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CICLO XXXI

Transcriptional profiling of Bovine Herpesvirus 4 infected bovine endometrial stromal cells

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SUMMARY

The research reported in this PhD thesis was published in an international scientific journal:

Virus-Mediated Metalloproteinase 1 Induction Revealed by Transcriptome Profiling of Bovine Herpesvirus 4-Infected Bovine Endometrial Stromal Cells.

Tebaldi G, Jacca S, Montanini B, Capra E, Rosamilia A, Sala A, Stella A, Castiglioni B, Ottonello S, Donofrio G.

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ABSTRACT

Bovine postpartum uterine diseases affect half of all dairy cattle after parturition, causing infertility by disrupting uterine and ovarian function. Female genital tract infections are a primary economic problem for efficient performance of dairy herds, as they cause reduced pregnancies as well as reduced milk production.

Although the etiology of bovine uterine diseases is mainly ascribed to bacterial pathogens, such as *Escherichia coli* and *Arcanobacterium pyogenes*, more extensive diagnostics have in many cases also detected the presence of Bovine Herpesvirus 4 (BoHV-4) in affected cattle, implying that co-infection may be involved in a possible vicious circle.

Following recognition of bacterial lipopolysaccharide (LPS) by the Toll-like receptor 4 (TLR4) expressed at the bovine endometrial epithelium level, expression of immune factors such as prostaglandin E (PGE) and Tumor necrosis factor alpha (TNF-α) are activated. This cytokine cascade triggers BoHV-4 replication by binding specific sites within the immediate early IE2 gene promoter. Conversely, Interferon gamma (IFN-γ) produced by the cells of the immune system, inhibits viral replication, always acting at the level of the IE2 gene promoter.

Although BoHV-4 is often isolated from animals affected by these uterine pathologies, and for this reason, consistently associated with postpartum metritis and chronic infertility in cattle, it is difficult to directly correlate the presence of the virus to the pathology in progress, partly because, the cellular pathways and the molecular mechanisms involved are not well defined, as well as which extracellular stimuli related to the intrauterine environment could influence the BoHV-4 replicative cycle.

In order to shed light on the true pathogenic role and to better investigate the molecular mechanisms of the physio-pathological response underlying the pathogen-host interaction, an experimental BoHV-4 infection was carried out with the aim to optimize infection conditions and a transcriptomic analysis of a pure bovine endometrial stromal cells population (BESCs) infected with BoHV-4 was performed.

The RNA-seq technology used for this purpose allowed to discover a set of differentially expressed genes (DE genes), among which 417 were up-regulated while 118 were down-regulated during BoHV-4 infection.

DE genes have been functionally classified through enrichment functional analysis on the basis of their involvement in various cellular pathways. Many pathways, related to cell proliferation and cell surface integrity, were found to be affected by BoHV-4 infection. Some DE genes that were found to be up-regulated also play a key role in pathogenesis as in the case of IL-8 and MMP-1.

MMP-1 up-regulation was not unexpected, as it belongs to the zinc-dependent endo-peptidases family involved in numerous and important cellular networks. Thanks to the ECM remodeling and immuno-modulation processes, MMPs assume a relevant connotation in the particular context of metritis, where under suitable conditions favor pathogen eradication, inflammatory state resolution as well as tissue anatomical and functional restoration.

Alternatively, pathogenic dysregulation and MMPs hyper-activation leads to host morbidity and mortality thereby favoring pathogen persistence and dissemination, preventing tissue healing and finally defining an immuno-pathological status.

By virtue of the fact that these enzymes play a pivotal role in maintaining the delicate balance between normal and hyper-activated immune response and for the putative involvement of MMP-1 in the bovine uterine diseases development, it was selected as a candidate gene for further studies as a key host factor involved in BoHV-4 pathogenesis.

The *in silico* observations were further corroborated by reverse transcription PCR, real time PCR, Western immunoblotting and finally a luciferase assay where a bovine MMP-1 specific promoter reporter construct was exploited.

BESC cells transfected with the reporter construct and subsequently infected with BoHV-4 showed an increasing luciferase expression with increasing viral load.

BoHV-4 replication is promoted by IE2 gene expression, while the IE2 protein product RTA/50 is able to transactivate both viral and host cell genes, such as those coding for the chemokine IL-8. IL-8 expression in infected BESCs was shown to increase in a time- and dose-dependent manner. This suggested a potential relationship between MMP-1 up-regulation, IE2 gene expression and viral replication.

Following BESCs transfection with an IE2 over-expressing construct, a direct correlation between RTA/50 over-expression and MMP-1 up-regulation was observed. This justified the abnormally high metalloproteinase levels in tissues, and suggested a possible connection to the defective endometrium healing and unresolved inflammation state.

These data, combined with those obtained by transcriptomic profile analysis, represent significant steps toward understanding the vicious cycle mentioned above, more precisely the existing interactions between virus, endometrial layer and host immune response, which sustain viral replication thereby eliciting tissue damage.

Elucidation of the molecular mechanisms and host cell pathways involved in this pathogenesis is central to the discovery of new targets for novel therapeutic treatments based on MMP-1 down-regulation.

RIASSUNTO

Le malattie bovine uterine colpiscono la metà delle bovine da latte dopo il parto, provocando infertilità attraverso la distruzione della funzione ovarica e uterina. Le infezioni del tratto genitale femminile rappresentano un problema economico primario inficiando sulle efficienti prestazioni delle mandrie da latte poiché causano riduzione delle gravidanze e della produzione di latte.

