Soraia Cristina Filipe Horta

Development of an indirect ELISA against Equine Herpesvirus Types 1 and 4

UNIVERSIDADE DO ALGARVE

Dissertation to obtain the Master's Degree in
Molecular and Microbial Biology

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Work carried out under the guidance of:

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Declaration of authorship of the work

I declare to be the author of this work, which is original and unpublished. Consulted authors and works are properly cited in the text and are included in the reference list included

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Abstract

The equine herpesviruses are important pathogens in the animal health context, namely in the equine's health. So far, nine types have been identified, of which herpesvirus type 1 (EHV-1) and type 4 (EHV-4) are clinically, economically, and epidemiologically the most relevant due to the pathologies associated.

The main objective of this work was to develop a peptide-based enzyme-linked immunosorbent assay (ELISA) for discrimination between EHV-1 and EHV-4. Selecting from the literature the immunogenic regions of both viruses, the tests were performed using a portion of glycoprotein E and a portion of glycoprotein G from each virus type.

The results obtained until the conclusion of this work are promising, however, they were not conclusive. Nevertheless, they open the way for future testing, in order to make these ELISA test applicable for the detection of antibodies against EVH-1 and EHV-4.

Keywords: Herpesvirus; EHV-1; EHV-4; Serology; ELISA; Peptide; Equine;

Resumo

Este trabalho experimental teve como principal objetivo o desenvolvimento de um teste ELISA que permitisse detetar e diferenciar o herpesvírus equino tipo 1 (EHV-1) e o herpesvírus equino tipo 4 (EHV-4).

Os herpesvírus equinos (EHVs) são vírus envelopados, com dupla cadeia de DNA que afetam todos os membros da família *Equidae* em todo o mundo (Wegdan *et al*., 2016; Slater, 2007), tendo sido, até ao momento, identificados e caracterizados nove tipos de herpesvírus. Os vírus dos tipos EHV-1, EHV-2, EHV-3, EHV-4 e EHV-5 infetam cavalos domésticos, enquanto os vírus dos tipos EHV-6, EHV-7, EHV-8 e EHV-9 estão associados a equídeos selvagens (Slater, 2007).

Estes vírus possuem ciclos de vida complexos, que envolvem a infeção de múltiplos tipos celulares em diferentes tecidos e, são ainda detentores de diferentes mecanismos de evasão que lhes permite escapar à resposta imunológica do hospedeiro (Slater, 2007). Uma das ferramentas mais poderosas destes vírus é a capacidade de permanecer em latência, estado esse em que os equinos transportam o vírus de forma assintomática durante longos períodos. (Ostlound,1993; Balasuriya *et al*, 2015 Slater, 2007).

Devido às suas características, os EHVs têm tido, a nível mundial e em todos os sectores da indústria equestre, um grande impacto económico e no bem-estar animal. (Slater, 2007; Milic *et al*., 2018).

Contudo, de entre os EHVs, o EHV-1 e EHV-4 são do ponto de vista clínico, económico e epidemiológico os agentes patogénicos mais relevantes (Patel & Heldens, 2005; Slater, 2007).

O EHV-1 é maioritariamente responsável por doenças respiratórias em animais jovens, aborto, morte neonatal e surtos extensivos de doença neurológica ou mielencefalopatia por herpesvírus equino (EHM), enquanto que o EHV-4 causa principalmente doenças respiratórias e raramente leva ao aborto (Ostlund, 1993; Balasuriya *et al*, 2015; Milic *et al*, 2018).

Os vírus EHV-1 e EHV-4 foram em tempos considerados e classificados como dois subtipos do mesmo vírus (Milic *et al*., 2018). No entanto, a utilização da sequenciação genómica permitiu diferenciar estes dois vírus que são, contudo, geneticamente muito semelhantes (Vandekerckhove *et al*., 2011; Azab *et al*., 2012; Wegdan, 2016; Milic *et al*., 2018).

Tendo em conta estas semelhanças entre os vírus, foram selecionadas para este trabalho, com base na literatura, duas regiões altamente imunogénicas que permitissem diferenciá-los sem que ocorresse reação cruzada. As regiões escolhidas foram uma porção do gene que codifica para a glicoproteína G (gG) e uma outra porção do gene que codifica para a glicoproteína E (gE).

As glicoproteínas do herpesvírus estão presentes no envelope do vírus e têm papéis funcionais importantes na patogénese da doença (Crabb&Studdert,1990; Molinková,2012), mediando a entrada do vírus na célula hospedeira através da adsorção e penetração (Crabb & Studdert, 1990; Patel & Heldens, 2005; Slater, 2007).

A maioria das diferenças entre EHV-1 e EHV-4 foram encontradas em 1993 por Crabb & Studdert na extremidade 3' do gene 70 que codifica a glicoproteína G. Comparando as sequências nucleotídicas de EHV-4 gG e EHV-1 gG, estes autores concluíram que as sequências possuíam 58% de identidade. No entanto, as regiões proteicas que incluíam os resíduos de aminoácidos 287 a 382 em EHV4 gG e 288 a 350 em EHV1 gG detinham apenas 21% de identidade. Por este motivo, esta região foi indicada como uma das mais promissoras na distinção de ambos os vírus. (Van Maanen, 2012; Thormann et al., 2012).

Mais tarde, Damiani *et al*. (2000) examinaram mutantes de supressão do heterodímero gI/gE em EHV-4, em condições *in vitro* e *in vivo*, concluindo que os genes intactos destas glicoproteínas, gI e/ou gE, são necessários para uma transmissão eficaz do vírus através da propagação de célula a célula e, consequentemente, existe um papel importante dos genes gI e gE na virulência do EHV-4. Com base nestas descobertas, Andoh *et al*. (2012) identificaram e descreveram epítopos imunogénicos de EHV-1 e EHV-4 fundados em gE.

Após o reconhecimento das regiões a utilizar, o presente trabalho foi desenhado de forma a obter estas proteínas recombinantes. Numa primeira fase, foram construídos dois vetores de expressão, cada um contento a porção imunogénica de gG de EHV-1 e EHV-4 para posterior transformação e expressão num organismo modelo neste caso, a bactéria *Escherichia coli.* E, em paralelo, o trabalho prosseguiu usando dois péptidos sintéticos de gE, um para cada um dos herpesvírus em estudo.

A fase seguinte deste trabalho englobou o desenvolvimento de um teste ELISA, teste esse que tem como base reações antigénio-anticorpo detetáveis por meio de reações enzimáticas e têm sido utilizados para identificar estados de infeção e/ou vacinação de forma rápida e precisa. O ELISA desenvolvido foi do tipo indireto onde a ligação antigénio-anticorpo é detetada por um segundo anticorpo marcado o que confere uma maior especificidade e sensibilidade ao teste.

No final, foi possível desenvolver e otimizar um teste ELISA indireto que permite a deteção de soros positivos e também a diferenciação de EHV-1 e EHV-4. No entanto, embora os resultados obtidos sejam promissores, trabalho futuro será necessário para testar um maior número de soros de forma a validar o teste ELISA e torná-lo apto para utilização em laboratório.

Palavras-chave: Herpesvírus; EHV-1; EHV-4; Serologia; ELISA; Péptido; Equino;

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Abbreviations

BHV – Bovine Herpes Virus

BSA – Bovine Serum Albumine

CF – Complement Fixing

CMI – Cell-mediated Immunity

CTL - Cytotoxic T cells

DIVA - Differentiating Infected from Vaccinated Animals

DNA - Deoxyribonucleic acid

dsDNA – Double-Stranded DNA

EHM – Equine Herpesvirus Myeloencephalopathy

EHV – Equine Herpesvirus

ELISA - Enzyme-Linked Immunosorbent Assay

gE – E Glycoprotein

gG – G Glycoprotein

IE - Immediate Early

IFN - Virus Control Interferons

IFNγ - Interferon-Gamma

Ig - Immunoglobulin

IPTG - Isopropyl β-D-1-thiogalactopyranoside

Kb - Kilobase

LAT – Late Transcript

LB – Luria-Bertani

MHC-I - Major Histocompatibility Complex Class I

mRNA – Messenger Ribonucleic Acid

NK – Natural Killer

NPC - Nucleopore complex

OD – Optical Density

ORF – Open Reading Frame

PCR – Polymerase Chain Reaction

RT-PCR – Real time – Polymerase Chain Reaction

Th1 – T Helper 1 Lymphocytes

TMB - 3,3',5,5'- tetrametilbenzidine

URT - Upper Respiratory Tract

VN - Virus Neutralizing

Objectives

The rapid identification and diagnostic of the disease is of maximum importance to prevent its spread and give a quick treatment response to the horse. The most used diagnostic method is ELISA screening for anti-EHV-1 and anti-EHV-4 antibodies in the serum of suspected animals. The aim of this work was to develop an indirect ELISA based on a recombinant protein that allows detection of antibodies against equine herpesvirus types 1 and 4. In summary, the main objectives of the work were:

- Construct two expression vectors each one containing the immunogenic portion of the EHV-1 and EHV-4 virus glycoprotein G.
- Express and purify the proteins of interest.
- Develop an indirect ELISA using plates coated with the expressed proteins.
- Optimize the reaction with respect to dilutions of the various reagents, incubation times and incubation temperatures using positive and negative sera for EHV-1 and EHV-4 viruses.
- Validate the developed ELISA by determining specificity, sensitivity, limit of detection, inter and intra-assay variability and Receiver Operating Characteristic (ROC) analysis.

1. Introduction

- **Herpesvirus**

Herpesviruses infect members of all groups of vertebrates and some invertebrates. The lineage of herpesviruses infecting mainly mammals and birds is subdivided into three groups or subfamilies, alpha (α), beta (β) and gamma (γ), according to some criteria as, tissue tropism, pathogenicity and behavior in tissue culture (Davison, 2002; Paillot *et al*., 2008).

Equine herpesviruses (EHVs) are double-stranded DNA (dsDNA) viruses of 125–245 kb (kilobase) that affect all members of the Equidae family worldwide. (Davison, 2002; Slater, 2007; Wegdan *et al*., 2016). Their virions are 200–250 nm in diameter, and the genome is packaged within an icosahedral capsid with approximately 125 nm in diameter, inserted in a matrix known as tegument which contains numerous virus-coded proteins, itself enfolded in a lipid membrane containing viral glycoproteins (Baker *et al*., 1990; Davison, 2002; Paillot *et al*., 2008) (Figure 1.1).

