles, pustules and ulcers on the vaginal and vestibular mucosa as well as on the skin of the penis, prepuce and perineal region (Allen and Umphenour, 2004). The disease has a considerable economic impact and has been described in most horse breeding populations worldwide (Barrandeguy and Thiry, 2010b; Tibary and Fite, 2007). EHV-3 is primarily transmitted through coitus although there are also strong evidences of non-coital, iatrogenic spreading by virus-contaminated objects (Allen and Umphenour, 2004, Blanchard et al., 1992; Barrandeguy et al., 2010a, Barrandeguy et al., 2010c). The state of latency with periodic EHV-3 reactivation and re-excretion as well as the subclinical infection in mares at the age of breeding have been recently demonstrated (Barrandeguy et al., 2008; Barrandeguy et al., 2010c). Treatment of ECE is generally limited to sexual rest until the lesions have healed to prevent further spread of the infection. Antiviral treatments, vaccination or other alternative treatments to prevent and control ECE remain largely unexplored (Barrandeguy and Thiry, 2010b).

Experimental transmission of ECE from infected mares to stallions by coitus and experimental reproduction of the clinical disease with material extracted from lesions have been previously successfully achieved (Girard et al., 1968, Monteverde et al., 1960, Krogsrud and Onstad, 1971). Nevertheless, an experimental reproduction model of ECE based on inoculation with titrated low cell-passaged virus is not available.

The aim of the present work was to develop a protocol of experimental EHV-3 infection in horses able to reproduce the lesions observed during the field outbreaks of ECE and to investigate the virus shedding and antibody response patterns in experimental conditions, both in seronegative (presumably naive) and seropositive (previously infected and latently infected) mares.

2. Materials and methods

2.1. Animals

2.1.1. First experimental infection: Two seronegative mares, G (gateada) and R (ruana), of an estimated age* of 8 and 5 years respectively, were used. The mares were kept in isolation from other animals during the experiment.

2.1.2. Second experimental infection: Previously infected mares R and G and two seronegative mares, A (alazana) and M (malacara), were used. The estimated age* of M and A was 10 and 7 years old respectively.

The mares were kept as two separate groups (seronegative and seropositive) isolated between them and also from other horses during the experiment. At the beginning of the experiment, mare M was 10-month pregnant.

Veterinarians specialised in equine practice were in char ge of the care, clinical examinations and sampling of the animals.

*The age of the mares was estimated by the presence or absence of permanent teeth and the aged-related changes in the incisors.

2.2. Inocula

2.2.1. First experimental infection: EHV-3 strain E/9283/07 isolated from lesions of a polo mare infected during an outbreak of ECE in an embryo transfer facility (Barrandeguy et al., 2008) was used as inoculum.

2.2.2: Second experimental infection: EHV-3 strain E/1333/07 isolated from lesions on the penis of an affected thoroughbred stallion was used as inoculum (Barrandeguy, unpublished data).

Both EHV-3 strains were plaque-purified three times on an EDerm cell line, after two passages in cell culture, before the final preparation of the experimental infection inocula. The inocula were titrated, aliquoted and kept a -70ºC until use. The virus titre was 10 6.5 TCID50/ml for the strain E/9283/07 (first experimental infection) and 10 6.66 TCID50/ml for the strain E/1333/07 (second experimental infection). These two strains are genetically distinguishable: the partial sequence of the gG gene revealed three base substitutions at positions 904, 1 103 and 1264, being strain E/9283/07 CAG and strain E/1333/07 ACT (Barrandeguy et al., 2009).

A back titration of the inocula was carried out after the experimental infection challenge.

2.3. Experimental design

The first experimental infection was performed in March-April 2009 and the second in October - November 2009. During the time frame between the first and second experimental infection, mares G and R remained in the same isolation conditions. Throughout the experiments the animals were kept and treated in accordance with the international standards regarding animal welfare (Anonymous, 1985), and the protocols were approved by the institutional animal care and use committee.

The inoculum (4 ml) was topically inoculated in the vagina, on the vulva and on the perineal skin surrounding the vulvar labia, using a swab. Before inoculation, the vulvar epithelium of the mares was abraded using a needle.

2.4. Clinical monitoring

The animals were daily monitored by a veterinarian; body temperature, general clinical signs and careful observation of the genital and perineal skin and mucosa were also recorded daily at the same moment of the day (in the morning), and a digital camera was used to record the evolution of the lesions. Careful attention was paid to the skin of the perineal region and vulvar labia and vagina. In addition, a clinical scoring system based on the presence or absence of congestion/tumefaction, vulvar discharge, pain at the moment of inspection, characteristics of the lesions (type, size, and number), and secondary bacterial infection or myiasis was used to quantify the severity of the lesions. The score of an individual mare was defined as the sum of all clinical signs presentA daily and a total clinical score were calculated for each animal at the end of the experiments.

2.5. Clinical samples

Samples for virus detection and titration were daily collected by roughly rubbing the perineal/vaginal area with an "in-house made" swab (PVS). After sampling, the clinical material obtained was poured down into 5 ml of viral transport medium supplemented with 5% fetal calf serum (Heuschele and Castro, 1992), and immediately transported on ice to the virology laboratory.

Unclotted blood was also daily collected using tubes containing EDTA for routine haematology tests. Blood for serum was obtained once a week (every Tuesday, for two months). The PVS and serum samples were stored at -70 and -20 ºC respectively, pending laboratory studies.

2.6. Laboratory analysis

2.6.1. EHV-3 detection and measurement of virus excretion by quantitative real time PCR

To detect and quantify EHV-3, a real time PCR tar geting the gG gene was carried out, as previously described (Barrandeguy et al., 2008), on DNA extracted from the PVS. Quantification of EHV-3 in the PVS was calculated by generating a standard curve with log dilutions of the reference strain containing 106.50 TCID50/ml and expressed as TCID50 equivalents.

2.6.2. Seroneutralisation test

Antibodies against EHV-3 in blood serum samples were detected by the seroneutralisation test, as previously described (Barrandeguy et al., 2008). Seroneutralising titres were expressed as the log10 of the reciprocal of the highest dilution that completely inhibited the EHV-3 cytopathic effect.

3. Results

Mares showed neither hyperthermia nor systemic clinical signs and the haematological pattern remained normal throughout both experiments.

The typical ECE lesions (vesicles, pustules, erosions and ulcers, of different type, size and number) were observed in all the mares (Figure 1).

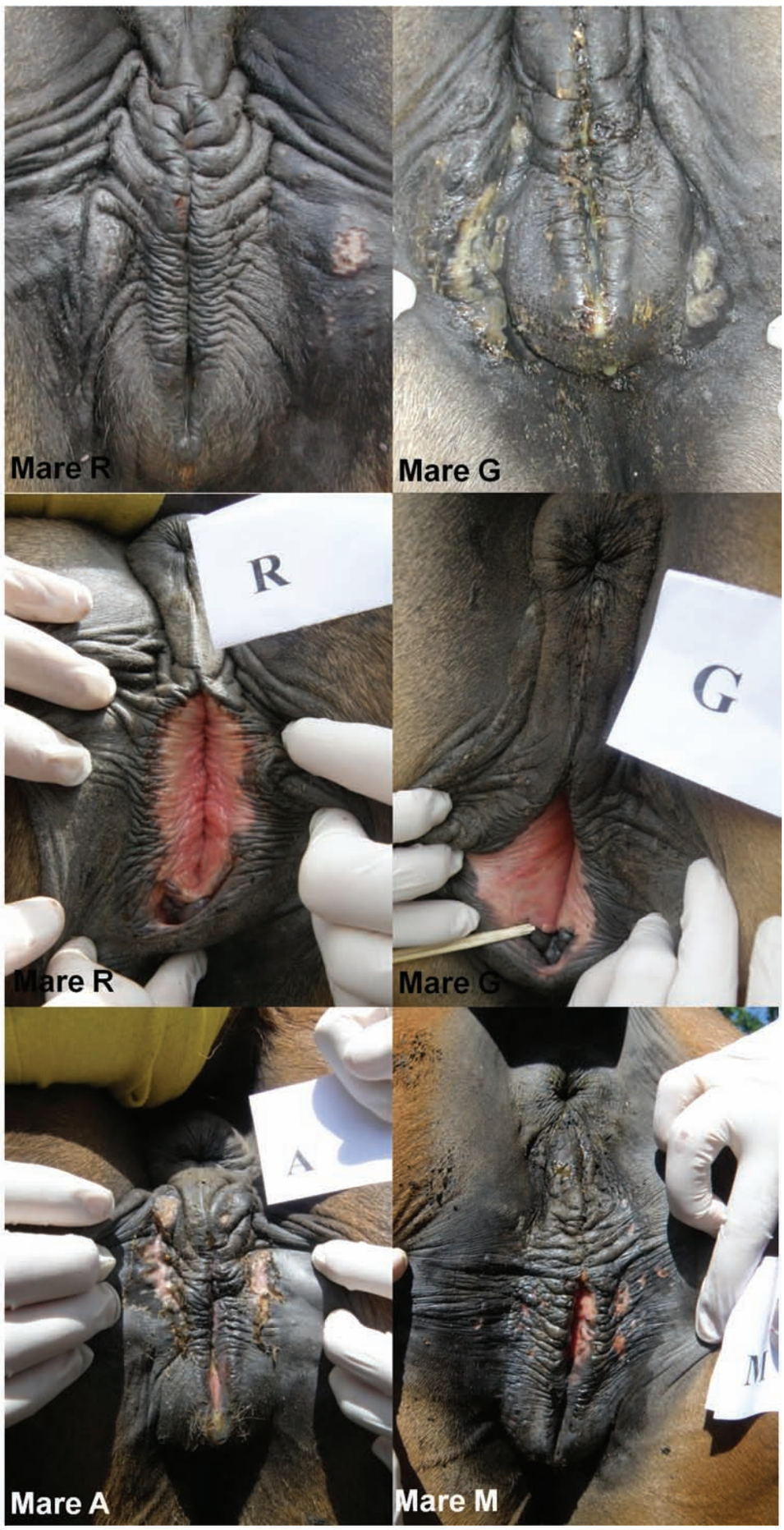

Figure 1: *Illustration of the lesions in the vulva and perineal skin observed in mares 8 days post infection.*

The clinical scores for each mare in the first and the second experiments are shown in Table 1a and 1b, respectively.