Sebbene l'eziologia delle malattie uterine dei bovini sia principalmente attribuita a batteri patogeni, come per esempio *Escherichia coli* ed *Arcanobacterium pyogenes*, studi diagnostici più approfonditi, hanno rilevato anche la presenza del Bovine Herpesvirus 4 (BoHV-4) in molti dei bovini colpiti, implicando quindi che l'infezione virale possa essere coinvolta in un possibile circolo vizioso.

A seguito del riconoscimento del Lipopolisaccaride batterico (LPS) da parte del recettore Toll-like receptor 4 (TLR4) espresso a livello dell'epitelio endometriale bovino, viene attivata l'espressione di fattori immunitari quali la Prostaglandina E (PGE) ed il fattore di Necrosi Tumorale α (TNF-α). Questa cascata di citochine innesca a sua volta, la replicazione di BoHV-4, interagendo con specifici siti all'interno del promotore dell'Immediate Early gene 2 (IE2). Viceversa, l'Interferon gamma (IFN-γ) prodotto dalle cellule del sistema immunitario, inibisce la replicazione virale, agendo sempre a livello del promotore del gene IE2.

Per chiarirne il vero ruolo patogenetico e studiare meglio i meccanismi molecolari della risposta fisiopatologica alla base dell'interazione ospite-patogeno, è stata condotta un'infezione sperimentale con BoHV-4 al fine di ottimizzare le condizioni di infezione ed in seguito è stata eseguita un'analisi trascrittomica di una popolazione di cellule stromali endometriali bovine pure (BESC) infettate con BoHV-4.

Per l'analisi trascrittomica, è stata applicata la tecnologia dell'RNA-seq che ha permesso di scoprire un insieme di geni differenzialmente espressi (geni DE), tra questi più precisamente, 417 sono risultati essere up-regolati mentre 118 down-regolati durante l'infezione da BoHV-4.

I geni DE sono stati successivamente funzionalmente classificati attraverso l'analisi funzionale dell'arricchimento in base al loro coinvolgimento nei vari pathways cellulari. Molti pathways, come per esempio quelli correlati alla proliferazione cellulare ed all'integrità della superficie cellulare, sono risultati essere influenzati dall'infezione con BoHV-4.

Inoltre, è stato interessante notare come alcuni geni DE, che si è osservato essere up-regolati, svolgano anche un ruolo chiave nella patogenesi, come nel caso del gene dell'Interleuchina 8 (IL-8) e della metalloproteasi di matrice 1 (MMP-1).

L'up-regolazione di MMP-1 non è un dato inaspettato, in quanto MMP-1 appartiene alla famiglia delle endo-peptidasi zinco-dipendenti che sono coinvolte in numerosi e importanti networks cellulari.

Grazie ai processi di rimodellamento della Matrice Extracellulare (ECM) ed immuno-modulazione, le metalloproteasi (MMPs) assumono una connotazione rilevante nel particolare contesto della metrite, dove in condizioni favorevoli facilitano l'eradicazione degli agenti patogeni, la risoluzione dello stato infiammatorio ripristinando funzionalmente e anatomicamente i tessuti coinvolti.

In alternativa, la disregolazione patogenica e l'iper-attivazione delle MMPs portano ad un incremento della morbidità e mortalità dell'ospite, favorendo così la persistenza e la disseminazione dei patogeni, prevenendo la guarigione dei tessuti e definendo infine uno stato immuno-patologico.

In virtù del fatto che questi enzimi svolgono un ruolo fondamentale nel mantenimento del delicato equilibrio tra risposta immunitaria normale ed iper-attivata e per il presunto coinvolgimento di MMP-1 nello sviluppo delle patologie dell'utero bovino, tale gene è stato selezionato come candidato per ulteriori studi come fattore chiave nell'ospite coinvolto nella patogenesi di BoHV-4.

Le osservazioni *in silico* sono state ulteriormente confermate mediante reverse transcription PCR, Real time PCR, Western immunoblotting ed infine un saggio di Luciferasi in cui è stato sfruttato un costrutto reporter caratterizzato dalla presenza dello specifico promotore bovino per MMP-1.

Le cellule BESC transfettate con il costrutto reporter e successivamente infettate con BoHV-4 hanno mostrato una crescente espressione della luciferasi all'aumentare della carica virale.

È noto che la replicazione di BoHV-4 è promossa dall'espressione del gene IE2, mentre il prodotto proteico di IE2, la proteina RTA/50 è in grado di transattivare sia geni virali che quelli delle cellule ospiti, come quelli per esempio che codificano per la chemochina IL-8.

È stato infatti dimostrato come l'espressione dell'IL-8 nelle BESCs infettate aumenta in maniera tempo e dose dipendente, suggerendo quindi una potenziale relazione tra l'up-regolazione di MMP-1, l'espressione del gene IE2 e la replicazione virale.

In seguito alla transfezione delle BESCs con un costrutto over esprimente IE2, è stata osservata una correlazione diretta tra l'over espressione della proteina RTA/50 e l'up-regolazione di MMP-1. Questo dato ha giustificato i livelli anormalmente alti di metalloproteinasi nei tessuti suggerendo inoltre una possibile connessione tra il deficit della guarigione endometriale e la permanenza dello stato infiammatorio.

Questi dati, in associazione a quelli ottenuti dall'analisi del profilo trascrittomico, rappresentano un significativo passo avanti verso la comprensione del circolo vizioso sopra menzionato; più precisamente sulle relazioni esistenti tra virus, strato endometriale e risposta immunitaria dell'ospite che sostengono la replicazione virale, incrementando così il danno tissutale.