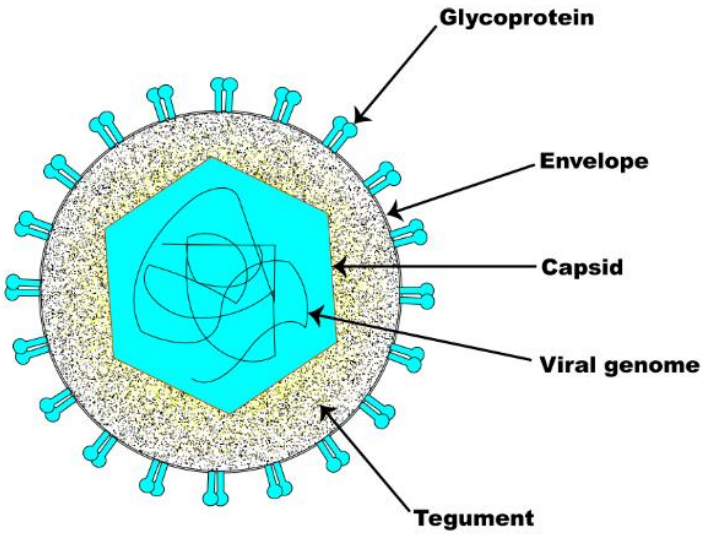

Figure 1.1 Schematic illustration of EHV structure. The basic architecture of EHV consists of an envelope, dsDNA genome enclosed within the capsid, and a tegument layer. Several glycoproteins are present on the surface of the envelope (Sowed as in Oladunni *et al*., 2019).

Thus far, nine EHVs have been characterized, EHV-1 to EHV-5 infect domestic horses, while EHV-6 to EHV9 are associated to wild equids (Slater,2007). In these nine types, six belong to the subfamily *alphaherpesviridae* and three to the *gammaherpesviridae* as shown in Table 1.1 (Davison,2002; Patel&Heldens,2005; Paillot *et al*.,2008).

Table 1.1 Equine herpesvirus classification (Adapted from Paillot *et al*., 2008).

Host Species	Name	Relationship	Subfamily	Disease
Equus caballus	EHV-1	Abortion virus	α	Respiratory, neurological, abortion
	EHV-2	Cytomegalovirus	γ	N/A
	EHV-3	Coital exanthema virus	α	Coital exanthema
	EHV-4	Rhinopneumonitis virus	α	Respiratory
	EHV-5	Cytomegalovirus	γ	N/A
Equus asinus	AHV-1/EHV-6	Related to EHV-3	α	Coital exanthema
	AHV-2/EHV-7	Related to EHV-2	γ	N/A
	AHV-3/EHV-8	Related to EHV-1	α	Rhinitis
Gazella thomsoni	GHV-1/EHV-9	Similar to EHV-1/8	α	Neurological

The equid herpesviruses have complex life cycles that involve infection of multiple cell types in different tissues, with different mechanisms for evasion of the host immune response (Slater, 2007). One of the most powerful tools of these viruses is the capability of being latent, a state in which horses carry virus in an asymptomatic form for extended periods (Ostlound, 1993; Slater, 2007). Latency provides a mechanism for the maintenance of viruses in the infected herd but also can be introduced into new herds through the movement of latently infected horses (Ostlound, 1993; Slater, 2007; Balasuriya *et al*, 2015). The reactivation of the virus is influenced by host and environmental factors that lead to recurrence of disease and viral transmission in contact with susceptible hosts (Balasuriya *et al*, 2015).

For these reasons, the EHVs have a major economic and welfare impact on all sectors of the horse industry worldwide through their direct clinical effects on the horse, including respiratory disease, abortion, and paralysis, and through their effects on horse breeding and competition (Slater, 2007; Milic *et al*., 2018; Oladunni *et al*., 2019).

However, equine herpesvirus type 1 (EHV-1) and equine herpesvirus type 4 (EHV-4) are clinically, economically, and epidemiologically the most relevant pathogens (Patel & Heldens, 2005; Slater, 2007).

- **EHV-1 and EHV-4 etiology**

Before 1981, EVH-1 and EVH-4 were considered as two subtypes of the same virus (Milic *et al*., 2018; Oladunni *et al*., 2019). Using genome sequence it was possible to confirm that the two types, EHV-1 and EHV-4, are closely related with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (Vandekerckhove *et al*., 2011; Azab *et al*., 2012; Wegdan, 2016; Milic *et al*., 2018).

EHV-1 and EHV-4 are both classified as members of the genus *Varicellovirus* in the *Alphaherpesviridae* subfamily (ICTV 2018b). Both are linear double stranded DNA viruses with a 145 to 150kb genome (Patel & Heldens, 2005; Milic *et al*., 2018; Reed & Toribio, 2004). EHV-1 genome contains 80 open reading frames (ORFs), of which four ORFs are duplicated, encoding a total of 76 unique genes. On the other hand, EHV-4 has 79 ORFs, with three of those duplicated (Maanen, 2002; Paillot *et al*., 2008; Oladunni *et al*., 2019).

Even though EHV-4 and EHV-1 are closely related, the genetic differences between the two viruses are sufficient to affect their host range and disease phenotypes (Patel & Heldens, 2005; Oladunni *et al*., 2019). EHV-1 is responsible for respiratory disease in young animals, abortion, neonatal death and extensive outbreaks of neurologic disease or equine herpesvirus myeloencephalopathy (EHM), while EHV-4 causes mainly respiratory disorders and rarely leads to abortion (Ostlund, 1993; Telford *et al*., 1998; Damiani *et al*., 2000; Balasuriya *et al*, 2015; Milic *et al*, 2018).

Currently, it is estimated that as many as 80% of all horses are latently infected with EHV-1, and seroprevalence for EHV-4 is even higher (Hussey, 2019).

- **Glycoproteins**

The glycoproteins of herpesviruses are present in the virion envelope and have important functional roles in the disease pathogenesis (Crabb & Studdert, 1990; Molinková, 2012). They are involved in virus-host cell interactions as mediation of the virion entry into the host cell via adsorption, penetration and cell fusion (Crabb & Studdert, 1990; Maanen, 2002; Damiani *et al*., 2000; Slater, 2007; Patel & Heldens, 2005). Due to their location, these molecules are in direct

contact with the host immune system being the principal target of the immune response (Molinkova, 2012; Von Einem *et al*., 2007). For this reason, the identification and understanding of immunological components is a requisite for effective intervention against the disease (Patel & Heldens, 2005).

There are eleven envelope-anchored surface glycosylated proteins on EHV-1 and EHV-4 that function as principal viral immunogens and are the major targets for virus-neutralizing antibodies (Slater, 2007). These eleven proteins are divided in those which are indispensable for the virus replication, glycoprotein B (gB), gD, gH, gK and gL and those which are not, gC, gE, gI, gG, gM and gp300 (Molinková, 2012; Slater, 2007; Patel & Heldens, 2005). Nevertheless, these "non-essential for replication" envelope glycoproteins play an important role in the expression of virulence (Damiani *et al*., 2000; Paillot *et al*., 2008).

- **Glycoprotein G**

The proteins with the closest homology between EHV-1 and EHV-4 include those involved in DNA replication and packaging, and capsid formation. Most differences were found in the 3' end of gene 70 encoding glycoprotein G. (Thormann *et al*., 2012; Van Maanen, 2012).

In 1993, Crabb & Studdert showed, by comparing the coding sequences of EHV-4 gG and EHV-1 gG, that these sequences were 58% identical however, peptide regions comprising amino acids residues 287 to 382 in EHV-4 gG and 288 to 350 in EHV-1 gG were only 21% similar. This finding suggested that this region may be used for differentiating these viruses.

Glycoprotein G (gG) is expressed early in the infection pathway and has been described to function as a broad-spectrum chemokine binding protein (Van de Walle *et al*., 2007). As chemokines play central roles in mediating inflammatory responses during viral infection (Van de Walle *et al*., 2007; Slater, 2007), this glycoprotein blocks their interaction with chemokine specific receptors and glycosaminoglycans. This interaction is required for endothelial transcytosis and correct presentation of chemokines to leukocytes (Bryant *et al*., 2003; Huang *et al*., 2005; Thormann *et al*., 2012), whereas their inhibition helps the virus in immune system suppression and evasion.

Recent studies affirm that EHV-1 gG deletion mutants showed a more pronounced inflammatory response when compared to wild-type virus (Von Einem *et al*., 2007; Thormann *et al*., 2012). Furthermore, *in vitro* chemotaxis experiments demonstrated that EHV-1 gG was

indeed able to inhibit migration of equine neutrophils, an observation that was not verified on EHV-4 gG, although the protein sequences shared an overall identity of 58%. Based on these results, it was proposed that the hypervariable region identified by Crabb & Studdert (1993) may contribute to EHV-1 gG to express a more severe phenotype (Van de Walle *et al*., 2007; Von Einem *et al*., 2007; Thormann *et al*., 2012).

- **Glycoprotein E**

The glycoprotein E (gE) form is associated with glycoprotein I (gI), forming a heterodimer that is involved in cell-cell spread (Slater, 2013), assisting viral envelopment into cytoplasmic vesicles that are sorted to epithelial cell junctions where the virus bind to the cellular receptors (Devlin *et al*., 2006). The EHV-1 gE has 550 amino acids while EHV-4 gE has 548 amino acids and weights approximately 95kDa (Damiani *et al*., 2000).

The gE deletion mutants of bovine herpesvirus type 1 (BHV-1) and pseudorabies virus, both of which as EHV-1 and EHV-4 belong to the subfamily Alphaherpesviridae, have been used as modified live virus vaccines (Andoh *et al*., 2013; Tsujimura *et al*., 2009). The lack of gE in these vaccines is a valuable marker for differentiation between a natural infection and antibodies induced by vaccine administration, using type specific enzyme-linked immunosorbent assay (ELISA) (Birch-Machin *et al*., 2000; Tsujimura *et al*., 2009; Andoh *et al*., 2013), that therefore may be used as DIVA (Differentiating Infected from Vaccinated Animals).

In 2000, Damiani *et al*. examined EHV-4 gI and gE deletion mutants under *in vitro* and *in vivo* conditions, concluding that intact gI and/or gE genes are necessary for efficient virus transmission through cell-to-cell spread and consequently, there is an important role of gI and gE genes in EHV-4 virulence. Based on these findings, in 2013, Andoh *et al*. identified and described immunogenic epitopes of EHV-1 and EHV-4 based on gE.

- **Life cycle**

Once attached to the cell, the virus has the capability to penetrate the cell by fusion of the virus envelope with the cell membrane or by endocytosis/phagocytosis, which release the nucleocapsid and tegument proteins of EHV-1 and EHV-4 into the cell (Frampton *et al*., 2007). As for other herpesviruses, such as herpes simplex virus, it is believed that most of the tegument

proteins dissociate from the capsid that is therefore transported along microtubules until near the nucleus (Frampton *et al*., 2007; Paillot *et al*., 2008). The association between the viral capsid and the viral nucleic acid, nucleocapsid, binds directly to the nucleopore complex (NPC) and the viral DNA is translocated into the nucleus while the nucleocapsid remains in the cytoplasm (Whittaker & Helenius, 1998). The transcription of the EHV-1/4 genome is requested with the expression of the immediate early (IE) gene (Gray *et al*., 1986; Garko-Buczynski *et al*., 1998; Paillot *et al*., 2008). The IE protein encoded is required for the transcription of the adjacent early and late genes regulating the activation and repression of gene expression (Gray *et al*., 1986; Garko-Buczynski *et al*., 1998).