	Days post-infection																			Total
Mares	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**Clinical**
Tumefaction[a]																				
R	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	11
G	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	12
Discharge[b]																				
R	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
G	0	0	0	1	2	2	2	2	3	3	3	3	2	1	0	0	0	0	0	24
Pain[c]																				
R	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	7
G	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	10
Lesion Type[d]																				
R	0	2	0	1	2	3	4	4	4	4	4	4	4	3	0	0	0	0	0	39
G	0	0	2	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	0	58
Lesion Size[e]																				
R	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	12
G	0	0	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	30
Lesion Number[f]																				
R	0	1	0	1	2	2	2	2	2	2	2	2	2	1	0	0	0	0	0	21
G	0	0	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	0	30
Others[g]																				
R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	8
TOTAL CLINICAL SCORE[h]																				
R	0	3	0	6	7	8	9	9	9	9	8	8	8	6	0	0	0	0	0	**90**
G	0	0	5	8	11	11	13	13	14	14	14	14	13	11	8	8	8	7	0	**172**

[a]Tumefaction: 0=none, 1=present

[b]Discharge: 0=none, 1=serous, 2=mucous, 3=purulent

[c]Pain: 0=none, 1=present

[d]Lesion Type: 0=none, 1=papula, 2=vesicle, 3=pustula, 4=erosions, ulcers

[e]Lesion Size: 0=none, 1=small (up to 1 cm), 2= large (more than 1 cm)

[f]Lesion Number: 0=none, 1=up to 4, 2=more than 4

[g]Others: 0=none, 1=secondary bacterial infection, myiasis

[h]Total Clinical Score.

Note: In the presence of scabs as a consequence of the healing process, in all lesions a score of 0 was attributed.

Table 1a: *Clinical scores applied to EHV-3-induced lesions observed in mares R and G during the first experiment.*

	Days post-infection																			Total
Mares	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**clinical**
Tumefaction[a]																				
R	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	7
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	11
M	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	10
Discharge[b]																				
R	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	0	0	0	1	0	2	2	2	3	3	3	3	1	0	0	0	0	0	0	20
M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pain[c]																				
R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	6
M	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	8
Lesion Type[d]																				
R	0	0	0	0	0	0	1	1	4	4	4	4	4	4	0	0	0	0	0	26
G	0	0	0	0	2	2	2	2	2	2	2	4	4	0	0	0	0	0	0	22
A	0	0	2	2	2	4	4	4	4	4	4	4	4	4	4	4	4	4	0	58
M	0	2	1	1	2	3	3	3	4	4	4	4	4	4	0	0	0	0	0	39
Lesion Size[e]																				
R	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	8
G	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	9
A	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	1	0	28
M	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	13
Lesion Number[f]																				
R	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	8
G	0	0	0	0	1	1	1	2	1	1	1	1	1	0	0	0	0	0	0	10
A	0	0	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	0	29
M	0	1	1	1	2	2	2	2	2	2	2	2	2	1	0	0	0	0	0	22
Others[g]																				
R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	8
M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOTAL CLINICAL SCORE[h]																				
R	0	3	3	1	1	1	4	4	6	6	6	6	6	6	0	0	0	0	0	**53**
G	0	0	0	0	4	4	4	5	4	4	4	6	6	0	0	0	0	0	0	**41**
A	0	0	5	6	7	12	13	13	14	14	13	13	11	9	8	8	8	6	0	**160**
M	0	4	4	5	7	8	8	8	9	9	8	8	8	6	0	0	0	0	0	**92**

[a]Tumefaction: 0=none, 1=present

[b]Discharge: 0=none, 1=serous, 2=mucous, 3=purulent

[c]Pain: 0=none, 1=present

[d]Lesion Type: 0=none, 1=papula, 2=vesicle, 3=pustula, 4=erosions, ulcers

[e]Lesion Size: 0=none, 1=small (up to 1 cm), 2= large (more than 1 cm)

[f]Lesion Number: 0=none, 1=up to 4, 2=more than 4

[g]Others: 0=none, 1=secondary bacterial infection, myiasis

[h]Total Clinical Score.

Note: In the presence of scabs as a consequence of the healing process, in all lesions a score of 0 was attributed.

Table 1b: *Clinical scores applied to EHV-3-induced lesions observed in mares R, G, M and A during the second experiment.*

The total clinical scores were 172 and 90 for the two seronegative mares included in the first experiment and 160 and 92, and 53 and 41 for the seronegative and seropositive mares respectively included in the second experiment (Table 2).

	Days post-infection																		Total
Mares	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	clinical
R	0	3	0	6	7	8	9	9	9	9	8	8	8	6	0	0	0	0	0	**90**
G	0	0	5	7	11	11	13	13	14	14	14	14	14	10	9	8	8	7	0	**172**
R	0	3	3	1	1	1	1	1	7	7	7	7	7	7	0	0	0	0	0	**53**
G	0	0	0	0	4	4	4	5	4	4	4	6	6	0	0	0	0	0	0	**41**
A	0	0	5	6	7	12	13	13	14	14	13	13	11	9	8	8	8	6	0	**160**
M	0	4	4	5	7	8	8	8	9	9	8	8	8	6	0	0	0	0	0	**92**

Table 2: *Total clinical scores of seronegative and seropositive mares after experimental infection with EHV-3 (first and second experiments).*

Virus shedding was detected by quantitative real time PCR in all mares. The duration and intensity of virus shedding for mares R and G in the first experiment and for mares A, M, R and G in the second experiment are shown in Figures 2a and 2b respectively. Excretion started 1 and 2 days post infection in the seronegative mares and 4 days post infection in the seropositive ones; at peak, virus excretion was as high as 105 and 104 TCID50/ml equivalents in the seronegative and seropositive mares respectively. Mean viral excretion lasted 15 days in the seronegative mares and 9 days in the seropositive ones.

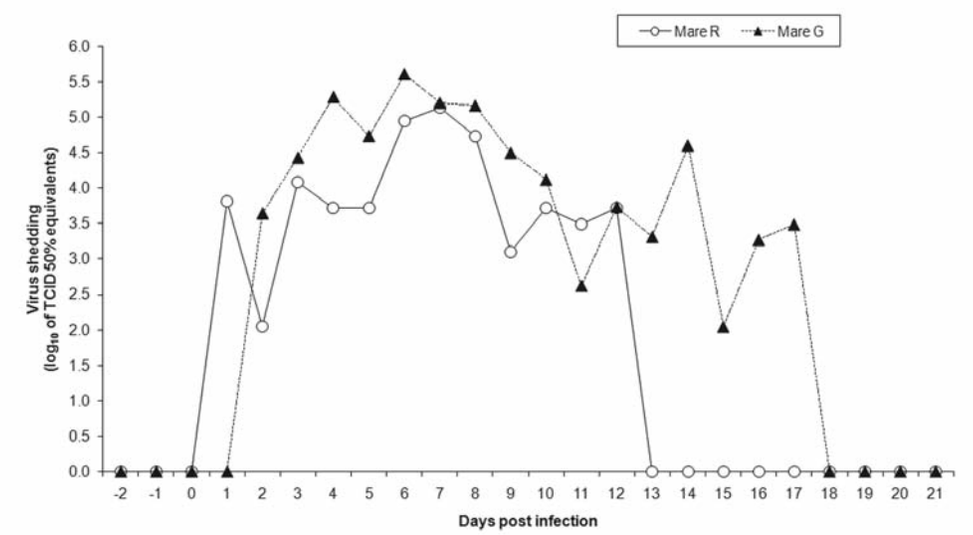

Figure 2a: *Virus shedding in seronegative mares R and G after experimental infection with EHV-3 (first experiment).*

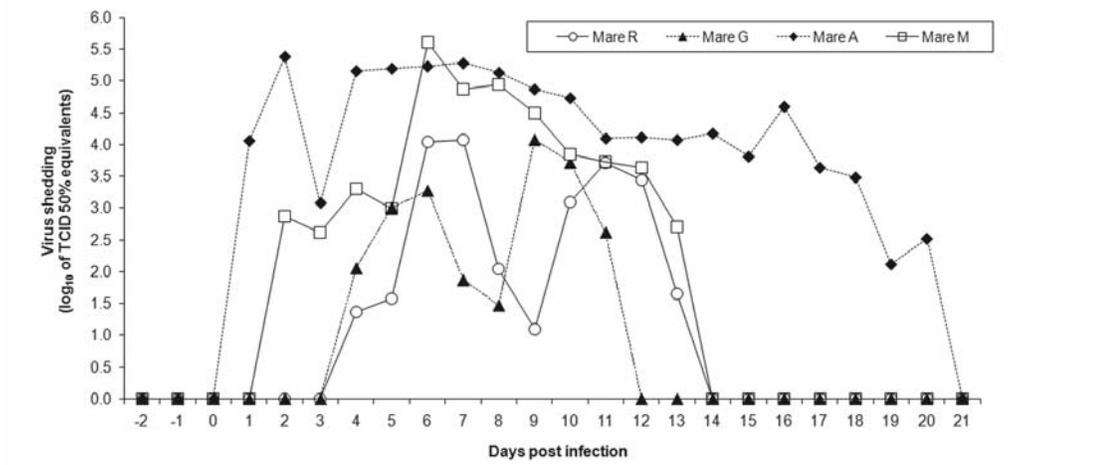

Figure 2b: *Virus shedding in seronegative mares A and M and seropositive mares R and G after experimental infection and re-infection with EHV-3 respectively (second experiment).*

Antibody response as measured by seroneutralisation was detected after infection in all the mares in both experiments, being the increase in antibody titre higher in the seronegative mares (Figures 3a and 3b). In the seronegative mares, neutralising antibodies were detectable 7 days after experimental infection and remained between 2.1 (log10 reciprocal of 1:256 serum dilution) and 2.7 (log10 reciprocal of 1:512 serum dilution) until the end of the study (two months). The antibody titre increased from 1.5 to 2.7 (log10 reciprocal of 1:32 and 1:512 serum dilution, respectively) in one of the seropositive mares and from 2.1 to 2.7 in the other, 7 days post infection.