La delucidazione quindi dei meccanismi molecolari e dei pathways delle cellule ospiti coinvolti in questa patogenesi è di fondamentale importanza per la scoperta di nuovi target terapeutici basati sulla down-regolazione di MMP-1.

HERPESVIRIDAE FAMILY

The word herpes derives from the ancient Greek “herpein” and is traced to Hippocrates who aimed define the creeping or spreading nature of the skin lesions [1,2]. Pathogenic herpesviruses had been discovered and characterized near the beginning of twentieth century and tissue cultures have been primarily employed to describe their physical, morphological and antigenic characteristics [3]. To date, through the progress in biotechnology and molecular biology fields, more than 100 different virus species were classified inside the “Herpesviridae” family by the International Committee of Taxonomy of Viruses (ICTV) [4]. This family comprises three distinct subfamilies appointed *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* whose host range is rather wide, ranging from mammals to birds, reptiles, amphibians, fishes, and mollusks [5]. The viruses differ significantly with respect to genetic sequence arrangement although they share many common features such as virion structure, replication cycle and biological properties including the ability to establish a latent infection in their hosts. The clinical manifestations of each infection vary considerably, according to cell tropism.

Herpes virions are amongst the most complex viral particles known, since they are characterized by the presence of more than thirty virally encoded proteins and cellular components [6].

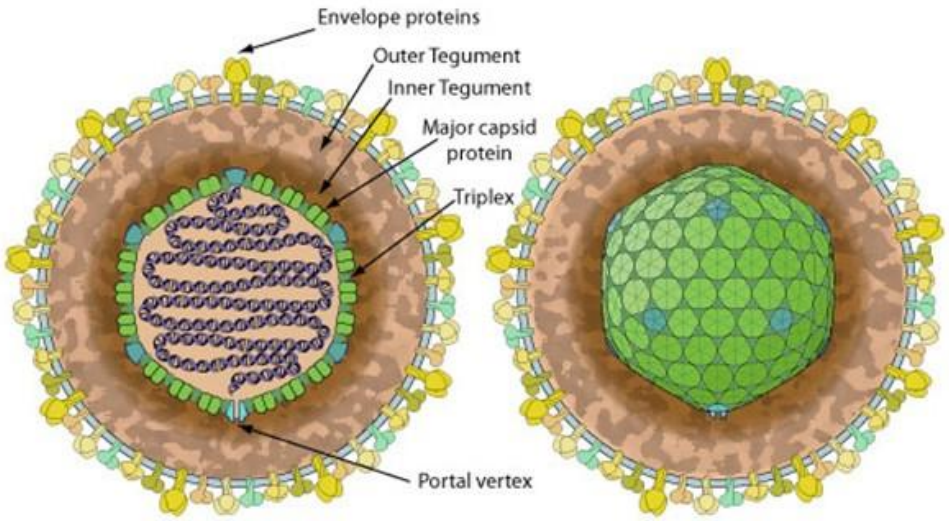


Figure 1. Schematic representation of the structure of the herpesvirus. Adapted from [7].

All viral particles have a size between 120 and 200 nm and a well-defined multilayered architecture. The spherical mature virion consists of four distinct components: a cell derived envelope, a tegument which links the envelope to the underlying capsid and a central core where viral genome is packaged [8].

The bilaminar envelope is the outer covering, acquired during viral egress from infected cells. Considering this provenance, it is not surprising that is composed of host cell-derived lipids containing virus-encoded glycoproteins [9,10].

Envelope proteins must be well assembled during virion maturation, as they are involved in the first step of infection during virus entry, mediating recognition and interaction with their cognate cellular receptors [11] as well as fusion of the virion envelope with the plasma membrane [12]. Beneath the envelope lies a proteinaceus compartment of variable thickness designated Tegument. Historically, it was defined as an amorphous layer, mostly with asymmetric or unstructured organization [8]. Only through increased scientific knowledge, especially cryoelectron microscopy analyses, it was discovered that the tegumentation process involves a complex network of protein-protein interactions through which it follows a highly ordered tegument proteins addition during virion assembly [13].

The inner tegument, in contact with the outer capsid surface, is arranged in an icosahedral symmetry conferring a highly ordered structure [8,10]. Unique to herpesviruses, the inner tegument fulfills several important roles including structural function, by linking the cytoplasmic tails of envelope glycoproteins to the nucleocapsid, ensuring in this manner, the integrity of viral particle [10].

The virus-encoded proteins localized here, promote the beginning of viral replication transactivating the viral immediate-early genes (IE genes) expression, modulating both host cell transduction and innate immunity (e.g., through the Virion Host Shutoff (VHS) protein) [2,13]. Immediately below is located the capsid, a 100-110 nm protein shell with T=16 icosahedral symmetry. Herpesvirus capsid assembly is a conservative process [14] beginning with assembly of pro-capsid, whose subunits are weakly linked due to protein condensation [15].