Early genes encode the proteins involved in stimulating virus replication while, late genes encode the viral structural proteins (Garko-Buczynski *et al*., 1998; Paillot *et al*., 2008). The immediate early proteins synthesized in the cytoplasm migrate into the nucleus to initiate transcription of early mRNA. Then, the synthesized proteins migrate through NPC into the nucleus to start replication of the virus. Late proteins synthesized in the cytoplasm migrate into the nucleus to form the nucleocapsid (Whittaker & Helenius, 1998). The nucleocapsid, surrounded by tegument proteins, leaves the nucleus by envelopment at the inner nuclear membrane that contains glycoproteins (Whittaker & Helenius, 1998; Paillot *et al*., 2008). This primary envelope is lost, and a second envelopment occurs at the cytoplasmic membranes which contain all the viral glycoproteins. The mature virus migrates through the secretory pathway via Golgi apparatus were the fusion of Golgi vesicles with the membrane allows the release to extracellular space, to infect other cells (Paillot *et al*., 2008).

- **Pathogenesis**

Although EHV-1 and EHV-4 respiratory infections are clinically indistinguishable, their pathogenesis is distinct (Hussey, 2019). Primary infection of EHV-1 starts in the epithelial cells of the upper respiratory tract (URT) where glycoproteins on the surface of the viral membrane bind to the host cell surface and allow fusion of the virus to the host cell (Reed & Toribio, 2004; Vandekerckhove *et al*., 2011; Thormann *et al*., 2012; Hussey, 2019). The virus replication occurs in the nucleus, and protovirions derive their envelope from the inner lamella of the nuclear membrane, this process results in rhinopneumonitis syndrome (Vandekerckhove *et al*., 2011; Thormann *et al*., 2012; Hussey, 2019). EHV-1 is detected in the local lymph nodes after 24 to 48 hours post infection (Slater, 2007; Azab *et al*., 2012; Hussey, 2019). Four to 10 days

post infection, the virus replicates in leukocytes of the respiratory tract lymph nodes and can enter the peripheral blood stream within monocytes and CD4+ and CD8+ T lymphocytes (Slater, 2007; Azab *et al*., 2012; Hussey, 2019). The virus is transported to other infection sites, without being destroyed, where the contact with the endothelium leads to endothelial cell infection, inflammation, thrombosis, and tissue necrosis, as well as secondary disease manifestations (Goehring *et al*., 2010; Thormann *et al*., 2012; Hussey, 2019). Severe infections may even lead to abortions, development of neurologic disease or equine herpesvirus myeloencephalopathy (EHM) (Vandekerckhove *et al*., 2011; Thormann *et al*., 2012; Hussey, 2019).

Although the pathogenesis of EHV-4 has not been completely revealed, it is known that the initial stage mimic EHV-1 infecting the epithelial cells of the upper respiratory tract and is associated with the lymphoid system (Vandekerckhove *et al*., 2011; Hussey, 2019). Usually, the infection and viral cell-to-cell spread does not extend to other areas. However, abortion cases associated with EHV-4 have been reported, suggesting that the virus can occasionally spread and overcome the host immune system (Figure 1.2) (Vandekerckhove *et al*., 2011; Oladunni *et al*., 2019).

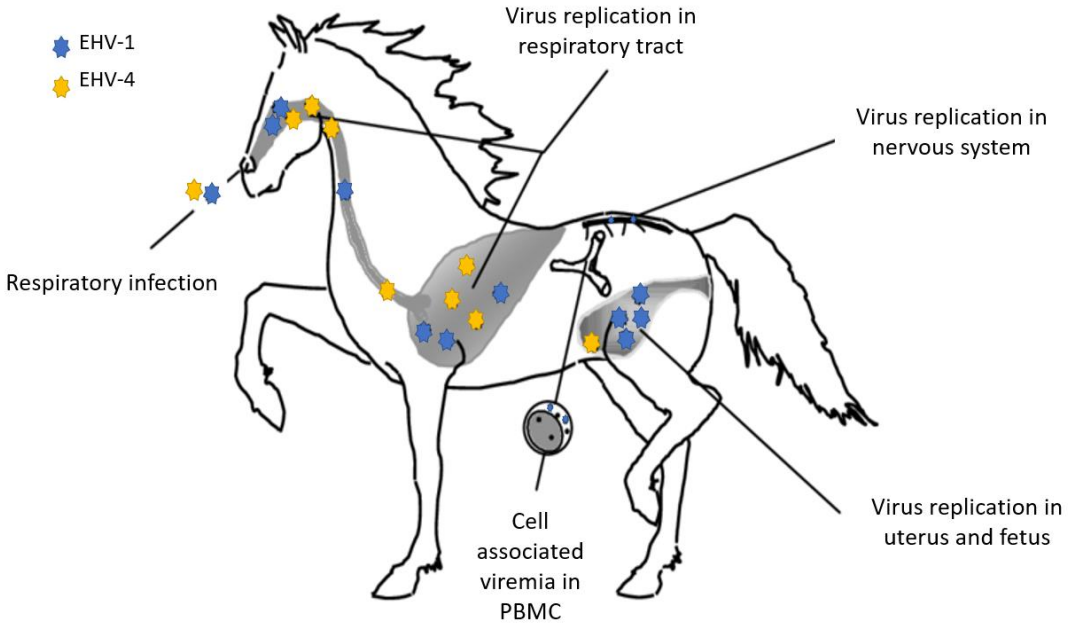

Figure 1.2. Pathogenesis of EHV-1 and EHV-4 infection in the horse (Adapted from Van der Meulen & Nauyink, 2003).

The severity of clinical disease associated with EHV-1 and EHV-4 infections is influenced by host and environmental factors, including age, physical condition, immune status, season, and if the infection is the result of primary exposure, reinfection, or reactivation of latent virus, as well as secondary bacterial infection of the respiratory tract, among others (Balasurya *et al*., 2015; Hussey, 2019).

- **Latency**

As mentioned, the ability of these viruses to become latent is one of the biggest advantages in terms of maintenance and spread, because this latent stage allows the virus to survive under unfavorable conditions. (Ostlound, 1993; Slater, 2007; Balasuriya *et al*, 2015). This stage allows the virus to protect itself from the antiviral effects of the cytotoxic T lymphocytes and neutralizing antibodies without the horse having any symptomatology (Reed & Toribio, 2004; Balasuriya *et al*, 2015). Plus, this stage allows the movement of infected horses and the introduction of the virus into new herds (Slater, 2007).

The prevalence of latent or undetected EHV-1 and EHV-4 infections may be influenced by factors such as geographical location, horse population, management practices, and the sensitivity of tests used for identification (Pusterla *et al*., 2009).

During latency, the linear genome form that is present in the infection stage adopts a transcriptionally repressed circular configuration associated with non-acetylated histone (Paillot *et al*., 2008; Gulati *et al*., 2015). The viral DNA translocates to the nucleus and the expression of the EHV-1 and EHV-4 genome is repressed except for the IE gene. (Maanen, 2002; Paillot *et al*., 2008). This results in the expression of the EHV latency associated transcript (LAT). However, the exact mechanism that controls this process is still under study (Slater, 2007; Paillot *et al*., 2008; Gulati *et al*., 2015; Oladunni *et al*., 2019).

The principal site of latency was initially controversial because latently infected cells do not express viral antigens and are thus not detectable immunologically (Slater, 2007; Paillot *et al*., 2008). However, using PCR and RT-PCR techniques it was possible to demonstrate, for both viruses, that latency is establish in lymphoid tissues, peripheral leucocytes and trigeminal ganglia (Maanen, 2002; Patel & Heldens, 2005; Gulati *et al*., 2015).

Reactivation and shedding of EHV-1 and EHV-4 has been associated with stressful events like weaning, castration, or transport (Maanen, 2002; Patel & Heldens, 2005). Occurs normally with

the translocation of the virus to the respiratory epithelial surface where it can be either neutralized by the host immune system or succeed in establishing infection (Slater, 2007).

The reactivation creates the opportunity for transmission to other horses, which is considered important in the epidemiology of EHV-1 and EHV-4 and might explain why these diseases can occur in closed populations. (Maanen, 2002; Patel & Heldens, 2005; Slater, 2007).

- **Transmission and clinical signs**

Viral transmission occurs by direct or indirect contact with infectious nasal secretions, aborted fetuses, placentas, or placental fluids (Maanen., 2002; Paillot *et al*., 2008). These last ones, related to abortion, are the major source of infection because they contain extremely high levels of live virus (Maanen., 2002; Paillot *et al*., 2008). The morbidity tends to be the highest in young horses sharing the same air space, however, the role of aerosol transmission depends on the amount of infectious virus present, climatic conditions, ventilation and distance between horses (Maanen., 2002).

The infection signs with EHV-1 and EHV-4 appear 2-8 days after exposure and are characterized by respiratory disease (Slater, 2007; OIE Terrestrial manual, 2017) namely, fever, depression, anorexia, coughing, nasal discharge, enlargement of mandibular lymph node and mucosal inflammation of upper respiratory tract (Damiani *et al*., 2000; Mizukoshi *et al*., 2002; Hussey, 2019).

Besides respiratory disease EHV-1 is associated with abortion and neurologic disease (Slater, 2007). Abortions may occur at any stage of gestation although it is more common on late stages in pregnancy (Molinkova, 2012; Reed & Toribio, 2004). Foals may become infected in utero and die or are born already showing some symptomatology (Wegdan *et al*., 2016; OIE Terrestrial manual, 2017).

The clinical signs observed as a result of EHV-1 EHM can be quite variable including symmetric ataxia, weakness of the pelvic limbs, urinary incontinence, loss of sensation, and motor deficits around the tail and perineal (Reed & Toribio, 2004; Holz *et al*., 2017; Hussey, 2019).

- **Diagnosis**

EHV-1 and EHV-4 clinical diagnosis is complicated due to the similarity between the symptoms caused by these viruses and the ones caused by other equine pathogens, beyond its capacity to be latent or cause reinfections (Ostlund, 1993; Slater, 2007; Balasurya *et al*., 2015).

Other difficulty behind these viruses identification is the antigenic similarity that makes it hard to distinguish one from another with the normal used serologic tests, since the available polyclonal antibodies of EHV-1 and EHV-4 are highly cross-reactive (Ostlund, 1993; Crabb *et al*., 1995; Van Maanen, 2002).

When a disease outbreak appear, clinical diagnosis and confirmation by means of rapid, sensitive, and specific laboratory tests is crucial to ensure a quick and effective response (Balasuryia *et al*., 2015). Nowadays, laboratory identification of EHV-1 and EVH-4 infection can be done by different methods including virus isolation, serologic typing, polymerase chain reaction (PCR), real-time PCR and sequencing (Van Maanen, 2002; Milic *et al*., 2018).

PCR can provide the identification of the virus using primers derived from conserved regions of the genome, which amplify products of similar size for each virus. To distinguish between EHV-1 and EHV-4 it is necessary hybridization using virus-specific probes or restriction endonuclease digestion (Reed &Toribio, 2004; OIE Terrestrial manual, 2017). Real-time PCR is a rapid, sensitive, specific, and quantitative method for the diagnosis of infectious diseases that allows, beyond the virus detection, quantify the viral title (OIE Terrestrial manual, 2017; Milic *et al*., 2018). Both these molecular techniques can be used with different samples: swabs, blood or abortion tissues (Maanen, 2002).

Other useful techniques include immunohistochemistry or immunofluorescence tests (Reed & Toribio, 2004; Patel & Heldens, 2005). Unfortunately, in some cases, it is impossible to achieve a definitive diagnosis without postmortem examination (Reed & Toribio, 2004).