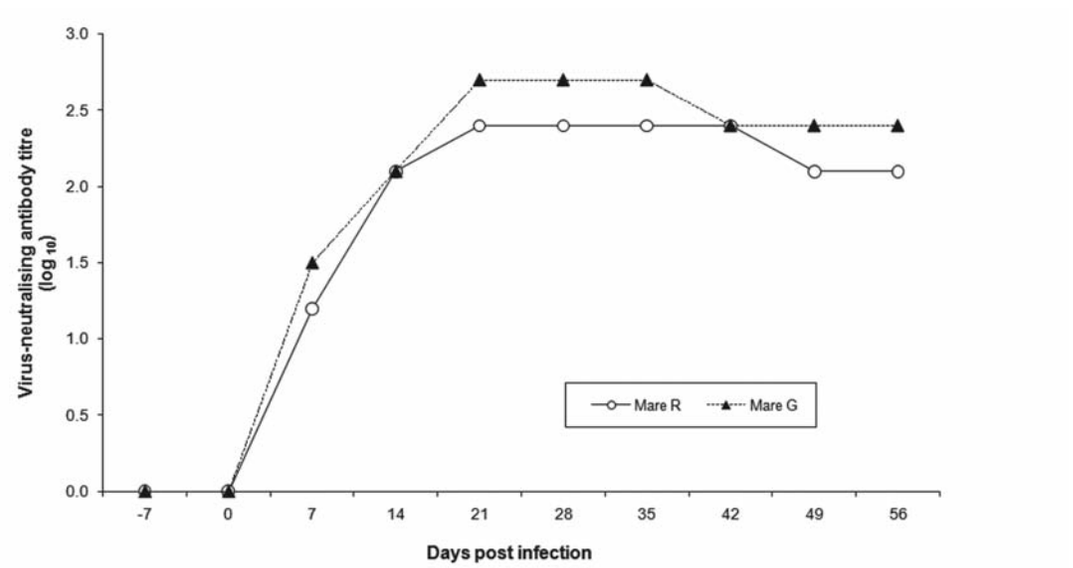

Figure 3a: *neutralising antibody response in mares R and G after experimental infection with EHV-3 (first experiment).*

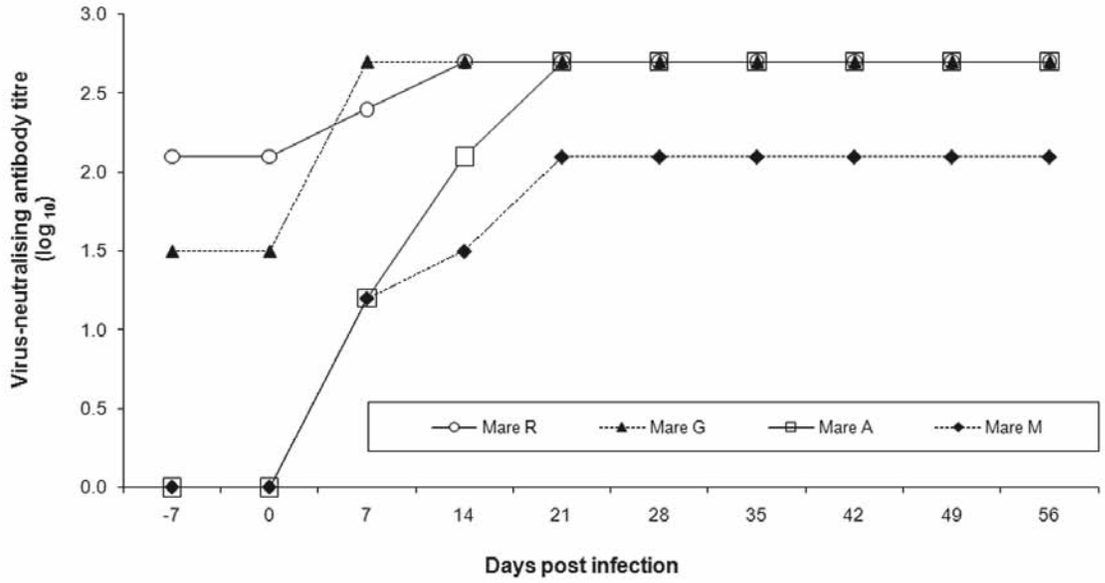

Figure 3b: *neutralising antibody response in mares R, G, A and M after experimental infection with EHV-3 (second experiment).*

4. Discussion

A topical experimental infection with EHV-3 was able to reproduce the ECE clinical signs closely similar to those previously described during field outbreaks (Barrandeguy et al., 2010a; Tibary and Fite, 2007; van der Meulen et al., 2006; Allen and Umphenour, 2004; Kleiboeker and Chapman, 2004; Seki et al. 2004). This experimental model was even able to reproduce milder clinical signs and virus shedding in seropositive (previously infected) mares. Significant neutralising antibody titres are an indication of the presence of an active specific immune response that contributes to the mitigation of clinical signs in positive mares. The underlying mechanisms are not known but mucosal and cell-mediated immune responses could play a role as in other herpesvirus infections (Roizman et al., 2007). In a previous transmission experiment, a mare with increased neutralising antibodies after a clinical EHV-3 infection 4 months before did not show any ECE typical clinical sign (Krogsrud and Onstad, 1971), whereas in our conditions, mild lesions were still observed. This may reflect differences in the level of the protective immune response between animals.

Like in other similar works (Dal Pozzo et al., 2009), the use of a scoring approach allowed us to appreciate the effects of infection in each individual, see the progression of the disease over time and compare its consequences between the mares. The total clinical score of mare G (first experiment) was highly similar to that of mare A (second experiment). These mares showed more severe ECE clinical signs than mares R (first experiment) and M (second experiment), all of them seronegative at the time of experimental infection. Even lower but similar total clinical scores, revealing mild disease presentation, were observed in mares R and G (both seropositive at the time of experimental infection) during the second experiment. The severity and duration of the disease vary considerably among individual horses; in uncomplicated cases, healing of the lesions is complete by 10 to 14 days (Allen and Umphenour, 2004). Virus shedding was detected in all mares but differences in the duration (days) and intensity (virus load) were found, being 15 and 9 days (average duration) and 105 versus 104 (virus excretion titre at peak) in seronegative and seropositive mares, respectively.

The presence of discrete clinical signs (total clinical scores of mares R and G in the second experiment was 53 and 41 respectively) in seropositive mares reflects the effect of an immune response that was, however, not enough to prevent the re-infection and virus shedding.

As previously reported, experimental infection had no effect on gestation (van der Meulen et al., 2006; Allen and Umphenour, 2004). Mare M, which was pregnant at the beginning of the experiment, carried its pregnancy to term and delivered a healthy foal 18 days post infection

Topical inoculation of EHV-3, as carried out in this work, closely mimics the situation that can occur in natural exposure to the virus by coitus and also that in the embryo transfer and artificial insemination practices (Allen and Umphenour, 2004; Barrandeguy et al., 2010d).

The accuracy and reproducibility of the experimental infection protocol described will be further evaluated using a larger number of animals and unifying the age of the mares as much as possible.

Although carried out in a small number of animals, the experimental protocol presented in this study consistently reproduced the lesions observed in natural outbreaks of ECE. It can thus be used to monitor the virus shedding patterns and neutralising antibody kinetics and could be very useful for future EHV-3 research including antiviral therapy and other preventive or therapeutic measures.

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4.2 Experimental reactivation of Equid herpesvirus 3 following corticosteroid treatment

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——————————— Equine Veterinary Journal, 2008, **40**, 593-595

Summary

State of latency , very well known for several herpesviruses, has been proposed for equid herpesvirus 3 (EHV-3) and supported by epidemiological observations. No detailed assessment about reactivation, patterns of excretion and re-excretion has been formally reported. An experimental reactivation study by corticosteroid treatment in previously naturally infected horses was therefore carried out. Two polo mares with clinical and virologically confirmed history of equine coital exanthema were injected with dexamethasone and prednisolone on 3 successive days. Clinical signs, body temperature and clinical samples for virological and serological studies were obtained daily. Mares did not show any systemic clinical sign or hyperthermia. EHV -3 shedding, seroconversion and the presence of a small lesion were observed in one of the mares under study two weeks after corticosteroid treatment. The results demonstrate that this virus exhibits a latency-reactivation behavior similar to that of the other alpha herpesviruses. Reactivation of latency may have an important bearing on the appearance of clinical signs in mares and/or stallions during the breeding season without the actual evidence of transfer from mares to stallion or vice versa.

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Experimental reactivation of equine herpesvirus-3 following corticosteroid treatment

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Keywords: horse; equine herpesvirus-3; coital exanthema; herpesvirus; latency; reactivation; corticosteroid

Summary

State of latency, well known for several herpesviruses, has been proposed for equine herpesvirus-3 (EHV-3) and supported by epidemiological observations. No detailed assessment about reactivation, patterns of excretion and re-excretion has been formally reported. An experimental reactivation study by corticosteroid treatment in previously naturally infected horses was therefore carried out. Two polo mares with clinical and virologically confirmed history of equine coital exanthema were injected with dexamethasone and prednisolone on 3 successive days. Clinical signs, body temperature and clinical samples for virological and serological studies were obtained daily. Mares did not show any systemic clinical signs or hyperthermia. EHV-3 shedding, seroconversion and the presence of a small lesion were observed in one of the mares under study 2 weeks after corticosteroid treatment. The results demonstrate that this virus exhibits a latency-reactivation behaviour similar to that of other alpha herpesviruses. Reactivation of latency may have an important bearing on the appearance of clinical signs in mares and/or stallions during the breeding season without the actual evidence of transfer from mare to stallion or *vice versa*.

Introduction

Equine coital exanthema (ECE), caused by equine herpesvirus-3 (EHV-3), is an acute, venereal disease characterised by the formation of papules, vesicles, pustules and ulcers on the vagina and vestibular mucosa as well as on the skin of the penis, prepuce and perineal region. The infection is transmitted primarily through coitus (Allen and Umphenour 2004).

Careful observations and frequent serological monitoring of a closed, pony-breeding herd strongly suggested the existence of latently infected animals from which EHV-3 was reactivated periodically and transmitted to cohorts (Burrows and Goodridge 1984). Latency of EHV-3 has not been demonstrated in field cases and the anatomical site that harbours latent infection is unknown (Allen and Umphenour 2004; Seki *et al*. 2004)

The investigation reported here was designed to examine reactivation of EHV-3 by corticosteroid treatment in previously naturally infected horses.

Materials and methods

Animals

Two polo mares (*Mares 55* and *110*), which had been involved in a natural outbreak of ECE in an embryo transfer facility 5 months previously and from whom EHV-3 (strain E/9283/07) had been isolated, were moved to experimental facilities and kept in isolation from other equids.

Experimental procedure

On June 20th 2007, both mares were injected with dexamethasone (Dex a vet)[1] 1 mg/kg bwt i.v. and prednisolone (Depo-Medrol)[2] 2 mg/kg bwt, i.m. on 3 successive days.

Nasal (NS), perineal/vaginal swabs (PVS), unclotted blood (for leucocyte separation and haematology) and blood for serum were collected daily as described by Edington and Bridges (1985) in an equine herpesvirus-1 (EHV-1) reactivation study.

Body temperature, general clinical signs and careful observation of the genital and perineal skin and mucosa were performed daily.

The experiment was conducted following international regulations regarding animal welfare.

Virus isolation

Monolayers of equine dermis (EDerm, ATCC No. CCL-57) and rabbit kidney (RK13, ATCC No. CCL-37) cells were used for virus recovery from swabs and leucocytes.

PCR analysis and DNA sequencing

DNA was extracted using the QIAamp DNA mini kit[3] according to the manufacturer's instructions. A primer pair capable of amplifying the partial-length glycoprotein G (gG) gene of EHV-3 was used as described by Dynon *et al*. (2001).

The unpurified PCR products were submitted for sequencing purposes to Macrogen Inc., Korea (http://www.macrogen.com). The obtained nucleotide sequence data were assembled and aligned using the BioEdit Sequence Alignment Editor V7.0.5 program (Hall 1999).