More in detail, the capsid is characterized by the presence of 162 capsomeres, of which, 150 are hexamers located at the faces and edges, whereas 12 are pentamers set at the vertices [16]. One of these latter vertices is hypothesized to be the way through which the genome enters and leaves the capsid [2]. Within this well-defined structure, is located the core which plays an important role containing and protecting the viral genome[8]. In 1972 Furlong et al., after electron microscopy of negatively stained capsids, observed that the core is characterized by a viral DNA molecule wrapped toroidally around a protein spindle [17,18]. Subsequently, exploiting the electron micrographs technique which allows to better preserve the viral morphology, it was shown that DNA is embedded at high density in liquid crystalline matrix which completely fills the capsid.[8,17,19,20]. Herpesvirus genome consists of linear, double-stranded DNA molecule with a ranging size from about 125 to 240 kbp. In general there is a considerable degree of variation in the DNAs size, structure and G-C nucleotide composition ranging from 32 to 75%, related to other viral species [21,22]. The majority of Gammaherpesvirinae show a lower G-C nucleotide composition compared to the other two subfamilies. This G-C content reduction in herpesvirus was considered to be indicative of latency in dividing cell populations where the latent genome is forced to replicate when host cells divide [17,21]. The Herpesvirus genome includes from about 70 to 200 ORFs (Open Reading Frame). Most of these coding sequences are conserved and arranged in 7 gene blocks encoding for capsid proteins, components of the DNA replication and packaging machinery, control proteins, nucleotide modifying enzymes, membrane and tegument proteins [5]**.**

Another structural peculiarity, which contributes to herpesviruses complexity, is the presence of both unique and long repeated sequences whose number and position differs among subfamilies, genus and species. Roizman and Batterson summarized the herpesviruses genome arrangements in a total of five possible genomic structures as depicted in Figure 2 [23].

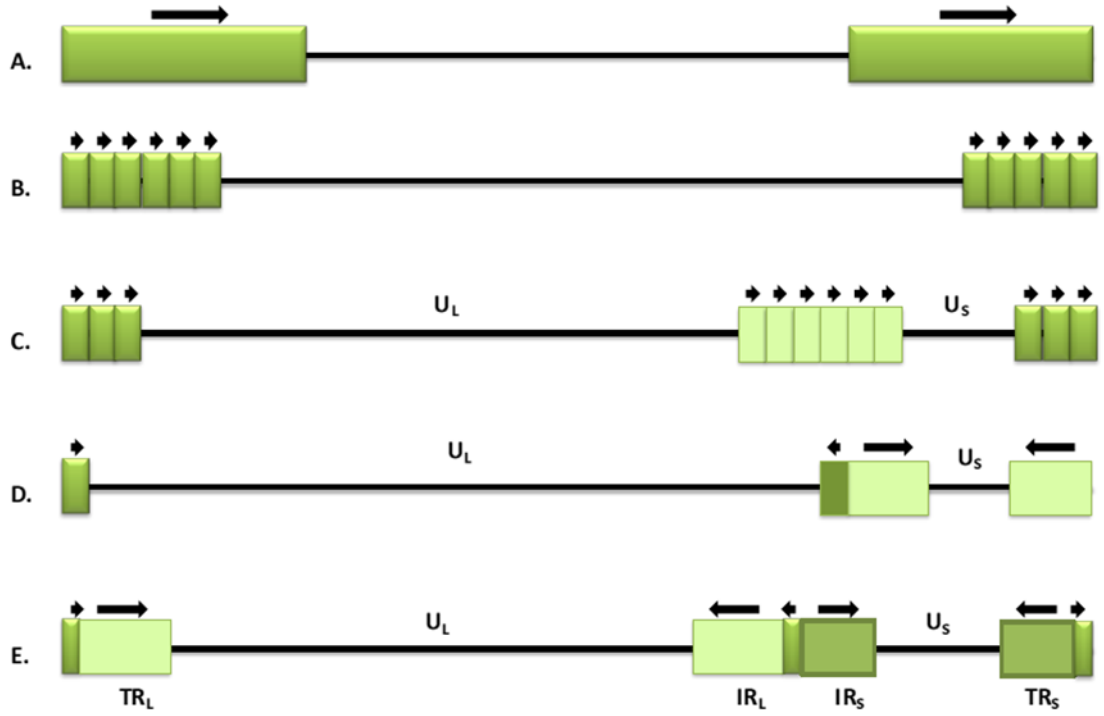


Figure 2. Schematic diagrams representing different genome organizations in herpesviruses. Unique regions are represented by lines, while repeated regions by rectangles. Rectangle of different colors stand for repetition of different sequences. The arrows indicate the orientation of the repetitions. UL: Unique long; US: Unique short; TRS: Terminal Repeat Short; IRL: Internal Repeat Long; IRS: Internal Repeat Short. Adapted from [24].

Structure A shows a unique sequence flanked by a direct repeat that could be larger than 10 kb (HHV-6), or small as 30 bp [Murid herpesvirus 1 (MuHV-1)]; rhadinoviruses in general present a type B structure, with one unique long region flanked by a variable copy numbers of tandemly repeated sequence of 0.8-2.3 kbp.

The class C structure is a derivative of class B, in which two unique regions are divided by a set of direct repeat and flanked by direct terminal repeats. Human herpesvirus 4 (HHV-4) has this arrangement.

Type D structure shows different elements at each terminus, which are present internally in inverted orientation and separate two unique regions. Human herpesvirus 3 (HHV-3) presents this kind of structure. The last one is the most complex genome structure in which terminal reiterations are also repeated in inverted orientations internally. Human herpes virus 1 (HHV-1) and Human herpesvirus 5 (HHV-5) are the viruses with a type E structure. Generally, closely related herpesviruses tend to share the same genomic structure, although exceptions are found [17]. The genome termini are neither covalently closed (as in the *Poxviridae*; [25]) nor covalently linked to a protein (as in the *Adenoviridae*; [26]) [17] but short repeated sequences are present at both ends, allowing viral genome circularization, presumably promoting the viral replication [20].