On the other hand, serological testing has been used for presumptive evidence of infection and/or vaccination status in animals due to its quick results. However, the use of polyclonal antibodies does not allow type-specific serological diagnosis due to the cross reactivity, (Van Maanen, 2002; Milic *et al*., 2018). The development of a type-specific enzyme linked immunosorbent assays (ELISA) detecting antibodies to immunodominant type-specific epitopes has allowed specific serodiagnosis of EHV-1 and EHV-4 infections, differentiating both virus (Van Maanen, 2002; Lang *et al*., 2013; Balasuryia *et al*., 2015).

- **Enzyme-linked immunosorbent assay (ELISA)**

In 1971, the Enzyme-linked immunosorbent assay or ELISA was developed by Perlmann and Engvall at Stockholm University in Sweden by modifying the radioimmunoassay (RIA) method (Aydin, 2015). Nowadays, it is a common laboratory technique which is usually used to measure the concentration of antibodies or antigens widely used in clinical diagnosis. It is described as a precise, sensitive, versatile, and quantifiable diagnostic method (Tiha & Ibrahim, 2015).

Basically, the specific link between an antigen and its antibody is traceable and allows the detection of very small quantities of antigens such as proteins, peptides, hormones, or antibody in a fluid sample (Gan & Patel, 2013).

On the ELISA method, the antigen utilized is bound to a solid phase and is allowed to bind to a specific antibody which is itself subsequently detected by a secondary, enzyme-coupled antibody that converts a colorless substrate (chromogen) to a colored product, this way reporting the ligation between antigen and antibody (Ma & Shieh, 2006; Yang & Ma, 2009; Gan & Patel, 2013).

Depending on how the test is designed, the assays may be classified as either competitive or noncompetitive (Engvall, 1980; Ma & Shieh, 2006; Yang & Ma, 2009). On competitive ELISA, unlabeled and labeled antigen (or antibody) compete for a limited number of antibody (or antigen) sites while on noncompetitive, that may be direct, indirect and sandwich ELISA, the antigen (or antibody) to be measured is first allowed to react with antibody (antigen) on a solid phase followed by measurement of the binding of enzyme-labeled immune reactant (Engvall, 1980) (Figure 1.3).

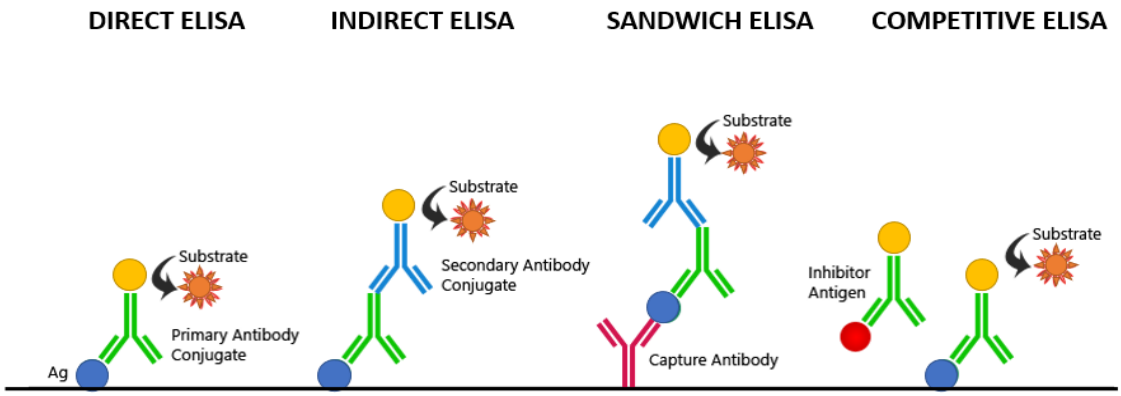

Figure 1.3. Schematic representation of the different types of ELISA (Adapted from Serrano-Pertierra *et al.*, 2020)

- **Recombinant proteins production**

Recombinant proteins are used in biological research to investigate enzyme activity and ligand binding interactions, develop diagnostic tests, pharmaceutical agents among other functions *in vitro* (Papaneophytou & Kontopidis, 2014).

The gene for the protein of interest is cloned into a vector and expressed in a model organism (Clark & Pazdernick, 2016). These expression vectors provide a strong promoter to drive expression of the cloned gene and contain genes for antibiotic resistance. These genes allow the selection of the recombinant vector and therefore of the recombinant protein (Clark & Pazdernick, 2016).

Although eukaryotic cells can be used to express eukaryotic proteins, bacteria are simpler to grow and manipulate genetically being this the principle advantage of express eukaryotic proteins in bacteria (Clark & Pazdernick, 2016).

Basically, the mRNA from the gene of interest must first be converted to cDNA to provide coding DNA without introns. Then, this cDNA is converted into dsDNA, which is then ligated to an expression vector, between a bacterial promoter and a bacterial terminator, and finally used to transform bacterial host cells, so the bacterial transcription and translation machinery may express the coding sequence (Figure 1.4) (Clark & Pazdernick, 2016).

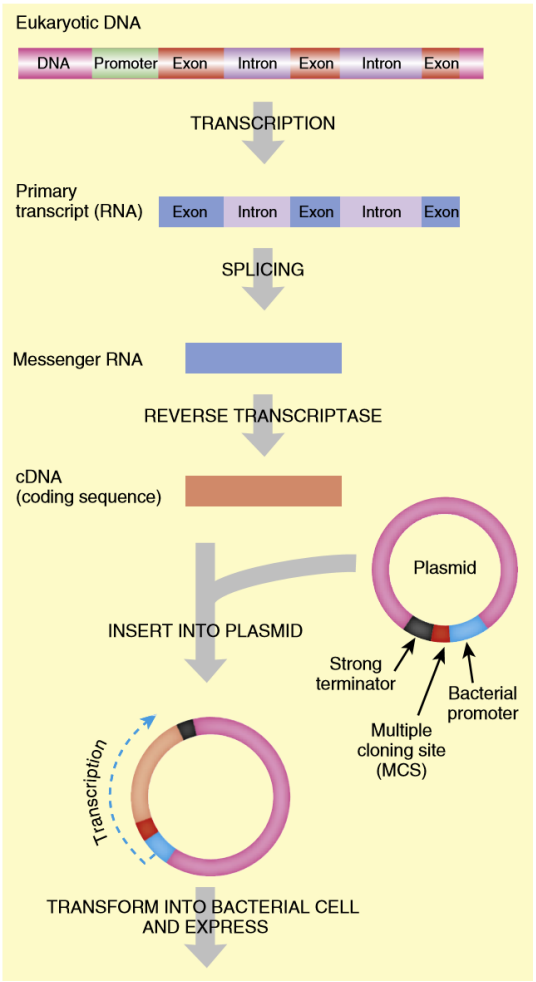

Figure 1.4. Schematic representation of the expression of eukaryotic gene in bacteria (Shown as in Clark & Pazdernick, 2016).

Several organisms can be used for recombinant proteins production besides bacteria as yeasts and microalgae (Ferrer-Miralles *et al*., 2015). However, the bacteria *Escherichia coli (E.coli)* is the most popular recombinant protein expression host (Andersen & Krumenn, 2002; Ni & Chen, 2009; Ferrer-Miralles *et al*., 2015).

- **Immune response**

Effective immunological protection against herpesviruses is the result of the cooperation of various immune mechanisms which prevents viral replication at each anatomical site of infection. These mechanisms are divided in innate and acquired immune responses (Chacón Salinas & Sanchéz Cruz, 2000; Breathnach *et al*., 2001).

The mechanisms of innate immunity act in the early stages of infection and prevent viral spread. In the virus control participate cytokines such as type I and II interferons (IFN), complement and natural killer (NK) cells. On the other hand, the acquired immune response occurs in the late phase of infection and includes the humoral and cellular immune responses. Antibodies prevent the spread of viruses to adjacent cells and tissues, neutralizing their infectivity, while cytotoxic T cells (CTL) kill infected cells, mainly by induction of apoptosis (Chacón Salinas & Sanchéz Cruz, 2000).

Between 1950 and 1960, assays were performed to measure serum antibodies. These studies allow the researchers to conclude that, complement fixing (CF) and virus neutralizing (VN) antibodies appear within 2 weeks after infection with EHV-1. However, CF antibodies are short lived and are not detectable past 3 months, whereas VN antibodies may last over one year (Kidd *et al*., 2006; Paillot *et al*., 2008; Ma *et al*., 2013; Oladunni *et al*., 2019).

The mucosal humoral immune response is believed to reduce virus shedding, (Breathnach *et al*.,2001; Paillot *et al*.,2008) however, the protective effect of circulating antibodies during infection is limited once a state of cell associated viremia is established and virus is protected while intracellular (Paillot *et al*.,2008; Oladunni *et al*.,2019).

Virus-specific cell-mediated responses are crucial components of immunity to herpesviruses. IFNγ (Interferon-Gamma) synthesis is one of the most used markers of cell- mediated immunity (CMI) to measure responses to herpesviruses (Kidd *et al*., 2006; Paillot *et al*., 2008). IFNγ promotes the increasing of viral peptides presentation and promotes the development of T helper 1 (Th1) lymphocytes. IFNγ is a key factor in the development of a response, being able to directly block pathogen replication, activate macrophages for the pathogen elimination and activate natural killer (NK) cells to lyse infected cells (Paillot *et al*., 2008).

- **Prophylaxis and treatment**

No specific therapy for the respiratory disease due to EHV-1 and EHV-4 infections exists. To prevent secondary bacterial infections prophylactic antibiotics can be administered and high fever can be treated palliatively with antipyretics (Maanen, 2002; Slater, 2007). Treatment of horses with neurologic disease is largely empirical since not enough experimental, or clinical evidence exists to confirm the success of the drugs used (Slater, 2007).

Regarding vaccines, one of the major difficulties associated with their development is the knowledge of the specific antigens adequate to elicit a high immune response (Reed & Toribio, 2004).

The first vaccine against EHV-1 was developed using infected equine fetuses and organs and was applied between 1943 and 1952. However, this inactivated vaccine does not provide complete protection against abortion and induces several side-effects as alloimmune hemolytic anemia (Maanen, 2002; Patel & Heldens, 2005; Slater, 2007).

At present, commercial inactivated combined EHV-1/EHV-4 vaccines are available and can be useful for reduction of respiratory clinical signs, influence viremia and appear to decrease abortion (Slater, 2007). However, no vaccine shows, until today, ability to protect against neurological disease (Kidd *et al*., 2016; OIE Terrestrial manual, 2017). Successful vaccination against EHV-1 and EHV-4 must protect against the development of respiratory disease, prevent the systemic dissemination and the virus reactivation because of humoral and cellular immune responses (Paillot *et al*., 2008).

Due to the high spread of the viruses, and difficult containment and treatment, preventive management measures need to be taken as soon as an animal is diagnosed (Maanen, 2002; Milic *et al*., 2018; Oladunni *et al*., 2019), for which reliable and expedite detection tools are paramount. These measures start by quarantining the infected and/or suspected horses, by isolating them for three weeks, while providing for their individual nursing and hygiene care (Maanen, 2002; Reed & Toribio, 2004; Pusterla *et al*., 2009).