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The sequences of gG gene of the EHV-3 strain isolate from *Mare 110* after reactivation experiment and that of strain E/9283/07 isolated from the same mare 5 months before were compared with the sequence of the 334/74 strain (Hartley *et al*. 1999) taken from GenBank (Accession No. AF081188).

Measurement of virus excretion by quantitative real time PCR

A quantitative real time PCR assay, similar to that developed by Hussey *et al*. (2006) to quantified EHV-1, was adapted to quantify EHV-3. The target sequence for real time PCR was a region of the EHV-3 gG gene as shown by Seki *et al*. (2004). Quantification of EHV-3 in PVS was calculated by generating a standard curve with log dilutions of the reference strain containing $10^{6.50}$ $TCID_{50}$/ml.

Seroneutralisation test

Serial 2-fold dilutions of sera were mixed with an equal amount of the EHV-3 reference strain containing 200 $TCID_{50}$/0.1 ml. After 60 min at 37ºC, an EDerm cell suspension was added and incubated at 37ºC in a CO_2 incubator during 3 days. Seroneutralising titres were expressed as the log_{10} of the reciprocal of the highest dilution that completely inhibited the EHV-3 cytopathic effect.

Results

Mares showed neither hyperthermia nor systemic clinical signs and the haematological pattern remained normal throughout all the observation period. A small and rounded area of erosion, up to 1 cm diameter, was observed on the left labia of the vulva in *Mare 110* on Day 19 after corticosteroid treatment and 5 days after the virus shedding was detected. The lesion was self-limiting and there was complete healing within 3 days. A cytopathic agent was isolated in EDerm cells, from PVS of *Mare 110*, on Day 14 after corticosteroid treatment and during the next 10 days. PCR reaction amplified the expected sequence of EHV-3 gG gene, with the same size as the reference strain, from Days 14–24 after corticosteroid treatment in *Mare 110*. The virus shedding quantified by real time PCR is shown in Figure 1.

The nucleotide homology of the amplified sequence of gG gene, was 100% between both isolates, E/9283/07 and *Mare 110*

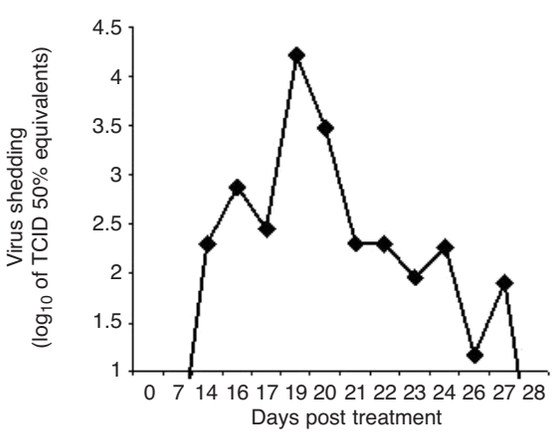

Fig 1: Estimated quantity of EHV-3 detected in perineal/vaginal swabs of Mare 110 *by real time PCR. The viral titres were calculated by generating a standard curve (correlation coefficient 0.997), with a previously titrated EHV-3 reference strain.*

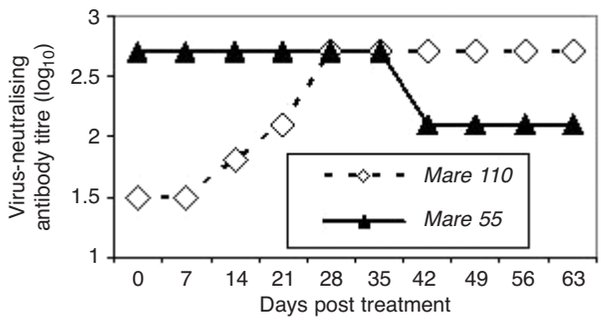

Fig 2: Neutralising antibody titres in Mares 110 *and* 55.

PVS, and 99.6% when compared with the reference strain AF081188, corresponding to 2 mutations: guanine for thymine in position 1265 and a cytosine for thymine in position 1301.

A significant (4-fold) increase in the antibody titre, from 1.5 (log_{10} reciprocal of 1:32 serum dilution) to 2.7 (log_{10} reciprocal of 1:512 serum dilution), was found in *Mare 110* at 28 days after corticosteroid treatment and 14 days after the beginning of virus re-excretion. In *Mare 55* the antibody titre fell slightly from 2.7 (log_{10} reciprocal of 1:512 serum dilution) to 2.1 (log_{10} reciprocal of 1:256 serum dilution) 42 days after corticosteroid treatment and persisted until the end of the observation period (Figure 2).

Discussion

In concordance with epidemiological observations, serological studies and in common with other members of the subfamily *Alphaherpesvirinae* (Slater *et al*. 1994; Edington *et al*. 1994) our results indicate that a state of latency is established after natural infection of EHV-3, inasmuch as the administration of corticosteroids in 2 animals resulted in the recovery of reactivated virus associated to 4-fold increase in neutralising antibodies in one of them.

These results were expected. The capacity of herpesvirus to establish latency and the reactivation of the virus by using corticosteroids is well known for other equine herpesviruses (Burrows and Goodridge 1984; Edington and Bridges 1985; Edington *et al*. 1994; Slater *et al*. 1994) but it is the first time this has been demonstrated for EHV-3.

When the nucleotide sequences of gG gene, from the virus isolated during natural outbreak of ECE (E/9283/07), was compared with that of the reactivated EHV-3 (110 PVS), the result showed that both viruses are identical, at least of the gene studied. In addition, only 2 base substitutions were observed in both viruses when compared with 334/74 (GenBank AF081188) reference strain gG sequence. These few nucleotide substitutions are consistent with the high degree of conservation of other equine herpesvirus genomes (Allen *et al*. 2004).

When the reactivation experiment started, the antibody titre was lower in *Mare 110*, where EHV-3 reactivation was observed and higher in *Mare 55* where no EHV-3 reactivation was detected. Even so, EHV-3 may have established a latent infection in *Mare 55* and reactivation might have occurred but in presence of a high level of a specific immune response, the reactivated virus could have been quickly eliminated and re-excretion not detected. This suggests that a high level of specific immune response, as identified by high antibody titres could diminish the amount of virus shedding and consequently the dissemination of the disease.

The results obtained in the present study confirm that EHV-3 establishes latency. The anatomical site of EHV-3 latency remains to be investigated. In concordance with previous observations (Seki *et al*. 2004), this work also emphasises the fact that reactivation of latency is not always accompanied by clinical signs; neither viraemia, nor EHV-3 nasal shedding was registered. However, mares in the clinical condition of *Mare 110* could be the origin of an outbreak of ECE during the breeding season. Reactivation may also explain the quite common experience in Thoroughbred stallions of clinical signs that appear during the breeding season without evidence of a particular mare being the origin of the infection.

These current results present experimental evidence that latent infection does occur with EHV-3 similarly with other equine herpesviruses, and that the experimental reactivation of virus can be induced by the administration of corticosteroids in the presence of low levels of circulating antibody.

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Manufacturers' addresses

[1]VETEC, Buenos Aires, Argentina.
[2]Pharmacia & Upjohn, Kalamazoo, Michigan, USA.
[3]Quiagen, Hilden, Germany.
[4]Applied Biosystems, Foster City, California, USA.

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4.3 Subclinical infection and periodic shedding of equid herpesvirus 3

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Summary

The aim of the present study was to estimate the prevalence of excretion of EHV-3 in mares without clinical symptoms under field conditions and the re-excretion patterns of the virus in two seropositive (presumably latently infected) mares maintained in isolation for 11 mo. The EHV-3 virus was detected in perineal-vaginal swabs by real time PCR in 14 (6%) of 220 thoroughbred mares without clinical symptoms at the time of breeding. In the two isolated mares, re-excretion of EHV-3 was demonstrated on two occasions, 3 mo apart (each for a 3-d interval) in one mare, and on only 1 d in the other mare. Antibodies against EHV-3 were identified by seroneutralization in 105 (48%) of the thoroughbred mares, and during the entire period in the two isolated mares. Therefore, the present study provided evidence of EHV-3 shedders in a healthy mare population under both field and isolation conditions. Furthermore, at least two periods of spontaneous EHV-3 reactivation and re-excretion in the presence of serum antibodies occurred in one mare in an 11-mo interval. These findings could assist in the design and implementation of measures to minimize the spread of EHV-3 and control ECE outbreaks.

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Subclinical infection and periodic shedding of equid herpesvirus 3

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Abstract

The temporary disruption of reproductive activities due to equine coital exanthema (ECE), caused by equid herpesvirus 3 (EHV-3), at thoroughbred breeding facilities and embryo transfer centres, has an appreciable economic impact. The aim of the present study was to estimate the prevalence of excretion of EHV-3 in mares without clinical symptoms under field conditions and the re-excretion patterns of the virus in two seropositive (presumably latently infected) mares maintained in isolation for 11 mo. The EHV-3 virus was detected in perineal-vaginal swabs by real time PCR in 14 (6%) of 220 thoroughbred mares without clinical symptoms at the time of breeding. In the two isolated mares, re-excretion of EHV-3 was demonstrated on two occasions, 3 mo apart (each for a 3 d interval) in one mare, and on only 1 d in the other mare. Antibodies against EHV-3 were identified by seroneutralization in 105 (48%) of the thoroughbred mares, and during the entire period in the two isolated mares. Therefore, the present study provided evidence of EHV-3 shedders in a healthy mare population under both field and isolation conditions. Furthermore, at least two periods of spontaneous EHV-3 reactivation and re-excretion in the presence of serum antibodies occurred in one mare in an 11 mo interval. These findings could assist in the design and implementation of measures to minimize the spread of EHV-3 and control ECE outbreaks.

Keywords: Equid herpesvirus 3; Equine coital exanthema; Latent infection; Viral excretion

1. Introduction

Equine coital exanthema (ECE), caused by equid herpesvirus 3 (EHV-3), is an acute, infectious, venereal disease resulting in the formation of papules, vesicles, pustules, and ulcers on the penis and prepuce of stallions, and on the vaginal and vestibular mucosa and perineum of mares. The virus is highly contagious, but causes only a local infection and clinical signs are relatively benign. The primary negative impact of ECE on equine breeding enterprises is the forced, temporary disruption of mating activities. In intensively managed stud operations with heavily-scheduled breeding dates for stallions, such disruptions may translate into notable end-of-season decreases in the number of mares bred by affected stallions [1–3]. The infection has an additional negative impact at artificial insemination and embryo transfer facilities, due to the extra time and precautions required to manage both donor and recipient mares and stallions in the face of an outbreak of ECE [4].

Virus reactivation from latently infected mares was recently reported, confirming speculation regarding the

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existence of clinically normal infected carrier animals [1,5]. It has been postulated that infected horses without clinical symptoms could transmit the virus to their breeding partners [1–3]. Epidemiological data suggest that either an infected visiting mare brought onto the stud farm for breeding, or a virus reactivated from a member of the resident stallion or mare population, may be the viral source of ECE outbreaks [1,6].