Herpesviruses are obligate intracellular parasites and to accomplish a productive “lytic” infection, the viral genome must be released into the host cell nucleus. For this to happen, the virus has to cross the cellular membrane firstly.

Enveloped viruses enter the cells through two different pathways. The first involves endocytosis followed by endosome acidification and fusion between viral envelope and endosome membrane [27].The second involves a precise sequence of events basically founded on the receptor binding step, an activation step and a membrane fusion step [28].

To date, it is believed that the majority of all herpesviruses follow the second pathway. To accomplish this goal, unlike the other enveloped viruses for which a single glycoprotein is enough, they appear to exploit a multiprotein entry mechanism.

The complexity of the herpesvirus entry process is represented by the fact that each employed glycoprotein, is multifunctional and could potentially interact with several receptors, other glycoproteins and undergo or induce conformational changes favoring membrane fusion [29].

The highly structurally conserved gB, gH and gL glycoproteins constitute the so called "core fusion machinery" and are present in all herpesvirus subfamilies whereas, in some cases, different herpesviruses, use several non-conserved accessory proteins to provide tropism, triggering the core fusion machinery, regulating membrane fusion [30,31].

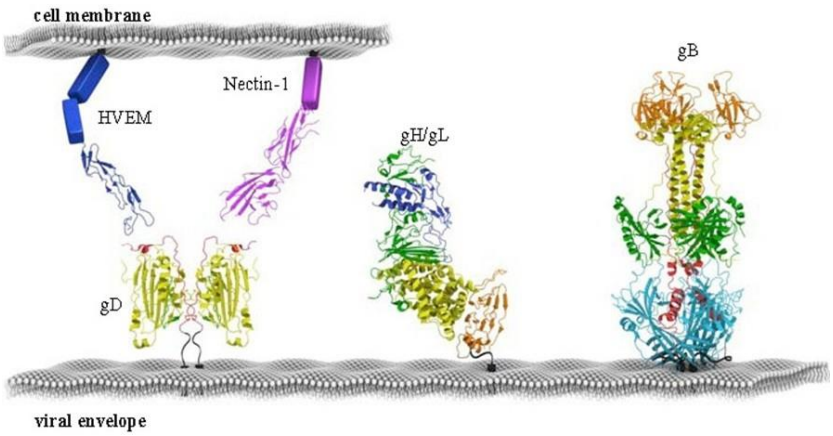


Figure 3. Conserved set of proteins required for HSV membrane fusion. Adapted from [32].

gD is used by alpha herpesviruses (HSV); UL128, UL130, and UL131 by Beta herpesviruses [Human cytomegalovirus (HCMV)] and Gamma herpesviruses [Epstein-Barr virus (EBV)] use the accessory protein gp42 [32]. More in detail: gH-gL heterodimer interacts with host cell receptors both directly and indirectly through additional viral proteins and cognate host receptors, giving rise to a membrane bridging structures leading to a virus-specific tropism.

Many different cell surface molecules can act as receptor favoring the attachment and entry. They include HVEM (Herpesvirus Entry Mediator), a member of the TNF receptor family; nectin-1 and nectin-2, belonging to the immunoglobulin superfamily and specific sites in heparin sulphate generated by 3 O-sulfotransferases activity [12].

As a result of these events, the "entry activator" gH-gL, promptly stimulates gB protein which in turn inserts the fusion loops (FLs) inside cell plasma membrane and undergoes a conformational change thus promoting membrane fusion [28,29].

At this point, the capsid is released into the host cell cytoplasm and transported close to the nuclear pore complexes (NPC) through microtubules (MTs). It has been highlighted that during this step, some of outer tegument proteins dissociate from nucleo-capsid, contributing to modify cell metabolism while on the contrary, the capsid-proximal tegument proteins remain associated until it reaches NPC [33,34].

The entry of viral DNA in association with α-TIF (α-gene Trans Inducing Factor) tegument protein into the nucleus, is followed by a transcriptional cascade, a highly complex process due to the large size of the genome.
Although Herpesviridae members differ in many aspects, the mechanism by which they replicate DNA during lytic infection is largely conserved [35]. This process as well as encapsidation, takes place in specific globular replication compartments as a consequence of cell nucleus reorganization following virus infection [36]. These compartments are involved in the basophilic nuclear inclusion bodies creation which are pathognomonic of herpesvirus infection as well as cell rounding and chromatin margination [37].

DNA synthesis firstly starts through a Theta mechanism, which is subsequently converted in a rolling circle mechanism thereby leading to the concatamers production. The processing of the latter in the final viral genomes length, occurs only during DNA packaging in the pre-formed immature capsid [16].

Every cellular factor that is useful to the virus is exploited while other factors and pathways are degraded or inactivated [36]. Since they lack their own translational apparatus, herpesviruses have developed several strategies to hijack cellular DNA and RNA polymerases, topoisomerases, histones, transcription factors and splicing factors. The fact that they are completely addicted to the host cell protein synthesis system, imposes restrictions that are pivotal to virus biology. Failure to translate viral mRNAs and to modulate host mRNA translation would have tragic consequences for virus replication, spread, and evolution [38]. Thanks to the tegumental viral protein VHS (Virion Host Shutoff) activity, the host mRNA translation is highly compromised in favor of the viral one resulting in the expression of over 80 viral proteins [37].
The transcriptional process guided by the host RNA polymerase II, leads to the production of three distinct class of proteins whose entire gene expression is finely controlled through a feedback mechanism.
Herpesvirus transcripts could have common initiation signals but different termination sites, different initiation sites but co-terminal ends, or differ in their initiation and termination sites but have partially collinear sequences [2].