2. Materials and methods

- **G glycoprotein (gG) amplification**

Fragments of G glycoprotein from EHV-1 and EHV-4 were amplified by Polymerase Chain Reaction (PCR) using the specific primers gG1-EcoRI – F and gG1-XhoI – R for EHV1 and gG4-EcoRI – F and gG4-XhoI – R for EHV4 (Table 2.1), which amplify a segment of approximately 189 and 288 base pairs (bp), respectively. The primers used were designed to add EcoRI and XhoI restriction sites at 5’ and 3’ ends of the fragment, respectively. The amplification reactions were done in a final volume of 25 µl with the followed final composition: 12.5 µl NZY II 2x colourless master mix containing dNTPs (NZYTech, Portugal) (0.2 U / µl), 0.5 µl of each primer (50 pmol/µl), 5 µl of DNA and sterile water. This reaction

was performed adding a negative control for each reaction (without template DNA). The temperature cycles applied in the C1000 thermal cycler (Bio-Rad, EUA) were as follows: 95ºC for 2 minutes followed by 50 cycles of 95°C for 30 seconds, 46°C for 30 seconds and 72 °C for 30 seconds, with a final incubation at 72°C for 5 minutes. The product of DNA amplification, via PCR, was then loaded on 1,5% agarose gel and subjected to electrophoresis.

Table 2.1 – Primers used for G glycoprotein (gG) portion amplification of EVH-1 and EVH-4 (Provided by INIAV).

Primer designation	**Primer sequence**
gG1-EcoRI – F (Forward)	5'-AAAAGAATTCGGTGACGAAACATACGA-3'
gG1-XhoI – R (Reverse)	5'-AAAACTCGAGTGGATGCCGTTCGACGC-3'
gG4-EcoRI – F (Forward)	5'-AAAAGAATTCGAAAATGAAACCTACAG 3'
gG4-XhoI – R (Reverse)	5'-AAAACTCGAGTGGAGGCCATGCAACAC-3'

- **DNA extraction in gel**

Fragments separated by agarose gel electrophoresis were excised from the gel and then extracted and purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega, EUA) following the manufacturer's instructions.

- **TA Plasmid ligation**

The purified PCR products were cloned into plasmid TA using The Original TA cloning Kit (Invitrogen, EUA), allowing for white-blue screening of the recombinants of interest. The ligation reaction was performed in a final volume of 10 µl with the composition of 2 µl vector (25 ng/µl), 2 µl of 5x ligation buffer, 1 µl T4 DNA Ligase (5 U/µl), 1 µl of PCR product, and sterile water. The reaction was incubated 1h at room temperature. The plasmids thus obtained, TA-EHV1 and TA-EHV4, were transformed into competent TOP 10 cells.

- **Transformation**

Two µl of each ligation reaction were added to the respective 50 µl of TOP10 competent cells (Invitrogen, EUA). The mixture incubated on ice for 30 minutes, followed by a heat shock that was performed at 42ºC for 30 seconds. A volume of 250 µl of LB (Luria-Bertani) at room temperature was added to each tube, followed by an incubation at 37ºC and 225 rpm agitation

for 1h. Next the cells were plated on LB medium plates containing kanamycin at a final concentration of 50 µg /ml and previously coated with 40 µl of IPTG (100 mM) and 40 µl of X-gal (40 mg/ml). Finally, the plates were incubated at 37ºC overnight.

- **Clone selection**

Three white colonies from each TA-EVH1 and TA-EVH4 plate were selected and transferred into liquid LB with Kanamycin (50 µg /ml). The cultures were incubated overnight at 37ºC with 225 rpm agitation.

- **Plasmid isolation by boiling method (miniprep)**

A volume of 2 ml of the cell culture were transferred into an Eppendorf and centrifugated at 8000 rpm for 2 minutes. The supernatant was rejected, and the pellet homogenized in the residual liquid and then resuspended in 750 µl of STET (5% Triton X-100, 8% sucrose, 50 mM EDTA, 50 mM Tris.HCl, pH 8.0) with 10 µl/ml of lysozyme (20 ng/ml) and 5 µl/ml of Rnase (10 mg/ml). After homogenization, the tubes were boiled in water for 4 minutes and centrifuged at 13000 rpm for 10 minutes at room temperature. The supernatant was transferred for a new tube and isopropanol was added in equal volume. The tubes were centrifuged at 4ºC and 13000 rpm for 5 minutes. The pellet was dried and resuspended in 50 µl of distilled water.

- **Enzymatic digestion for insert verification**

The restriction reactions were performed in a final volume of 15 µl containing 1 µl of EcoRI 10 000 U/µl (NZYtech), 2 µl XhoI 10 000 U/µl (NZYtech), 1.5 µl of 10x buffer, 10 µl of DNA and distilled water. The reactions were incubated overnight at 37 °C. The presence of the vector and of the DNA fragment were confirmed by loading on 1.0% agarose gel followed by electrophoresis. The DNA fragments of interest, corresponding to the TA-EVH1 and TA-EVH4 inserts were excised and then extracted and purified using the Wizard SV Gel and PCR Clean -Up System kit (Promega) following the manufacturer's instructions.

- **Enzymatic digestion for pET-28a (+) obtaining**

Plasmid pET-28a (+) was obtained from cultures containing pET-N8t (a fragment of N8 neuraminidase encoding gene of avian influenza virus). The restriction reaction was performed in a final volume of 10 µl containing 1 µl of EcoRI 10 000 U/µl (NZYtech), 2 µl XhoI 10 000 U/µl (NZYtech), 1 µl of 10x buffer, 5 µl of DNA and distilled water. The reactions were incubated overnight at 37°C. The presence of the vector and of the DNA fragment were

confirmed by loading on 1.0% agarose gel followed by electrophoresis. The fragment corresponding to pET-28a (+) were excised and then extracted and purified using the Wizard SV Gel and PCR Clean -Up System kit (Promega) following the manufacturer's instructions.

- **Preparation of MRF' *E. coli* competent cells**

An initial inoculum was performed placing 5µl of MRF' *Escherichia coli* cells in 5 ml of liquid LB medium without antibiotic and incubating at 37ºC overnight with 225 rpm agitation. 250 µl of the initial inoculum were transferred for 25 ml of the LB liquid medium and incubated at the same conditions until optical density (OD) at 600 nm between 0.45 - 0.55 was reached. The cells were incubated on ice for 10 minutes and were centrifuged at 4ºC for 10 minutes at 3000 rpm. The supernatant was discarded, and the pellet resuspended in 5 ml of cold 50 mM $CaCl_2$. The centrifugation was repeated as well as the following steps and then the cells were incubated on ice for 30 minutes. A last centrifugation was performed at 4ºC for 5 minutes at 2000 rpm. The supernatant was discarded, and the pellet resuspended in 1 ml of cold 100 mM $CaCl_2$ with 15% of glycerol. Aliquots were made and stored at -80ºC.

- **pET Plasmid ligation**

The purified DNA products obtained, gG1 and gG4, were ligated to plasmid pET28 a(+) (Novagen). The ligation reaction solution was performed in a final volume of 10 µl with the composition of 5 µl vector, 2 µl 5x ligation buffer, 1 µl T4 DNA Ligase Express Link (5 U/ µl) and 2 µl of insert DNA. The reaction was incubated 1h at room temperature. The plasmids thus obtained, pET-EHV1 and pET-EHV4, were transformed into competent MRF' *Escherichia coli* cells.

- **Transformation**

50 µl of cells were transformed with each plasmid. 10 µl of ligation reaction were added to the competent cells MRF' that were incubated on ice for 30 minutes. Then, a heat shock was performed at 42ºC for 30 seconds, followed by the addition of 250 µl of LB at room temperature and 1h incubation at 37ºC with 225rpm agitation. In the end, the cells were plated on LB medium plates containing kanamycin at a final concentration of 50 µg /ml. The plates were incubated at 37ºC overnight. The obtained colonies were transferred to liquid LB medium with kanamycin (final concentration 50 µg /ml) and DNA was extracted by the boiling method miniprep, as described before.

- **Sequencing**

DNA fragments were sequenced using the BigDye Terminator v1.1 cycle Sequencing Kit (Applied Biosystems). The reaction mixture consisted of 2 µl of Ready Reaction Mix, 1 µl 5x sequencing buffer, 0,5 µl (5 pmol/µl) of primer, T7 Prom or T7 Term, 2 µl of DNA and sterile water to perform a final volume of 10 µl. The thermal cycler program used consisted of 1 minute at 96 °C, followed by 25 cycles of 10 seconds at 96 °C, 5 seconds at 47ºC for T7 Prom primer and 54ºC for T7 Term primer and a final incubation at 60ºC during 30sec. The DNA was then precipitated by the addition of 50 µl 95% ethanol, 2 µl EDTA (125 mM) and 2 µl sodium acetate (3M), followed by centrifugation for 20 minutes at 4ºC and 13000 rpm. The supernatant was pipetted and discarded, 200 µl 70% ethanol was added, stirred, and centrifuged for 7 minutes at 13000 g and 4°C. Finally, the supernatant was discarded and the pellet dried. The pellet was resuspended in 20 µl Hi-Di Formamide. The reading was performed on 3130 Genetic Analyzer (Applied Biosystems).

Table 2.2 – Primers used for PET-gG-EHV1 and PET-gG-EHV4 sequencing (available at PET28-NOVAGEN manual)

Primer designation	Primer sequence
T7 Prom (Forward)	5'-TAATACGACTCACTATAGG-3'
T7 Term (Reverse)	5'-GCTAGTTATTGCTCAGCGG-3'

ELISA gE DEVELOPMENT

- **Peptides**

Peptides from an immunogenic portion of EHV-1 and EHV-4 glycoprotein E, gE1 and gE4, respectively, were synthesized by NZYTech, based on Andoh *et al*. description in 2012 (Table 2.3).The gE1, and gE4 peptides were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/mL and stored at -80ºC. A work dilution was performed at a final concentration of 500 µg/mL and stored at -20ºC.

Table 2.3 – Synthetic peptides sequence

Peptide designation	Peptide sequence
gE1	KKPPKQPQPRLRVKTPPPVTVPQVPVKTHTDFV
gE4	KKPPTLPRVHVKTPPPILVPQVTPEAHTDFI

- **Serum samples**

Horse sera were obtained from the INIAV serum library. These sera were previously divided into EHV-1 and EHV-4 positives and negatives using ELISA RINO (INgezim RINO 14.HVE.K.1). Positive ones were subjected to seroneutralization for EHV-1 in order to differentiate both viruses.