The basis for controlling the impact of outbreaks of ECE in breeding establishments is to prevent the spread of infection by immediate cessation of mating activities of clinically affected stallions and mares, heightened vigilance for early recognition of new clinical cases, and strict adherence to breeding shed hygiene procedures designed to eliminate mechanical transmission of the virus [1].

The aim of the present study was to estimate the prevalence of excretion of EHV-3 in mares without clinical symptoms under field conditions and to characterize re-excretion patterns of the virus in seropositive (presumably latently infected) mares maintained in isolation. It was expected that these data would contribute to assessing the risk of EHV-3 infection during mating in the absence of detectable ECE lesions.

2. Materials and methods

2.1. Animals and clinical examination

2.1.1. Field study

This study was carried out in a horse facility, La Mission, San Andres de Giles, Buenos Aires province, Argentina, where eight shuttle stallions from the USA, Ireland, and France were brought for the 2007 breeding season. A total of 1080 mares from 69 breeding farms visited the facility to be bred (natural mating) by shuttle stallions.

All mares booked at the stallion station were clinically evaluated before mating; those with systemic or local clinical signs or lesions suspicious of infectious diseases were segregated and rejected for breeding.

2.1.2. Isolation study

Two EHV-3 seropositive polo mares (Mares A and B), which had been included in a previously described EHV-3 reactivation study [5], were used to investigate the spontaneous reactivation and re-excretion of the virus over the time. To this end, they were kept isolated from other horses from August 2007 to June 2008. Rectal temperature, general conditions, and careful observation of the genital and perineal regions were recorded daily. All mares were kept in accordance with the international standards regarding animal welfare [7], and the protocol was approved by the institutional (INTA) animal care and use committee.

2.2. Clinical samples

Samples for virus detection and titration consisted of swabs from the perineal and vaginal regions (PVS) in viral transport media (Minimum Essential Medium, GIBCO BRL, Grand Island, NY, USA), supplemented with 5% fetal calf serum and 8% penicillin-streptomycin [8]. Serum samples were also collected for antibody detection and quantification. In the field study, PVS and serum samples were obtained from 220 mares immediately before preparation (i.e., washing genitalia and wrapping the tail) for breeding. The sampling time frame was from August to November 2007. In the two isolated mares, PVS were collected daily and serum samples once a week, from August 2007 to June 2008. The PVS and serum samples were stored at −80 and −20 °C respectively, pending laboratory studies.

2.3. Virus detection and measurement of virus excretion by quantitative real time PCR

To detect and quantify EHV-3 in both the field and isolation studies, a quantitative real time PCR assay targeting the gG gene was conducted, as previously described [5], on DNA extracted from PVS.

2.4. Seroneutralization test

Antibodies against EHV-3 in blood serum samples were detected by the seroneutralization test, as previously described [5].

3. Results

3.1. Field study

Shedding of EHV-3 was demonstrated in 14 (6%) of the 220 mares without clinical symptoms, by real time PCR, whereas EHV-3 specific antibodies were detected by a seroneutralization test in 105 (48%). In six of the 14 mares, EHV-3 shedding was detected in the absence of detectable antibodies, whereas in the remaining eight, the virus was present in PVS simultaneously with serum antibodies. In 97 mares, no virus shedding but antibodies were detected, and in 109, no antibodies and no virus were found. The amount of excreted virus, as quantified by real time PCR, ranged between 1.2 and 4.6 $\log_{10}$ tissue culture infectious dose 50% ($TCID_{50}$) equivalents, whereas the antibody titer ranged from negative to 2.4 ($\log_{10}$ reciprocal of 1:256 serum dilu-

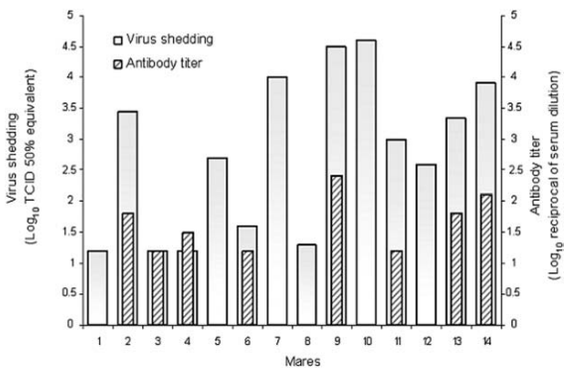

Fig. 1. Level of EHV-3 shedding detected using real time PCR and serological status of the 14 infected mares (field study). Note that numbers on the horizontal axis represent individual mares.

tion) (Fig. 1). In mares negative for EHV-3 shedding, the antibody titer ranged from negative to three ($\log_{10}$ reciprocal of 1:1024 serum dilution).

3.2. Isolation study

No clinical abnormalities, hyperthermia, or ECE lesions were recorded in the mares throughout the study (11 mo interval). Shedding of EHV-3 was detected by real time PCR in PVS from October 23rd to October 25th 2007, and from February 4th to February 6th 2008 in Mare A, and on March 6th 2008 in Mare B. As quantified by real time PCR, the virus titer shed by Mare A ranged between 3.2 and 4.0 $\log_{10}$ $TCID_{50}$ equivalents in the first reactivation and re-excretion period, and from 4.2 to 3.6 $\log_{10}$ $TCID_{50}$ equivalents in the second. The virus titer shed by Mare B on the only day of reactivation and re-excretion detected was 2.6 $\log_{10}$ $TCID_{50}$ equivalents. In both mares, antibody titers remained relatively consistent, between 2.4 ($\log_{10}$ reciprocal of 1:256 serum dilution) and 2.7 ($\log_{10}$ reciprocal of 1:512 serum dilution) throughout the observation period (data not shown).

4. Discussion

This study, conducted under field conditions, provided clear evidence for the existence of EHV-3 shedders in mares without clinical symptoms and revealed that almost 50% of the adult, breeding-age mares were seropositive and presumably latently infected. Despite the small number of animals included (only two), the study in mares maintained in isolation demonstrated that at least two periods of EHV-3 spontaneous reactivation and re-excretion were possible in the same animal in an 11 mo interval.

Although the existence of EHV-3 subclinically infected horses has been suspected from epidemiological and serological data [1,6,9–15], to our knowledge, this is the first study that clearly demonstrated reactivation of infection and shedding of virus by clinically healthy mares without corticosteroid pre-treatment. Latency and reactivation are critical features of the epidemiology of herpesvirus infections [1,16,17]. After Equid Herpesvirus 1 primary infection, latency is established both in the lymphoreticular system and in the trigeminal ganglion [1,17]. Based on indirect field evidences and the biology of other members of the herpesviridae family, the latency stage in EHV-3 infections was inferred [1,16,17] and recently demonstrated by reactivation and re-excretion of the virus from one seropositive mare after corticosteroid treatment [5]. Based on this information, and assessment of the virological and serological data for each mare included in our field study, we inferred that 6 mares were acutely infected (positive for virus but negative for antibodies), 8 were in the reactivation and re-excretion stage (positive for both virus and antibodies), 97 were in the latency stage (negative for virus but seropositive), and 109 were naïve mares (negative for virus and antibodies) fully susceptible to infection (Table 1).

Bearing in mind that in breeding stations and embryo transfer centres there are intensively managed reproductive operations, heavily-scheduled breeding dates for stallions, and widespread movement of donor and recipient mares, the potential risk for venereal and iatrogenic transmission of EHV-3 with a consequent outbreak of ECE is of concern. Despite the presence of mares shedding EHV-3 and the other conditions favourable for EHV-3 transmission described above, no ECE signs or lesions were detected in any of the eight shuttle stallions used for the breeding services at La Mission during the 2007 season. It has been reported that once re-excretion of EHV-3 infection has occurred

Table 1
Apparent stage of infection with EHV-3, based on virological and serological status (field study).

Infection stage	EHV-3 shedding*	EHV-3 antibodies†	No. mares
Re-excretion after reactivation from latency	+	+	8
Acute	+	−	6
Latency	−	+	97
Naïve (uninfected)	−	−	109

* Presence or absence of shedding was based on real time PCR of swabs collected from the perineal and vaginal regions.
† Based on seroneutralization test for EHV-3 antibodies.

with clinical signs, ECE poscoital infection rates were up to 100% [1,18]. However, the rate of transmission from infected mares without clinical symptoms to stallions and the minimum infectious dose has not been established. We can assume that factors like exhaustive hygiene procedures, washing the mares' genitalia with plain water and soap immediately before the breeding service, a "low" virus load (4.6 $\log_{10}$ $TCID_{50}$ equivalents was the highest virus titer detected) in mares without clinical signs, and thorough rinsing of the stallion penis and prepuce with warm water after each mating may have reduced the risk of acquiring an EHV-3 infection, thus preventing the clinical manifestation of ECE in those stallions.

In mares maintained in isolation, only short periods of virus reactivation and re-excretion (3 d for Mare A and 1 d for Mare B) were detected in comparison with the 10 d virus shedding periods recorded in a previously reported corticosteroid reactivation study [5], as well as during the experimental reproduction of the disease [19]. The virus load was also lower (4.2 $\log_{10}$ $TCID_{50}$ as maximum for Mare A and 2.6 for Mare B) than those detected in PVS obtained from mares suffering clinical ECE (4.4–5.6 $\log_{10}$ $TCID_{50}$ equivalents), as well as during the experimental reproduction of the disease (6.0 $\log_{10}$ $TCID_{50}$ equivalents) [19].

The EHV-3 reactivation and re-excretion in the mares maintained in isolation were not related to any detectable increase/decrease in neutralizing antibodies. Furthermore, in the field study, the antibody level and the EHV-3 shedding titer did not show a direct relationship, i.e., the highest virus shedding titers were found in both seropositive and seronegative mares. Since the opposite situation, i.e., a low virus shedding titer in seropositive and seronegative mares, was also found, the serological status does not provide any predictive value.

Spontaneous reactivation of EHV-3, like that of other herpes viruses, has been associated with various stress factors and local or systemic stimuli (e.g., physical or emotional stress, hyperthermia, hormonal imbalance) [1,16]. In the mares under field conditions, multiple situations, such as the transportation, the disruption of the group, the frequent gynecological examinations, exposure to light to hasten cyclicity, etc., are potential causes of stress. Nevertheless, we were unable to identify any evident stress factor associated with reactivation and re-excretion in the mares included in the isolation study.

In conclusion, in the horse population investigated, almost half of the adult horses were EHV-3 seropositive, presumably latently infected. Based on our data, these animals are likely to express at least one reactivation-re-excretion phase every 11 mo. Viral excretion without clinical signs can therefore occur and contribute to the venereal and mechanical transmission of EHV-3. These findings should be considered when implementing measures to minimize the spread of EHV-3 during mating, and in designing control programmes for equine coital exanthema.