Immediate early proteins (IE), otherwise known as alpha proteins, are the first protein class produced within 2-4 hours post infection and are involved both in transcriptional regulation, in beta protein synthesis control and simultaneously their own expression. IE proteins inhibit cellular DNA synthesis and arrest cell cycle. Beta proteins include DNA polymerase, helicase, thymidine kinase and transcription factors taking part in DNA replication. It was observed that are also involved in IE gene down-regulation. Alpha and beta proteins together are able to regulate the gamma genes expression. Most of the Gamma proteins, also known as late proteins, are structural component whose production begins after viral DNA synthesis and takes part in capsid assemblation inside the nucleus [2,16,20,37].

Virion assembly and maturation continues in the cytoplasm [39]. Virion assembly and egress represent an essential stage of herpesviral lytic replication, since are indirectly involved in herpesvirus pathogenesis [13].

It's known that this event proceeds from the nucleus to the cytoplasm but the exactly mechanism trough which this happens, has been questioned for a long time [40]. Several models have been proposed: the first one, also known as the single envelopment pathway, was supported by Johnson and Spear study [41], where virion leaves the nucleus crossing outer nuclear membrane by becoming encased in vesicles–vacuoles characterized by immature oligosaccharides . Along the exocytic or secretory pathway, vescicles reach the Golgi apparatus where viral glycoproteins undergo a maturation process. The mature virion is now ready to leave the cell environment, fusing its own membrane with the plasma membrane. Stackpole proposed the second and most widely accepted model within the last 10–15 years, called the envelopment-deenvelopment–reenvelopment pathway [42–44].

The capsid initially buds at the inner nuclear membrane (envelopment step), once in the perinuclear space, the viral particle fuses its membrane with the outer nuclear membrane, releasing naked capsid into the cytoplasm (deenvelopment step). Here cytoplasmic capsid move along microtubules to vesicles derived from the trans Golgi network or endosomes [45], where a second, and final, round of envelopment takes place. There, capsid acquires its lipid envelope and the tegument layer (reenvelopment step) [44].

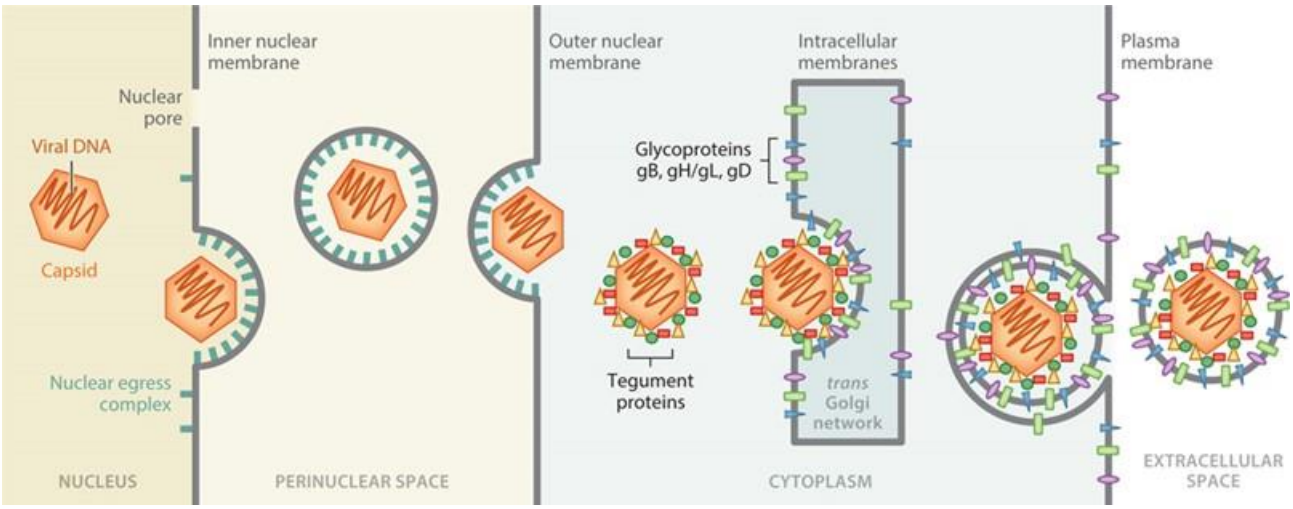


Figure 4. Schematic representation of herpesvirus egress. Adapted from [44].

The production and release of viral progeny is generally followed by irreversible host cell damage.

One of the herpesvirus hallmark is the ability to persist in their host after primary infection thereby establishing a latency state. Although the term latency is conventionally referred to the silent maintenance of the virus inside the body, not detectable by normal virological procedures [46], recent evidence shed light on that it is more dynamic and complicated than initially believed. In this state, viral genome is circularized and maintained in episomal manner within host cell nucleus, where viral gene expression is thought to be silenced or at most limited to the production of few proteins [47]. Albeit latency establishment is characterized by viral DNA strictly associated to particular histone types and finely checked by host cell epigenetic mechanism, it was observed that virus express a limited and distinct set of viral proteins and non-coding RNA known as the latency-associated transcript (LAT) [39]. This behavior is the result of a delicate balance through which virus try to avoid immune system and to keep itself for a long-term period especially when host

cell environment is not suitable for productive lytic infection establishment. In this manner they could reactivate as soon as a better condition is restored [47].