- **ELISA development**
    - **Testing gE peptide and sera concentration**

Synthetic peptides were diluted to 5 µg/ml, 50 µg/ml, 100 µg/ml and 250 µg/ml with adsorption buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6), and 50 µl were added to each well of 96-well microplates. After incubation at 37°C for 2h, the plates were placed at 4°C overnight. After washing five times with PBS containing 0.05% Tween 20 (PBS-T), 100 µl of 0,1% bovine serum albumin (BSA, SIGMA) in PBS was added and incubated at 37°C for 1 h. Washing with PBS-T was performed and 100 µl of sera diluted in the proportions of 1:5 and 1:20 in dilution buffer (2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, 500 mM NaCl, 0.05% Tween 80) was added to the wells. The plate was incubated 1h at 37°C. After new wash, the plate was incubated with 100 µl of 1:5000 peroxidase-conjugated goat anti-equine IgG(T) (BIO-RAD) diluted in PBS-T at 37°C for 1h. Following five washes with PBS-T, 100 µl of substrate 3,3',5,5'-Tetramethylbenzidine liquid (TMB) (SIGMA) was added to each well. After incubation for 15 min at room temperature in the dark, the enzymatic reaction was stopped by adding 100 µl of sulfuric acid stop solution (INGENASA, Spain) to each well. The absorbance was measured at 450 nm on SUNRISE spectrophotometer (Tecan).

    - **Testing blocking buffers**

Plates were coated with 50 µl of 5 µg/ml gE1 and gE4 synthetic peptides. After incubation at 37°C for 2h, the plates were placed at 4°C overnight. After washing five times with PBS-T, different blocking buffers were tested namely, 5% powdered milk (Molico), 5% BSA (SIGMA), 5% sucrose (Merck) and 5% powdered milk plus 5% sucrose all diluted in PBS-T. The tested sera were diluted in a proportion of 1:10 in each one of the blocking buffers and 100 µl were added to each of the respective well. The incubation was performed at 37°C for 1 h. After wash, the incubation was repeated with 100 µl of 1:10000 peroxidase-conjugated goat anti-equine IgG(T) (BIO-RAD, EUA). Following five washes with PBS-T, 100 µl of substrate

TMB (SIGMA) was added to each well. After incubation for 15 min at room temperature in the dark, the enzymatic reaction was stopped by adding 100 µl of sulfuric acid stop solution (INGENASA, Spain) to each well. The absorbance was measured at 450 nm on SUNRISE spectrophotometer (Tecan).

- **Testing Anti-equine concentration**

Plates coated with 50 µl of 5 µg/ml gE1 and gE4 were washed five times with PBS-T. 100 µl of sera diluted in 5% sucrose in a proportion of 1:5 and 1:10 were added to the wells that were incubated at 37°C for 1h. After washing, 100 µl of the conjugated were added in different dilutions namely, 1:5000, 1:8000 and 1:10000 in 5% sucrose blocking buffer. The plates were incubated 1h at 37ºC. Following five washes with PBS-T, 100 µl of substrate TMB was added to each well. After incubation for 15 min at room temperature in the dark, the enzymatic reaction was stopped by adding 100 µl of stop solution to each well. The absorbance was measured by a spectrophotometer with a 450-630nm filter.

A fourth test was performed testing the sera diluted in 5% sucrose plus the conjugated in 2% sucrose and the sera plus the conjugated in 2% sucrose. All other conditions were maintained.

3. Results and Discussion

- G glycoprotein (gG) amplification

Fragments of G glycoprotein from EHV-1 and EHV-4 were amplified by PCR using the specific primers described earlier. After agarose gel electrophoresis it was possible to confirm the amplification of two fragments, one of 189 bp corresponding to gG-EHV1 and another with 288 bp corresponding to gG-EHV4 (Figure 3.1), as expected.

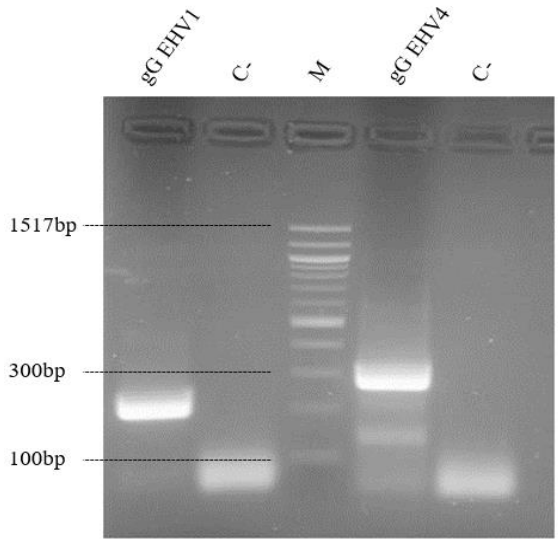

Figure 3.1- Agarose gel electrophoresis of the PCR products of G glycoprotein of EHV-1 and EHV-4. Marker (M) used was Quick-Load 100 bp DNA Ladder from BioLabs. Negative control was performed with the PCR mixture without DNA.

- Plasmid TA construction, cell transformation and clone selection

The PCR products obtained were cloned in TA plasmid (Invitrogen) followed by transformation into TOP10 competent cells. The white colonies obtained by white-blue screening, containing the plasmids with the sequences of interest were inoculated in liquid LB medium with kanamycin for posterior plasmid extraction (miniprep) (Figure 3.2).

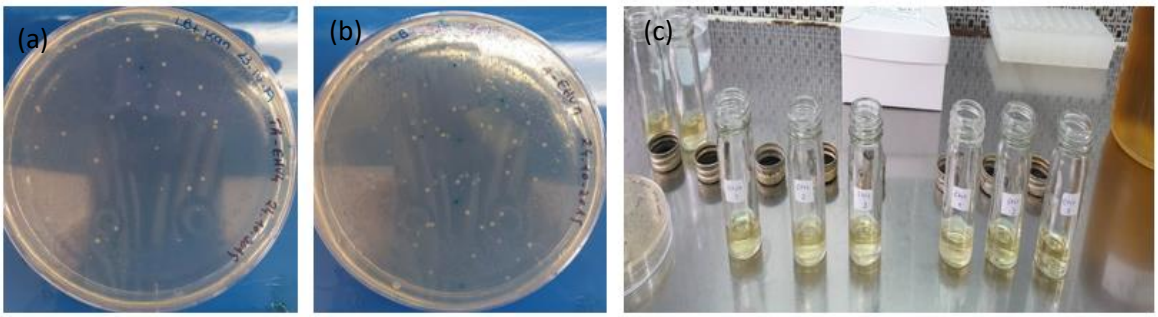

Figure 3.2 – Colonies obtained after cell transformation with the TA plasmid. (a) TA-gG-EVH4 and (b) TA-gG-EVH1. (c) Clone selection and passage to liquid LB medium with kanamycin.

The plasmids obtained, TA-gG-EHV1 and TA-gG-EHV4, were then checked by enzymatic restriction with EcoRI and XhoI.

- Enzymatic digestion

The plasmids TA-gG-EHV1 and TA-gG-EHV4 were digested by the restriction enzymes EcoRI and XhoI (Figure 3.3). These enzymes flank the gene of interest, since they were included in the fragment by the amplification primers that were designed for that purpose. Both enzymes do not cut inside the fragments.

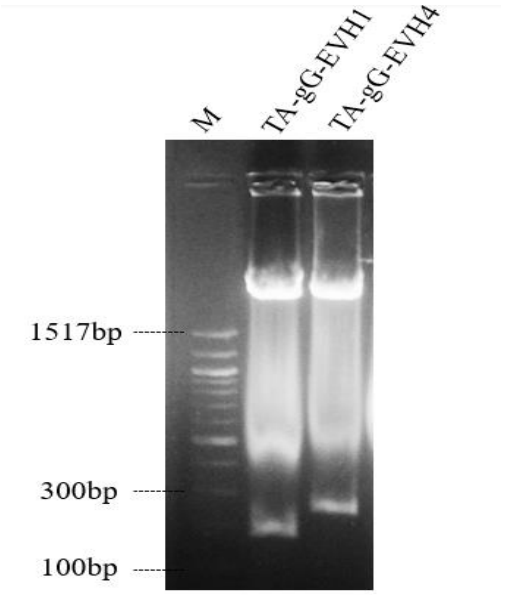

Figure 3.3 – Agarose gel electrophoresis of digested plasmids TA-gG-EHV1 and TA-gG-EHV4. Marker (M) used was Quick-Load 100 bp DNA Ladder from BioLabs.

Two fragments can be observed for each plasmid, one corresponding to the TA fragment or vector (bands with higher molecular weight) and the other corresponding to gG encoding gene fragments. The fragments of 189 bp and 288 bp, corresponding to gG-EHV1 and gG-EHV4, respectively were excised and purified.

Plasmid pET-28a (+) was obtained from cultures containing pET-N8t (a fragment of N8 neuraminidase encoding gene of avian influenza virus) previously obtained in the laboratory. pET-N8t was also cleaved with the restriction enzymes mentioned. The cleavage was verified by 1% agarose gel electrophoresis (Figure 3.4) and the separation of the fragments from the plasmid was confirmed. The fragment of lower molecular weight corresponds to N8 encoding gene, while the fragment of higher molecular weight corresponds to pET28a(+) backbone. The two upper bands were excised, mixed, and purified.

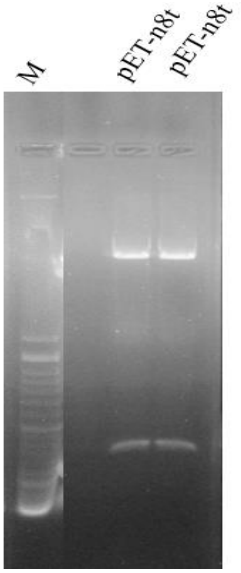

Figure 3.4– Agarose gel electrophoresis of digested plasmid pET-n8t. Marker (M) used was NZYDNA Ladder VI from Nzytech.

- Plasmid pET ligation, cell transformation and clone selection

The fragments of interest were inserted in the pET plasmids obtained using T4 DNA ligase and the resulting recombinant plasmids were transformed into MRF' *E. coli* competent cells. The colonies obtained were inoculated in liquid LB medium with kanamycin for posterior plasmid extraction (Figure 3.5).

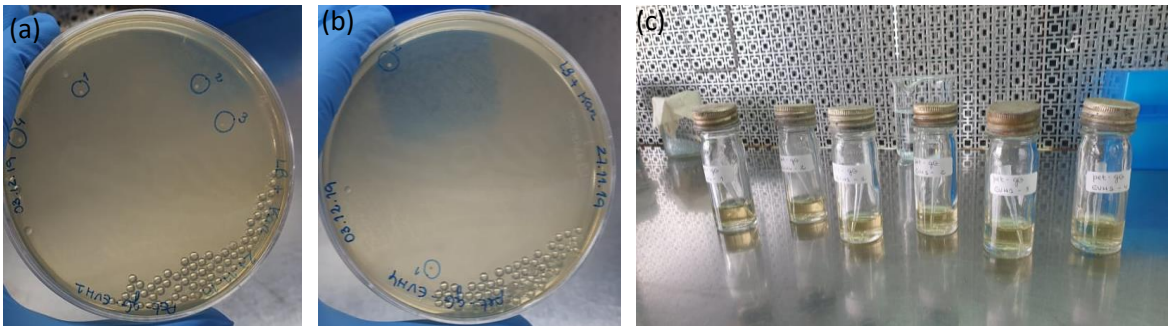

Figure 3.5 – Colonies obtained before cell transformation with the pET plasmid. (a) pET-gG-EVH1 and (b) pET-gG-EVH4. (c) Clone passage to liquid LB medium with kanamycin.