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Chapter 5

General discussion

Virological aspects and pathogenesis
of natural and experimental equid herpesvirus 3
infection in horses

Aspects virologiques et pathogénie
de l'infection naturelle et expérimentale
du cheval par l'herpèsvirus équin 3

Equine coital exanthema (ECE) caused by EHV-3 affects mares and stallions worldwide (Barrandeguy et al., 2010; Tibary and Fite, 2007; Van der Meulen et al., 2006; Allen and Umphenour, 2004; Kleiboeker and Chapman, 2004; Seki et al. 2004). EHV-3 infections and ECE outbreaks disrupt breeding activities, cause economic losses to mare and stallion owners, compromise welfare and are costly to deal with. The analysis and description of natural infections, their consequences for the equine industry and the study of the viral strains involved in such outbreaks of ECE constitute the first topic in this discussion. Secondly, aspects related with the pathogenesis of EHV-3, mainly the subclinical and latent infection seen under natural and experimental conditions, will be discussed.

Natural infections of horses with equid herpesvirus 3

ECE is a venereal disease and most of the cases originate from such way of transmission. However , other minor routes of transmission have been identified, especially the respiratory one, by dissemination among horses, and also in another case most likely through medical equipment.

The clinical signs and lesions, except for the lymphadenopathy, found in affected mares in an embryo transfer facility were consistent with ECE and confirmed by virological and serological tests. ECE is a venereal disease and natural breeding does not take place at embryo transfer facilities. Thus, since no contact between stallions and mares occurred, it could be hypothesized that the viral spread among mares in both donor and recipient areas was a consequence of contamination by means of the gloves or ultrasonography scanner used. As in other reported field outbreaks (Van der Meulen et al., 2006; Allen and Umphenour, 2004; Kleiboeker and Chapman, 2004; Seki et al., 2004; Cochard et al., 2002) the index case could not be identified but it seems that a subclinically infected mare, without visible noticeable lesions, was the primary source of the EHV-3 infection. Likewise, how the infection moved from the donor to the recipient area or vice versa remains unknown. In agreement with previous reports (Barrandeguy et al., 2010; Allen and Umphenour , 2004; Couto and Hughes, 1993; Blanchard et al. 1992), a possible explanation is that iatrogenic transmission of infection by virus-contaminated objects took place.

It is well known that infectious diseases can be disseminated between animals, premises or even countries by fresh or cryopreserved semen and embryos (T imoney, 2007; Samper and Tibary, 2006; Metcalf, 2001). Although the embryo was the only "common piece" between the donor and recipient mares at this embryo transfer facility, the transmission of EHV-3 via infected embryos was not likely to happen during this outbreak.

Another interesting and unreported finding during this outbreak was lymphadenopathy.

Lymphadenopathy, a consistent feature of this ECE case but not previously reported associated with ECE, seems to be an additional negative consequence of EHV-3 infection.

The lymphadenopathy observed could be a lesion specifically associated with the EHV-3 infection in the embryo transfer practice due to the particular reproductive procedures. Several factors such as EHV-3 and ECE lesions, a secondary bacterial infection, repeated transrectal palpation in donor and recipient mares, mechanical (transrectal palpation) and chemical irritation (lubricants) of the area could contribute to this. At embryo transfer facilities, lymphadenopathy had the most relevant negative clinical consequence of ECE outbreaks because it caused constipation, pain and reluctance of the mares to be inspected. Lymphadenopathy provides a new concern associated with ECE. Whether the enlargement of the anorectal lymph nodes is due to the EHV-3 infection or to a secondary bacterial infection needs to be elucidated.

Interestingly, as previously mentioned, the index case could not be identified, but considering that EHV-3 induces latent infections (Barrandeguy et al., 2008; Allen and Umphenour , 2004), spontaneous reactivation and re-excretion related to stress could have occurred in one of the mares and the infection could have then been iatrogenically transmitted from it to the cohorts. It is another example that latency is a key strategy of herpesviruses to be perpetuated in nature (Pellets and Roizman, 2007; Roizman et al., 2007).

The outbreak of rhinitis caused by EHV-3 described constitutes another example of non-venereal

transmission of EHV-3 and also highlights the importance of biosecurity in veterinary practice. The development of lesions in the mouth and nostrils as a consequence of EHV-3 infection (Crandell and Davis, 1985; Gibbs et al., 1970) as well as the EHV -3 transmission by contaminated objects have been previously reported (Allen and Umphenour, 2004). Nevertheless, the occurrence of several cases of unilateral rhinitis in horses in training evidences undescribed consequences of EHV-3 infections and also illustrates how easily infectious diseases can be disseminated. Again, in this case, although the origin of infection could not be identified, a spontaneous and subclinical reactivation of EHV -3 from a naturally infected horse, which led to excretion of the virus in nasal secretions, may have been the initial source of contamination of the endoscope.

A characterization study was carried out in order to find tools to follow the virus circulation in horse populations. Argentinean EHV-3 field isolates were distinguished from other strains obtained in geographically distant regions (Costa et al., 2009, Hartley et al., 1999) by means of RE patterns, plaque size and gG gene partial sequencing. Although differences were found between them, neither the RE pattern nor the "genotype" in the gG sequence could be associated with any particular clinical manifestation of EHV-3 infections. Nevertheless; RE assays and gG gene sequencing were able to detect differences between field isolates allowing its use as a tool for molecular epidemiology. In addition, with the current development of molecular biology technology, the gG gene sequencing seems to be the best approach for molecular epidemiological studies. Further research, together with the determination of the full EHV-3 genome sequence, will provide an alternative genome region to be studied in order to add knowledge on EHV-3 diversity.

Pathogenesis of Equid herpesvirus 3 infections

A topical experimental infection with EHV-3 was able to reproduce the ECE clinical signs closely similar to those previously described during field outbreaks (Barrandeguy et al., 2010a; Tibary and Fite, 2007; van der Meulen et al., 2006; Allen and Umphenour, 2004; Kleiboeker and Chapman, 2004; Seki et al. 2004). The data obtained from those experiments contribute to the knowledge of the infection and disease since the virus shedding patterns and the antibody kinetics had not been described previously . A remarkable aspect of the EHV-3 infection evidenced in the present experiments is that mares showing only mild signs of disease, which could be unnoticed in field conditions, shed virus for 8 to 10 days. Those subclinically infected mares could play a crucial role in the initiation of ECE outbreaks. As previously described (Barrandeguy and Thiry, 2010; Allen and Umphenour, 2004; Blanchard et al., 1992), a specific immune response is acquired after infection. This immune response can account for the mild clinical signs observed in seropositive mares after experimental infection. Nevertheless, such immunity is not enough to prevent the infection and virus shedding 5 months after the primary infection. Furthermore,recurrent clinically evident ECE was observed previously mainly in aged mares and stallions (Allen and Umphenour, 2004; Blanchard et al., 1992).

The experimental protocol outlined in this thesis consistently reproduced the lesions observed in natural outbreaks of ECE. It can thus be used to monitor the virus shedding patterns and neutralising antibody kinetics and could be very useful for future EHV-3 research including antiviral therapy and other preventive or therapeutic measures.

In another experiment the state of EHV-3 latency was demonstrated for the first time; the virus was isolated from perineal/vaginal swabs obtained 15 days after the treatment of one mare with high doses of corticosteroids. This mare had suffered ECE five months before during an outbreak of disease in an embryo transfer centre (unpublished data). These results were not unexpected. The capacity of herpesvirus to establish latency and the reactivation of the virus by using corticosteroids is well known for other equine herpesviruses (Burrows and Goodridge 1984; Edington and Bridges 1985; Edington et al. 1994; Slater et al. 1994). The molecular characterization of these isolates showed that both were identical.

Although the EHV-3 latency stage was unequivocally demonstrated, which is the anatomical site of

latency and how EHV-3 reaches the site of latency are points to be elucidated. EHV-3 replication is limited to the stratified epithelium of epidermal tissue present within the skin or at mucocutaneous mar gins and systemic dissemination of EHV-3 has never been reported (Allen and Umphenour, 2004). As pointed out in the experimental reproduction of the disease previously discussed and in concordance with previous observations (Seki et al. 2004), this work also emphasizes the fact that reactivation from latency is not always accompanied by clinical signs. Mares in the clinical condition of this could be the origin of an outbreak of ECE during the breeding season.

Therefore, latency and reactivation are critical features of the epidemiology of herpesvirus infections (Allen and Umphenour., 2004; Roizman et al., 2007; Lunn et al., 2009). Subclinical infected animals have been suspected to be the primary source of virusin most of the outbreaks of ECE described (Barrandeguy et al., 2010; Tibary and Fite, 2007; Van der Meulen et al., 2006; Allen and Umphenour, 2004; Kleiboeker and Chapman, 2004; Seki et al. 2004). The study conducted under field conditions provided clear evidence of the existence of EHV-3 shedders in mares without clinical signs and revealed that almost 50% of the adult breeding-age mares were seropositive and presumably latently infected. At the same time, spontaneous reactivation with re-excretion was demonstrated to occur at least twice a year in two seropositive mares kept in isolation.

Bearing in mind that in stud farms, breeding stations and embryo transfer centres there are intensively managed reproductive operations, heavily-scheduled breeding dates for stallions and a widespread movement of donor and recipient mares, and that some of those animals are shedding virus without showing any clinical signs, the potential risk for venereal and iatrogenic transmission of EHV -3 with a consequent outbreak of ECE is of concern. It has been reported that once re-excretion of EHV3 infection has occurred with clinical signs, ECE postcoital infection rates are up to 100% (Allen and Umphenour , 2004; Evermann et al., 1983). However, the rate of transmission from infected mares without clinical symptoms to stallions and the minimum infectious dose has not been established.

Spontaneous reactivation of EHV-3, like that of other herpesviruses, has been associated with various stress factors and local or systemic stimuli (e.g., physical or emotional stress, hyperthermia, hormonal imbalance) (Allen and Umphenour, 2004; Roizman et al. 2007). In stud farms, breeding stations and reproductive centres, multiple situations, such as the transportation, the disruption of the group, the frequent gynecological examinations and the exposure to light to hasten cyclicity, are causes of stress, which can lead to EHV-3 reactivation.

During this work, significant progress that could be useful for a better understanding of the pathogenesis and for the evaluation of the economic impact of EHV-3 infections was made.

Preventing transmission of the virus from subclinically infected animals remains a challenging area of research. Efforts should be focused on prevention of virus shedding using topical vaccines, local "neutralisation" of the virus by antibody enrichment ointments or local treatment with effective antiviral drugs. Another strategy worth to further study is the detection of EHV-3 shedding mares to segregate them from the breeding service; thus, the development of field tests for rapid screening, or pen-side tests such as lateral flow, should be a priority. Latency, reactivation and re-excretion and virus shedding by clinically healthy mares are critical features of EHV-3 epidemiology, prevention and control.