Alphaherpesvirinae

The main features of this subfamily are short replication cycle (12-48 hours), a variable host range, the capacity to grow rapidly in a wide variety of cell culture causing lysis with the formation of syncytia and inclusion bodies. They typically establish latent infection in sensory nerve ganglia. Some examples belonging to this subfamily are Human Herpesvirus-1 (HHV-1 otherwise known as Herpes Simplex virus 1), Human Herpesvirus-2, Varicella-zoster virus (VZV otherwise known as HHV-3, Human Herpesvirus-3), Bovine Herpesvirus-1 (BoHV-1) and Caprine Herpesvirus-1 (CpHV-1) [16,37]

Betaherpesvirinae

This subfamily is distinguished by a slow replication cycle, close host range and latency in secretory glands, monocytes, kidney and other tissues. Unlike alphaherpesvirus, cytopathic effect is represented by the presence of inclusion bodies with considerable enlargement of the affected cells (cytomegaly phenomenon) [16]. Four genera belong to this subfamily: roseoloviruses, probosciviruses, muromegaloviruses and cytomegaloviruses (HCMV), also Known as Human Herpesvirus-5 (HHV-5) [37]. This last one has a high prevalence and is responsible of severe and fatal diseases in immunologically immature or compromised patients, like mental retardation in foetus, pneumonitis in transplanted or AIDS-affected patients. This viral class presents the major evolutionary and genetic diversity if compared to the other two [26].

Gammaherpesvirinae

Members of this subfamily have a restricted host range, latency in lymphocytes or in lymphoid tissues occasionally involving in oncogenesis; furthermore many of them do not easily replicate in cultured cells [48]. Two genera belong to this subfamily: Lymphocryptovirus (or gamma-1-herpesvirus) and Rhadinovirus (or gamma-2-herpesvirus). One of the most important member of Lymphocryptovirus is Human Herpesvirus-4 also known as Epstein Bar Virus (EBV) which is the etiological agent of infective mononucleosis, and it is also associated with oncogenic potential in lymphoid tissues. Within the Rhadinovirus genus belong a wide number of viruses of interest for medicine, biomedical research and veterinary medicine, like for example Saimiriine Herpesvirus-2 (SaHV-2) in monkeys, Equid Herpesvirus-2 (EHV-2) in equines, Murid Herpesvirus-4 (MuHV-4 or Murine gammaherpesvirus-68, MuHV-68) in mice, Bovine Herpesvirus-4 (BoHV-4) in cattle and Human Herpesvirus-8 (HHV-8 or Kaposi's Sarcoma associated Herpesvirus, KSHV) in man [48].

Generally, Herpesviruses have a global distribution and they produce lesions of varying severity in both animals and men, ranging from localized vesicular eruptions on surface epithelia to diffuse wide spread damage in the respiratory, digestive and genital mucosal tract; from localized giants cell proliferation in glandular epithelium to necrosis of liver,

lymphoid and other tissue; from specific neural damage to diffuse meningo-encephalitis. In addition to this, they are able to determine lesions in the female genital tract representing a possible danger in case of pregnancy; in fact the infection of the fetus may lead to fetal death, abortion or in new borned diseases [24].

BOVINE HERPESVIRUS-4 (BoHV-4)

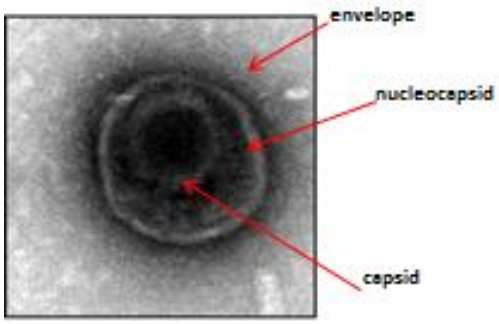


Figure 5. BoHV-4 particle as appear at electronic microscope. Adapted from [49].

GENERAL ASPECTS: nomenclature and classification

Bovine Herpesvirus 4 (BoHV-4) is a member of the Herpesviridae family and now, thanks to recent molecular data, it is possible to say with certainty that it is a *Gammaherpesvirus* belonging to *Rhadinovirus* genera.

The first discovered BoHV-4 dates back to 1963 in Western Hungary when Bartha and coworkers isolated a herpesvirus-like agent from bovine with pneumo-enteritis and kerato-conjunctivitis diseases. Its inclusion in the Herpesviridae family occurred only after viral particle morphologic properties observation under electron microscope.

In 1971 in United States, Mohanty encountered a herpesvirus strain highly similar to that discovered by Bartha. Since these isolates were antigenically closely related, sharing similar restriction patterns [50–53] and cross-hybridization [54], the discovery of two herpesvirus prototype strains designated like Movar 33/63-like (for European one) and DN 599-like (for American one) respectively, has been hypothesized.[55–58].

From its first discovery, several antigenically related herpesviruses were isolated with worldwide distribution from a variety of clinical syndromes as well as from apparently healthy cattle [59]. Since they showed several features which were not common to other herpesviruses, like for example latency sites, persistence in serum, dividing cells necessary for virus replication and the ability to infect a wide host range without causing illness [57], the exact classification in this family was uncertain and confuse.