- **Enzymatic digestion**

To confirm cloning, the resulting plasmids were cleaved with EcoRI and XhoI, in order to separate the inserted fragment and verify if it has the expected molecular weight (Figure 3.6).

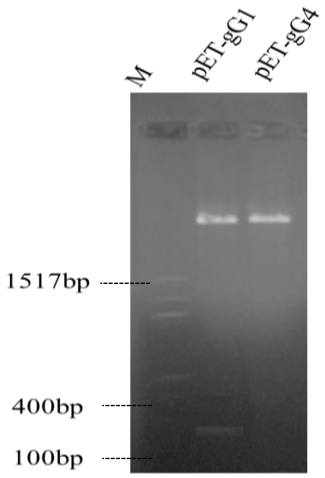

Figure 3.6 – Agarose gel electrophoresis of digested plasmids pET-gG1 and pET-gG4. Marker (M) used was Quick-Load 100 bp DNA Ladder from BioLabs.

Two fragments can be observed for pET-gG1 plasmid, one corresponding to the pET fragment, with higher molecular weight, and the other corresponding to gG1 fragment of 189 bp. However, only the band corresponding to the pET is possible to be seen in the pET-gG4 clone. This result indicates that the clone does not have the insert under study. Therefore, only pET-gG1 was obtained.

- **Sanger sequencing**

Sanger sequencing was performed to confirm the correct ligation between the interest fragment (gG1) and the expression vector pET using the universal primers complementary to the plasmid and primers complementary to the insert. The plasmid pET-gG1 sequenced, confirmed to have the sequence of interest correctly cloned. The sequence obtained can be observed in table 3.1.

Table 3.1 – Sequence from the pET-gG1 plasmid obtained from the selected clone.

Plasmid	**Consensus sequence**
pET-gG1	ATGGGTCGCGGATCCGAATTCGGTGACGAAACATACGACACCA TCCGCGCAGAAGCAAAGAATTTAGAGACCCACGTACC CTCAAGTGCTGCAGAGTCGTCTCTAGAAAACCAATCGACACAG GAGGAGTCTAACAGCCCCGAAGTTGCCCACCTGCGAA GCGTCAACAGCGATGACAGTACACACACGGGGGGTGCGTCGA ACGGCATCCACTCGAGCACCACCACCACCACCACTGA

In order to develop a more specific and sensitive ELISA, a second approach was tested simultaneously. In this, synthetic peptides from another immunogenic portion of both EHV-1 and EHV-4 were used, namely gE1 and gE4 from E glycoprotein described by Andoh *et al*. (2013).

ELISA

- **gE peptide and sera concentration**

The first approach in the development of the ELISA reaction was the determination of the optimal antigen concentration. This was performed by testing different concentrations of the antigen for the coating of the wells, namely 5 µg/ml, 50 µg/ml, 100 µg/ml, and 250 µg/ml. This test was performed using two different concentrations of serum, 1:5 and 1:20, since the detection limit was unknown. The plate scheme is represented in Fig.3.7 with the correspondent absorbance values obtained indicated in table 3.2.

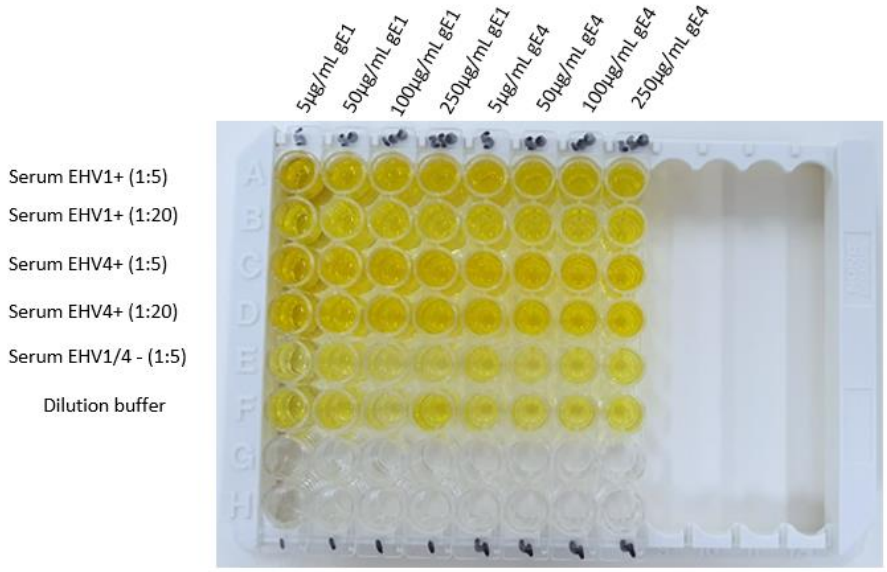

Figure 3.7 Well plate scheme for gE peptide and serum concentrations testing. Four peptide concentrations were tested for each virus (5 µg/ml, 50 µg/ml, 100 µg/ml, and 250 µg/ml). Serum was diluted 1:5 and 1:20 in serum dilution buffer, as indicated in Materials and Methods. Negative controls were performed with a negative serum (Serum EHV1/4 -) and serum dilution buffer.

Table 3.2 – Absorbance results at 450-630 nm from the comparative ELISA between the antigen concentration and the serum dilutions.

	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***
A	1.9040	1.6100	1.7160	1.6680	1.8590	1.4790	1.4070	1.4350
B	1.1590	1.0660	1.1700	0.9660	1.0810	1.1490	0.9550	1.3310
C	2.3840	2.4690	2.4800	2.4810	2.4620	2.4530	2.4290	2.5440
D	2.0350	1.7920	1.9040	1.7450	1.9160	1.9330	1.7990	2.0690
E	0.3760	0.5610	0.3580	0.3910	0.6390	0.4370	0.6000	0.7000
F	0.6550	0.6720	0.3900	0.9090	0.5630	0.5910	0.6170	0.7390

Clear differences were observed between positive and negative sera. However, a high background was observed, with high absorbance values obtained when EHV-1 serum is tested with EHV-4 antigen and contrariwise, indicating that cross reactions have occurred. As expected, the absorbance values obtained were higher when the serum samples were diluted 1:5 instead of 1:20, but the differences observed were not very high, and for that reason, an intermediate dilution of serum samples of 1:10 was chosen for the subsequent steps.

Regarding the concentration of the coating antigen, no significant differences were observed between the different concentrations. It was expected that higher absorbance values were

obtained with higher concentrations of the antigens, what was not observed, indicating that even in the lower concentration the antigen was not the limiting factor. For economic reasons, the lower antigen concentration of 5 µg/ml was chosen for the subsequent experiments.

- **Blocking buffers**

In the previous assay, the blocking step was performed before the addition of the serum samples, as previously described. However, a high background was observed, and for that reason the dilution of the serum samples in blocking buffer was tested. Four blocking solutions were experimented with the aim of reducing this background**.** The blocking buffers tested were 5% powdered milk, 5% BSA, 5% sucrose and, finally, 5% powdered milk plus 5% sucrose, all of them in PBS-T. The plate scheme is showed in Fig. 3.8 and the correspondent absorbances values obtained are indicated in Table 3.3.

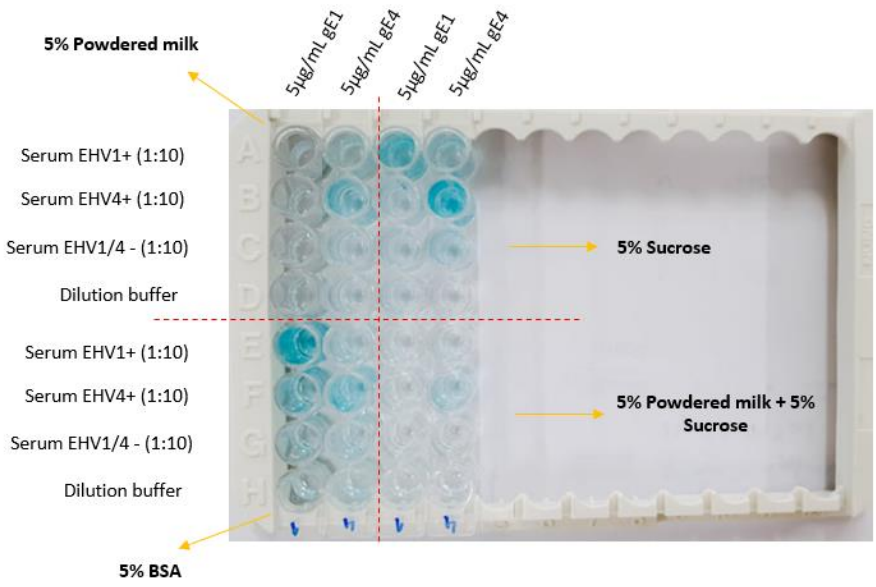

Figure 3.8 – Well plate scheme for blocking buffer evaluation. Four blocking buffers were tested: 5% powdered milk, 5% sucrose, 5% BSA and 5% powdered milk plus 5% sucrose. One positive EHV-1 and one positive EHV-4 serum samples were used. Negative controls consisted in a negative serum (serum EHV1/4 -) and dilution buffer.

Table 3.3 – Absorbance results at 450-630 obtained from the comparative ELISA between blocking buffers composition.

	1	***2***	***3***	***4***
A	0.1680	0.1210	0.4680	0.2510
B	0.1830	0.3990	0.1910	0.7830
C	0.1360	0.1500	0.1870	0.2950
D	0.1710	0.1100	0.1500	0.0650
E	0.6620	0.2110	0.0590	0.0980
F	0.3570	0.5420	0.0760	0.3230
G	0.1950	0.2420	0.0390	0.0820
H	0.1070	0.1500	0.0570	0.0630

Since the aim of this work is the development of a specific ELISA to differentiate between EHV1 and EHV4 positive samples, it was expected that serum EHV1+ originated a positive result with the EHV1 antigen and a negative result with the EHV4 antigen. The opposite result was expected for EHV4+ serum sample. Regarding the negative serum and the dilution buffer, it was expected that minimum absorbance values were achieved. The results obtained showed that powdered milk blocked not only the nonspecific but also the specific ligations, originating very low absorbance values even when homologous sera were used. This blocking was even more noticeable when sucrose was added to the powdered milk. Similar results to the expected were obtained when 5% BSA or 5% sucrose were used as blocking buffer. With both blocking buffers, the absorbance value obtained with EHV1+ serum sample was much higher with the EHV1 antigen (0.662 with BSA; 0.468 with sucrose) than with the EHV4 antigen (0.211 with BSA; 0.251 with sucrose). The opposite result was obtained with the EHV4+ serum sample that originated an absorbance value much higher with the EHV4 antigen (0.542 with BSA; 0.783 with sucrose) than with the EHV1 antigen (0.357 with BSA; 0.191 with sucrose). The negative serum sample and the dilution buffer originated low absorbance values in both cases.

This experiment was repeated, and the results obtained were similar (data not shown). However, the absorbance values for the negative controls were slightly lower when 5% sucrose was used, and therefore this was the blocking buffer chosen for subsequent experiments.