Chapter 6

Conclusions and perspectives

Virological aspects and pathogenesis
of natural and experimental equid herpesvirus 3
infection in horses

Aspects virologiques et pathogénie
de l'infection naturelle et expérimentale
du cheval par l'herpèsvirus équin 3

Equid herpesvirus-3 infections and ECE are still a threat for the equine industry. EHV-3 was found to be the causal agent of several field outbreaks of ECE in thoroughbred breeding farms, embryo transfer facilities and even at the training racecourse. The veterinarians reported the disease as a true sanitary problem and are demanding alternative preventive measures. In addition, EHV-3 was detected as a not rare event in clinically healthy mares, which constitutes the most relevant finding from an epidemiological perspective. The population of EHV-3 latently infected mares, which reaches up to 50% at the time of breeding, deserves special attention, and as reactivation of latent virus is not preventable, those mares can spontaneously reactivate the virus and become a source of infection for highly valuable horses like "shuttle stallions", with the consequent economically negative impact on the equine enterprises.

Finally, there is an important need for finding out additional preventive measures including "pen side" diagnostic tools that allow the detection of subclinically EHV-3 shedding mares in order to segregate them from natural breeding and give them an appropriate antiviral treatment before they are covered by the stallions.

Chapter 7

Summary - Résumé

Virological aspects and pathogenesis
of natural and experimental equid herpesvirus 3
infection in horses

Aspects virologiques et pathogénie
de l'infection naturelle et expérimentale
du cheval par l'herpèsvirus équin 3

Equine coital exanthema (ECE), caused by equid herpesvirus 3 (EHV-3), is a contagious venereal disease characterised by the formation of painful papules, vesicles, pustules and ulcers on the external genitalia of both mares and stallions. EHV -3 is an alphaherpesvirus, distinct from the other equine herpesviruses, endemic in most horse breeding populations worldwide. EHV-3 is primarily transmitted through coitus, although there is also evidence supporting the possibility of non-coital spreading through infected fomites and contacts other than coitus. The infection does not usually result in systemic illness. Epidemiological observations and serological monitoring suggest the existence of latently infected animals from which EHV-3 is periodically reactivated and transmitted to cohorts but latency of EHV-3 has not been formerly demonstrated.

The negative impacts of ECE on equine breeding enterprises are the forced, temporary disruption of the mating activities of mares and stallions, the additional care and supportive treatment in affected horses, and the risk of virus spread by either fresh or frozen semen as well as by artificial insemination and embryo transfer practices. In intensively managed stud operations, which have heavily-scheduled breeding dates for thoroughbred stallions, breeding disruptions may translate into significant end-of-season decreases in the number of entries into the mare book of affected stallions. Also, delayed foaling dates and/or reduced pregnancy rates may occur in mares that miss breeding opportunities due to the disease. Similarly, in the face of an ECE outbreak in artificial insemination and embryo transfer centres, both donor and recipient af fected mares show such discomfort that they are reluctant to be inspected, inseminated or transferred, with the consequent loss of opportunity to become pregnant. The additional time and necessary precautions required to manage the donor and receptor mares due to the presence of the disease also have a substantial negative impact.

Because ECE is a not a notifiable disease and the diagnosis is made on the basis of typical clinical signs, most cases and outbreaks of ECE remain unnoticed and its true prevalence and economic impact is difficult to assess and is probably underestimated.

Therefore, as several aspects of EHV-3 infection are largely unknown and it has severe economic consequences to the horse industry , the general aim of this doctoral study was to increase the knowledge about the biology of EHV -3 infection. The specific objectives were to investigate the iatrogenic transmission of infection and to set up a protocol for experimental reproduction of the disease, to study the reactivation and re-excretion patterns from latency, to evaluate the epidemiological importance of subclinical infections, and to hypothesise about the ECE economic consequences in the current context of the equine industry.

During the occurrence of an outbreak of ECE in an embryo transfer centre, approximately 32% (n=35) of the donor mares and 25% (n=125) of the recipient mares showed typical ECE lesions around the anus and on the perineal skin, discomfort, and anorectal lymphadenopathy. EHV-3 was detected in 7 (58%) of the affected mares and specific antibodies in 23 (88%) of the convalescent mares. Since no natural breeding had taken place on the affected mares, it could be hypothesised that the virus spread was a consequence of contamination by means of the gloves or the ultrasonography scanner used. Lymphadenopathy provides a new concern associated with ECE.

EHV-3 was isolated from nasal swabs obtained during an outbreak of unilateral rhinitis af fecting approximately 40 out of 2000 thoroughbred horses. The fact that an endoscopic examination had been performed in the week previous to the onset of the lesions to evaluate the respiratory tract function was a common finding in all the horses affected. EHV-3 was demonstrated as the etiologic agent of the unilateral rhinitis observed in those 40 thoroughbred horses. The endoscope used for respiratory tract examination was identified as the most likely cause of the spread of the infection. This case is an example of non-genital iatrogenic transmission and reinforces the importance of strict application of hygienic measures in order to reduce the risk of spread of infectious diseases.

The virus isolated from this field outbreak as well as that isolated from others were characterized by means of restriction endonuclease (RE) fragment patterns, plaque size and gG gene partial nucleotide sequencing. In the 25 isolates included in the study , different RE patterns were found: two with *Bam*HI (one of them identical to the one of the reference strain), two with *Hind* III (both different from the one of the reference strain) and one with Eco RI (dif ferent from the one of the reference strain). The plaque size was homogeneous between the isolates, and 1.64 and 2.88 times larger than that of the reference strain. Three base substitutions in the gG gene were found at positions 904, 1103 and 1264, which resulted in strains CAT (Australia), AAT (the United States and Brazil), CAG (Ar gentina) and ACT (Argentina). The RE pattern and the nucleotide sequence of the gG gene obtained revealed that there are genetically distinguishable EHV-3 strains in circulation. Not only the RE patterns, as previously described, but also the nucleotide sequence of the gG gene, could be useful tools for epidemiological studies. The biological implications of these changes are still unknown.

Two sets of experimental infection with EHV-3 were carried out under controlled conditions. In the first experiment, two seronegative mares were topically inoculated in the vagina and perineal area with EHV-3. The same protocol was followed in the second experiment in two seronegative and two seropositive mares (the mares which had been included in the first experiment were used six months later). Clinical samples consisted of swabs from the vagina and perineal area, and blood samples were obtained for virological, serological and haematological studies. A scoring system was designed and used for daily clinical evaluation and rectal temperature records from each mare. Neither hyperthermia nor haematological changes were recorded in the mares analyzed. Typical ECE lesions were observed in seronegative animals: the clinical score was 172 and 90 (average score: 131) for the mares included in the first experiment and 160 and 92 (average score: 124) for the mares included in the second experiment. Only slight lesions were observed in the seropositive mares, being the clinical score 53 and 41 (average score: 47). Also, differences were detected in the duration and intensity of virus shedding, being 15 and 9 days (duration) and 105 versus 104 (the highest virus load detected) in the seropositive and seronegative mares, respectively.

In one study designed to demonstrate EHV-3 latency and to study reactivation and re-excretion patterns, virus shedding, seroconversion and the presence of a small ECE lesion were observed in one out of two previously naturally infected mares after corticosteroid treatment. EHV-3 was isolated from perineal vaginal swabs of one of the mares, both on day 14 after corticosteroid treatment and along the following 10 days. A small and rounded area of erosion was observed on the left labia of the vulva of the same mare on day 19 after corticosteroid treatment and 5 days after the virus shedding was detected. A significant (four-fold) increase in the antibody titre was found in the mare which shed the virus 28 days after corticosteroid treatment and 14 days after the beginning of virus re-excretion. In concordance with epidemiological observation and serological studies, and in common with other members of the subfamily Alphaherpesvirinae, this study indicates that a state of latency is established after natural infection of EHV-3.

A study was carried out to estimate the prevalence of excretion of EHV-3 under field conditions. The virus was detected in perineal-vaginal swabs by real time PCR and specific antibodies were identified by seroneutralization in 14 (6%) and 105 (48%) respectively of 220 thoroughbred mares without clinical signs at the time of breeding. In order to assess the re-excretion patterns of spontaneous reactivation, two seropositive (presumably latently infected) polo mares were kept in isolation for 1 1 months. Virological investigations on perineal vaginal swabs obtained on a daily basis revealed re-excretion of EHV-3 on two occasions, 3 months apart (each for a 3-day interval) in one of the mares, and on only 1 day in the other mare. Antibodies against EHV-3 were detected with only slight variation during the entire period in both mares. Clear evidence of the existence of EHV -3 shedders in a healthy mare

population under both field and isolation conditions is provided. Furthermore, despite the small number of animals included (only two), the study in mares kept in isolation demonstrated that at least two periods of EHV-3 spontaneous reactivation and re-excretion in the presence of serum antibodies were possible in the same animal in an 11-month interval.

In conclusion, EHV-3 infections and ECE are still a threat for the equine industry In the present study, EHV-3 was found in several field outbreaks of ECE in thoroughbred breeding farms; the disease was reported by the veterinarians as a true sanitary problem and thus demands additional preventive measures. In addition, EHV-3 was detected as a not rare event in clinically healthy mares, which constitutes the most relevant finding from an epidemiological perspective. ECE is also a sanitary problem of concern for embryo transfer and routine veterinary practices.

The population of EHV -3 latently infected mares which reach up to 50% at the time of breeding deserves special attention. Reactivation of the latent virus is not preventable and those mares can spontaneously reactivate the virus and become a source of infection for highly valuable horses like « shuttle stallions », with the consequent economically negative impact on the equine enterprises.

Finally, there is an important need for finding out additional preventive measures including « pen side » diagnostic tools that allow the detection of subclinically EHV-3 shedding mares in order to segregate them from natural breeding and give them an appropriate antiviral treatment before being covered by stallions.

L'exanthème coïtal équin (ECE), causé par l'herpèsvirus équin 3 (EHV-3), est une maladie vénérienne contagieuse caractérisée par la formation de papules douloureuses, de vésicules, de pustules et d'ulcères sur les organes génitaux externes des juments et des étalons. L'EHV-3 est un alphaherpèsvirus, et à la différence des autres herpèsvirus équins, celui-ci est endémique dans les élevages de chevaux. L'EHV-3 est principalement transmis par le coït, mais il y existe aussi l'évidence d'une possible transmission non-coïtale propagée par le biais de contages et d'autres contacts. Généralement, l'infection ne provoque pas de maladie généralisée. Des observations épidémiologiques et la surveillance sérologique ont suggéré l'existence d'animaux infectés de manière latente et à partir desquels l'EHV-3 était réactivé périodiquement et transmis aux animaux de la même cohorte, mais la latence de l'EHV-3 n'a pas été démontrée.