- **Anti-equine concentration**

The anti-equine serum was tested in three different dilutions, 1:5000, 1:8000 and 1:10000 to determine which concentration originated a better result. Serum samples were tested at dilutions 1:5 and 1:10. Figure 3.9 shows the plate scheme and the correspondent absorbances are indicated in table 3.4.

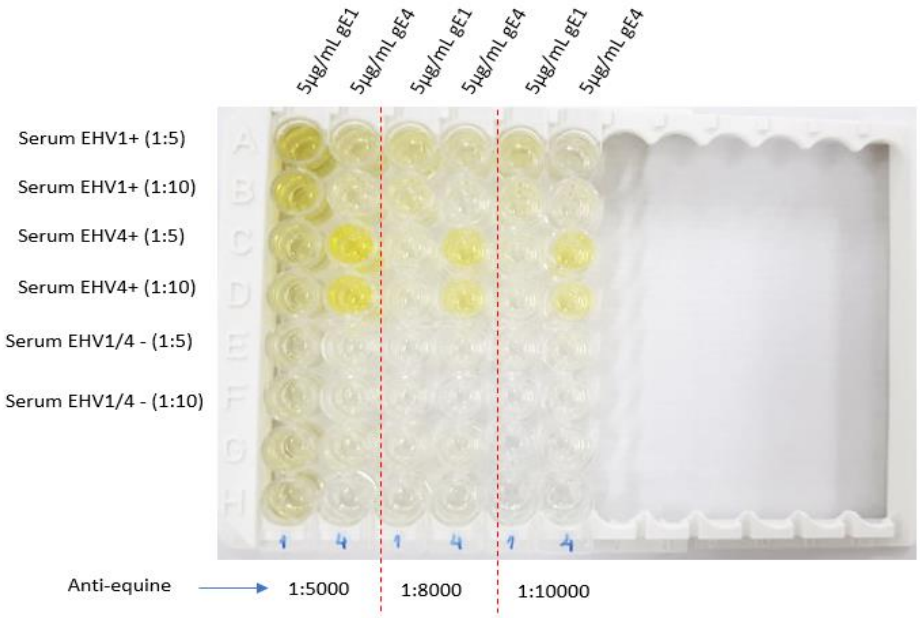

Figure 3.9 – Well plate scheme for anti-equine concentration testing. The conjugate was tested diluted at 1:5000, 1:8000 and 1:10000 in PBS-T. One positive serum sample for each virus was tested in two dilutions (1:5 and 1:10 in PBS-T). A negative serum (Serum EHV1/4 -) was used as negative control.

Table 3.4 – Absorbance results at 450-630 from the comparative ELISA using the anti-equine IgM conjugate in three different dilutions.

	1	***2***	***3***	***4***	***5***	***6***
A	0.3670	0.1610	0.1960	0.1200	0.1770	0.0860
B	0.3490	0.1380	0.1780	0.0650	0.1400	0.1010
C	0.1930	0.5750	0.0960	0.3550	0.0820	0.3450
D	0.1640	0.4830	0.0810	0.3110	0.0640	0.2780
E	0.1010	0.0570	0.0310	0.0510	0.0390	0.0700
F	0.1050	0.0680	0.0380	0.0270	0.0210	0.0580

For the three concentrations of anti-equine serum tested, higher absorbance values were obtained for EHV1+ serum sample with EHV1 antigen than with EHV4 antigen, while for the EHV4+ serum sample, higher absorbance values were obtained with the EHV4 antigen than with the EHV1 antigen, as expected. However, the differences observed were much higher when the 1:5000 dilution was used, indicating that this is the most favorable concentration of anti-equine. The negative serum sample originated low absorbance values in the three dilutions.

The results obtained demonstrated that with the 1:5000 dilution it was possible to detect more clearly the ligation between the antibody and the antigen, as observed by the stronger reaction occurred between the peroxidase in the secondary antibody and the substrate, resulting in higher absorbance values.

- **Anti-equine serum and serum samples dilution**

Several approaches were used in order to reduce nonspecific reactions and consequently the background. As already referred, a previous blocking step was carried out in the first experiments, but it was found that the blocking is more effective when performed simultaneously with the addition of the serum samples. In order to reduce even more the nonspecific reactions, dilution of the anti-equine secondary antibody in the blocking buffer was also tested. For that reason, another experiment was performed diluting serum samples in 2% and 5% sucrose, while maintaining the dilution of the conjugate in 2% sucrose, as shown in Figure 3.10 and Table 3.5.

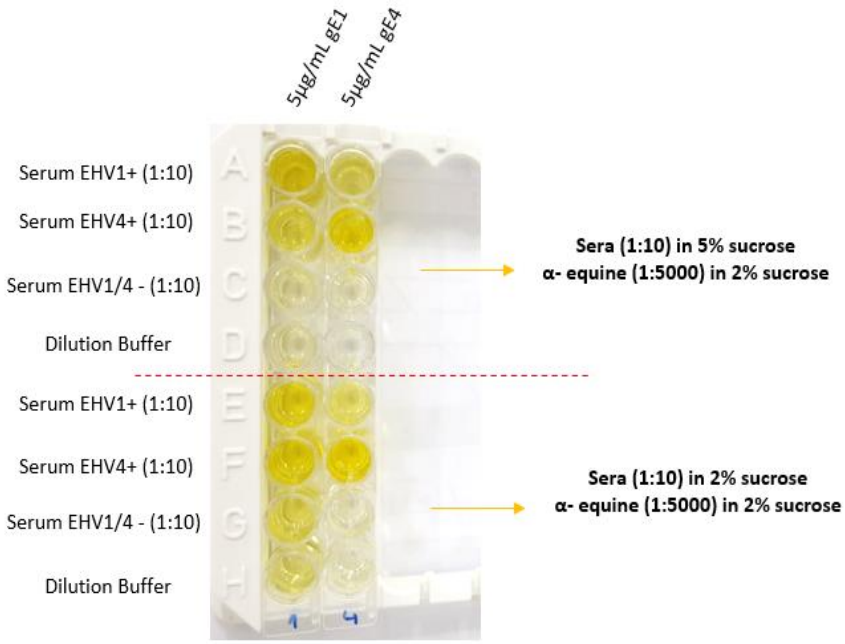

Figure 3.10 – Well plate scheme for sera and anti-equine blocking. One positive serum sample for each virus was used and the negative controls were performed with a negative serum (serum EHV1/4 -) and dilution buffer.

Table 3.5 – Absorbance results at 450-630 from the comparative ELISA using the blocking solution in different concentrations (2% and 5%) to dilute the serum sample and the anti-equine serum.

	1	***2***
A	0.9770	0.3590
B	0.4660	1.3160
C	0.1400	0.1110
D	0.1230	0.0420
E	0.9460	0.3860
F	1.1240	1.7320
G	0.4130	0.1090
H	0.2890	0.1020

Observing the results in Table 3.5, it is possible to see that the serum dilution in 5% sucrose blocking buffer and the dilution of the anti-species in 2% sucrose blocking solution allow to distinguish between the positive sera for each virus combined with lower absorbance values in the negative controls.

In general, the tests performed allowed the optimization of the procedure, necessary to obtain the ELISA test using the gE portion of herpesviruses 1 and 4. The optimized ELISA reaction comprises the following steps:

- Wells coating with 50 µL of 5 µg/ml gE1 or gE4 protein diluted in adsorption buffer.
- Incubation of 2h at 37ºC followed by incubation overnight at 4ºC.
- Washing 5 times with PBS-Tween.
- Dilution of the samples in a 1:10 ratio with 5% sucrose blocking buffer in PBS-T (100 µl/well).
- Incubation for 1h at 37ºC.
- Washing 5 times with PBS-Tween.
- Addition of 100 µl of peroxidase-conjugated anti-horse immunoglobulins diluted in 2% sucrose in PBS-T in a 1:5000 proportion.
- Incubation for 1h at 37ºC.

- Washing 5 times with PBS-Tween.
- Addition of 100 µl of TMB.
- Incubation at room temperature for 15minutes.
- Stop of the reaction with 100 µl of sulfuric acid.
- Reading of absorbance at 450-630 nm.

To validate and implement the technique, a high number of positive and negative serum samples should be tested. It would be necessary to determine the sensitivity and specificity of the test, as well as the repeatability and reproducibility. In order to verify if the developed ELISA could discriminate between EHV-1 and EHV-4 positive serum samples it would be necessary to test a high number of sera with known value both with EHV-1 and EHV-4 antigens. However, until the end of this experimental work, serum samples were not available, and therefore it was not possible to validate the ELISA reaction.

4. Conclusion

The main objective of this work was the development of an ELISA reaction for the identification and discrimination of antibodies against equine herpesvirus, types 1 and 4 (EHV-1 and EHV-4). Two approaches were assayed: one based on recombinant glycoprotein G and the other based on synthetic glycoprotein E peptides. For both approaches, gG and gE fragment proteins of EHV-1 and EHV-4 virus types were intended to be obtained and all of them should be used as coating antigen for the development of the ELISA test. However, the proposed objectives were only partially completed.

For the production of recombinant proteins, an attempt with an expression vector was done, using pET-28a (+) as vector and a DNA fragment of an immunogenic portion of glycoprotein G. The respective sequences for EHV-1 and EVH-4 should be different enough to allow to distinguish between antibodies against these two types of virus. It was possible to obtain the expression vector encoding the glycoprotein G of EHV-1 (gG1), but not that encoding the glycoprotein G of EHV-4 (gG4), during the time of the work. Unfortunately, the experimental work had to end before the expected and for that reason, neither the pET-gG4 was constructed, neither the gG1 recombinant was produced and purified from pET-gG1. The other approach was the use of synthetic glycoprotein E peptides (gE1 and gE4) as coating antigens for the development of the ELISA tests. Several parameters were optimized, namely the concentration of the coating antigen, the serum dilution, the composition and concentration of the blocking buffer and the concentration of the secondary antibody. Also, times and temperatures of incubation were tested. Positive EHV-1 and EHV-4 serum samples were used, as well as a negative serum for both virus types. Two promissory ELISA tests were developed. In the test performed with gE1 as coating antigen, the serum containing antibodies against EHV-1 gave rise to a high absorbance value, while the serum containing antibodies against EHV-4 originated a low absorbance value. The opposite was observed in the test developed with gE4 as coating antigen, as expected. The absorbance values obtained with the negative serum sample were low in both tests. In summary, the two tests proved to be able to distinguish between positive and negative sera, under the conditions described above despite the existence of nonspecific reactions.

The ELISA tests should be validated in terms of specificity, sensitivity, reproducibility, repeatability, and determination of the limit detection. The evaluation of reproducibility and repeatability, as well as the limit of detection, for which, only one serum sample would be

needed was not possible to perform, since, as already referred, the experimental work ended before the expected due to the pandemic caused by the SARS-CoV-2 virus. For the evaluation of the specificity and sensitivity, a high number of positive and negative serum samples of known value would be necessary, and they were not available, which hampered that evaluation.

The difficulty in obtain the vectors and the limitations on available sera, made it impossible to obtain conclusive results and, consequently, an ELISA that could be used in the laboratory in the time allotted for this experimental work. Additional work will be required in order to overcome these obstacles.

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