Les impacts négatifs du ECE dans les élevages de chevaux sont provoqués par l'interruption forcée et temporaire des activités de reproduction des juments et des étalons; des soins supplémentaires et le traitement de soutien pour les chevaux affectés, et le risque de propagation du virus par le sperme soit frais ou congelé, ainsi que par l'insémination artificielle et les pratiques de transfert d'embryons. Parmi les activités intensives de l'élevage, avec des jours de reproduction prévus de manière précise pour les étalons pur-sang, l'interruption de la reproduction peut provoquer à la fin de la saison une diminution significative du nombre de juments fécondées par les étalons affectés. Par ailleurs, le poulinage tardif ou la réduction du taux de gestation peut se produire chez les juments qui n'ont pas été saillies à cause de cette maladie. De même, dans le cadre d'une épidémie à ECE en insémination artificielle et dans les centres de transfert d'embryons, les juments affectées, autant les donneuses que les receveuses, éprouvent une telle douleur qu'elles ne veulent pas être examinées, inséminées ou subir le transfert d'embryons, ce qui provoque la perte de la possibilité d'être gestante. Face à la présence de cette maladie, le délai de temps additionnel et les précautions nécessaires pour traiter les juments donneuses et receveuses, ont aussi un impact négatif considérable.

Étant donné que l'ECE n'est pas une maladie à déclaration obligatoire et que le diagnostic est fait sur base de signes cliniques typiques, la majorité des cas et d'épidémies d'ECE sont inaperçus, si bien que la prévalence réelle et l'impact économique sont difficiles à évaluer et sont probablement sous-estimés.

Par conséquent, comme plusieurs aspects de l'infection à EHV-3 sont inconnus et qu'il y a des conséquences économiques graves pour l'industrie du cheval, le but général de cette étude de doctorat est d'enrichir les connaissances sur la biologie de l'infection par l'EHV-3. Les objectifs spécifiques sont l'étude de la transmission iatrogène de l'infection, la mise en place d'un protocole de reproduction expérimentale de cette maladie, l'étude des profils de réactivation de la latence et de reexcrétion, ainsi que d'évaluer l'importance épidémiologique des infections subcliniques, et aussi d'émettre des hypothèses sur les conséquences économiques de l'ECE dans le contexte actuel de l'industrie équine.

Pendant une épidémie d'ECE dans un centre de transfert d'embryons, environ 32 % (n=35) des juments donneuses et 25 % (n=125) des juments receveuses présentaient des lésions d'ECE typiques autour de l'anus et sur la peau du périnée. Elles éprouvaient aussi de la douleur et une lymphadénopathie anorectale. L'EHV-3 a été détecté dans 7 (58 %) des juments affectées et des anticorps spécifiques dans 23 (88 %) des juments convalescentes. Comme on n'avait pas réalisé de monte naturelle sur les juments affectées, on peut supposer que la propagation du virus a été la conséquence d'une contamination transmise par les gants ou de l'utilisation d'un appareil scanner ultrasonographique. La lymphadénopathie est une nouvelle lésion associée à l'ECE.

L'EHV-3 a été isolé à partir des prélèvements nasaux obtenus pendant une épidémie de rhinite unilatérale qui a affecté environ 40 sur 2000 chevaux pur-sang. La constatation qu'un examen endoscopique réalisé une semaine avant l'apparition des lésions pour évaluer la fonction des voies respiratoires fut un élément en commun chez tous les chevaux affectés. L'EHV-3 a été démontré comme l'agent étiologique de la rhinite unilatérale observée parmi les 40 chevaux pur-sang. L'endoscope utilisé pour l'examen des voies respiratoires a été identifié comme la cause la plus probable de la propagation de l'infection. Ce cas est un

exemple d'une transmission iatrogène non génitale et renforce l'importance d'une stricte application des mesures hygiéniques pour réduire le risque de propagation des maladies infectieuses.

Les virus isolés dans l'épidémie précédemment décrite ainsi que dans d'autres cas ont été caractérisés par l'analyse des profils obtenus par digestion par endonucléases de restriction (RE), la taille des plages de lyse et le séquençage nucléotidique partiel du gène codant la glycoprotéine gG. Parmi les 25 isolats inclus dans l'étude, des profils de RE différents ont été observés, deux avec *Bam*HI (l'un d'eux identique à celui de la souche de référence); deux aussi avec *Hind* III (les deux différents de celui de la souche de référence) et un avec Eco RI (différent de celui de la souche de référence). La taille des plages de lyse était homogène entre les isolats, 1.64 et 2.88 fois plus grande de celle de la souche de référenceTrois substitutions nucléotidiques ont été identifiées dans le gène gG au niveau des positions 904, 1 103 et 1264, ce qui permet de définir des souches de type CAT (Australie), de type AAT (États-Unis et Brésil), de type CAG (Ar gentine) et de type ACT (Argentine). Le profil de RE et la séquence nucléotidique du gène gG obtenus ont révélé une circulation des souches d'EHV-3 génétiquement différenciables. Non seulement les profils de RE, précédemment décrits, mais aussi la séquence nucléotidique du gène gG, pourraient constituer des outils pratiques pour des études épidémiologiques. Les implications biologiques de ces changements sont encore inconnues.

Deux études de reproduction expérimentale de l'infection par l'EHV -3 ont été ef fectuées en conditions contrôlées. Dans la première expérience, deux juments séronégatives ont été inoculées avec l'EHV-3, localement au niveau des régions vaginale et périnéale. Le même protocole a été suivi dans la deuxième expérience a été réalisée sur quatre juments, deux séronégatives et deux séropositives (les juments qui avaient participé à la première expérience ont été utilisées six mois plus tard). Les échantillons cliniques consistaient en des prélèvements vaginaux et périnéaux et du sang a été prélevé pour les analyses virologiques, sérologiques et hématologiques. Les scores cliniques et la température rectale ont été enregistrés quotidiennement pour chaque jument.

Les juments n'ont pas montré d'hyperthermie ni de changements hématologiques. Des lésions d'ECE typiques ont été observées chez les juments séronégatives ; les scores cliniques ont été 172 et 90 (score moyen : 131) pour les juments inclues dans la première expérience et 160 et 92 (score moyen : 124) dans la deuxième expérience. Seules des lésions légères ont été observées chez les juments séropositives, avec des scores cliniques de 53 et de 41 (score moyen : 47). Des différences ont été aussi observées dans la durée et l'intensité de l'excrétion virale avec des durées de 15 et 9 jours et des charges virales de 105 et de 104 équivalents CCID50 (en plus haute charge virale détectée) chez les juments séropositives et séronégatives, respectivement. Un protocole de reproduction expérimentale d'ECE a été mis en place et est prêt à être utilisé pour des recherches futures. L'immunité post infection mesurée par les taux d'anticorps neutralisants a réduit l'intensité des signes cliniques. Cependant, elle n'a pas empêché la réinfection et l'excrétion virale.

Dans une étude visant à démontrer la latence de l'EHV -3 et à étudier les profils de réactivation et de réexcrétion de l'EHV-3, une excrétion virale, une séroconversion et la présence d'une petite lésion d'ECE ont été observées après traitement aux corticostéroïdes chez l'une des deux juments qui avaient été antérieurement infectées de manière naturelle. L'EHV-3 a été isolé des prélèvements vaginaux et périnéaux d'une jument, au jour 14 après le traitement aux corticostéroïdes et durant les 10 jours suivants. Une petite lésion érosive arrondie a été observée sur la vulve de la même jument au jour 19, après traitement aux corticostéroïdes et 5 jours après la détection de l'excrétion du virus. Une augmentation significative (quatre-fois) du titre en anticorps a été observée chez le jument qui avait présenté une excrétion virale 28 jours après le traitement avec des corticostéroïdes et 14 jours après le début de la réexcrétion virale. En accord avec les observations épidémiologiques et les études sérologiques et de façon similaire aux autres membres de la sous-famille des Alphaherpesvirinae, cette étude démontre qu'un état de latence est établi après une infection naturelle à EHV-3.

Une étude a été menée pour estimer la prévalence de l'excrétion de l'EHV-3 en conditions de terrain. Le virus a été détecté dans des prélèvements vaginaux et périnéaux en PCR temps réel chez 14 (6 %) des 220 juments pur-sang et des anticorps neutralisants chez 105 (48 %) de ces juments sans signes cliniques au moment de la reproduction. Afin d'évaluer le profil de réexcrétion au cours d'une réactivation spontanée, deux juments de polo séropositives (probablement infectées de manière latente) ont été isolées pendant 11 mois. Des recherches virologiques dans des prélèvements quotidiens vaginaux et périnéaux ont révélé une réexcrétion du EHV-3 à deux occasions, à 3 mois d'intervalle (chacune durant 3 jours) chez une jument, et seulement un jour chez l'autre jument. Des anticorps envers l'EHV-3 ont été détectés avec seules de légères variations durant toute la période chez les deux juments. L'existence d'excréteurs d'EHV-3 a donc été démontrée dans une population de juments saines dans les deux conditions, de terrain et expérimentale. Par ailleurs, malgré le faible nombre d'animaux inclus (seulement deux), l'étude avec les juments maintenues en isolement a démontré qu'au moins deux périodes de réactivation et de réexcrétion spontanée d'EHV -3 étaient possibles chez le même animal dans un intervalle de 11 mois, en présence d'anticorps spécifiques.

En conclusion, les infections à EHV-3 et l'ECE restent une menace pour l'industrie équine. Dans cette étude, l'EHV-3 a été observé dans de nombreuses épidémies d'ECE dans des haras de pur-sang ; la maladie est reconnue par les vétérinaires comme un problème sanitaire réel ; ils sont demandeurs de mesures de prévention additionnelles. En outre, l'EHV-3 a été détecté dans une proportion significative de juments cliniquement saines, ce qui constitue un résultat très important d'un point-de-vue épidémiologique. L'ECE est aussi une préoccupation sanitaire pour le transfert d'embryons et pour la pratique vétérinaire en général.

La population de juments infectées de manière latente par l'EHV-3, dont la fréquence atteint 50 % au moment de la reproduction, mérite une attention particulière et, comme la réactivation du virus latent ne peut pas être prévenue, ces juments peuvent réactiver le virus de manière spontanée et devenir une source d'infection pour des chevaux de haute valeur comme les étalons navettes («shuttle stallions») avec un impact économique négatif pour les entreprises équines.

Finalement, il convient de mettre au point des mesures de prévention additionnelles, telles des moyens diagnostiques utilisables sur site (« pen side test ») qui permettent la détection des juments qui font une excrétion subclinique de l'EHV-3 afin de leur interdire la mise à la reproduction naturelle et de leur donner un traitement antiviral approprié avant de les autoriser à la monte naturelle.

Chapter 8

References

Virological aspects and pathogenesis
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Aspects virologiques et pathogénie
de l'infection naturelle et expérimentale
du cheval par l'herpèsvirus équin 3

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