For at least 20 years these various isolates were called in the literature with different names as “orphan herpesvirus”, “Movar-type herpesviruses”, Bovid cytomegalovirus”, “Bovid herpesvirus 3 (BHV-3)”, “Bovid herpesvirus 5 (BHV-5)” by some American authors. The first effort in order to limit the nomenclature confusion was made by Bartha in 1987 who suggested to group all these “Movar-type” isolates sharing serological, biological and genetic similarities under the name of “Bovid Herpesvirus-4 (BHV-4)”. More recently, the official Bovine Herpesvirus 4 (BoHV-4) designation was universally established by the International Committee on Taxonomy of Viruses in 2000 [60–62]. Another source of

confusion and misunderstanding was related to the fact that BoHV-4 was initially classified into Betaherpesvirinae subfamily for its biological properties in cell cultures resembling those of human cytomegalovirus [51].

This initial hypothesis was corroborated by the fact that the all studied BoHV-4 strains, showed identical cytopathogenic as well as plaquing properties, and the restriction enzyme pattern of their DNA was virtually identical to that of a previously described bovine cytomegalovirus [51].

An *in vitro* growth attenuation was also observed as well as the presence of both cytoplasmic and nuclear high density inclusion bodies and the formation of giant cells after infection [61,62]. However, subsequent studies and the progress in molecular biology field, have shed light on that BoHV-4 is genetically more similar to gammaherpesvirus. Noteworthy, was the fact that the genome size is smaller than Betaherpesvirinae and has a B type structure which is a gammaherpesvirus hallmark [63].

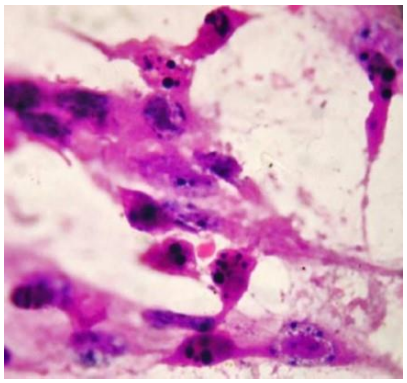


Figure 6. Intranuclear inclusion bodies in MDBK cells. May Grünwald-Giemsa (magnification 1000X). Adapted from [64].

In contrast to alpha and Betaherpesviruses, the genome is generally deficient in CpG dinucleotides, which is another gammaherpesviruses hallmark (during latency state) as well as the presence of thymidine kinase, absent in Betaherpesviruses [17]. In addition to that, all BoHV-4 genes analyzed so far, encode amino acid sequences which have a higher identity percentage with gammaherpesviruses Epstein-Barr virus (EBV) and Herpesvirus Saimiri (HVS) proteins than to the homologous products of the Alphaherpesviruses varicella-zoster virus and herpes simplex virus type 1 or the Betaherpesvirus human cytomegalovirus.

Even though BoHV-4 genome organization shows a similar co-linear arrangement with those of the EBV and HVS, this aspect is markedly evident in HVS suggesting that BoHV-4 is phylogenetically more closely related to gamma-2 herpesviruses (or *Rhadinovirus*) than gamma-1 herpesviruses (or *Lymphocriptovirus*) [62,65].

GENOME

Over the last years, thanks to molecular biology techniques development, the entire BoHV-4 genome was completely sequenced [66–68]. This process, in association with phylogenetic analyses, allowed to confirm the biological properties previously observed i.e. BoHV-4 is a gamma herpesvirus belonging to rhadinovirus genera and that is more similar to HVS than EBV [69].

The genome structure is typical of group B herpesviruses [70] with a double stranded DNA molecule of 144± 6kb characterized by the presence of a central Long Unique Region (LUR) of approximately 108 kb, with lower G+C content and flanked by a high G+C content region designated as polyrepetitive DNA tract (prDNA) or Heavy DNA (H-DNA) or terminal tandem repeats.

The 58% of the total genome is organized into five blocks of genes [57], numbered from 1 to 5 according to the 5' to 3'orientation whose overall arrangement is identical for all subfamily members but different among subfamilies. [69].

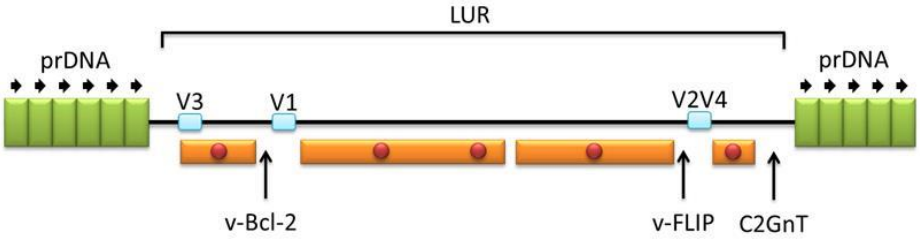


Figure 7. BoHV-4 genomic organization (the orange bars represent the 5 conserved blocks within the LUR; V1-V4 the variable regions between strains). Adapted from [62] .

More precisely, within the 1, 2 and 4 blocks are grouped genes which are conserved among all three herpesvirus family and only within the 3rd and 5th are present genes which are typical of gammaherpesviruses, in particular in Saimiriine Herpesvirus 2 (SaHV-2) and EBV genomes.

It was also observed that both the genetic content and length of the "interblock" regions are specific of each virus [62,64].

The bovine herpesvirus-4 (BoHV-4) group is represented by antigenically related viruses collection, distinct from the other bovine herpesviruses, and all of which follow this schematic genomic organization [59]. Even though, all of them follow this schematic genomic organization, three major differences: two in the LUR and one in the prDNA unique region were detected. The genome analysis exploiting *EcoRI*, *BamHI*, *HindIII* restriction endonuclease activity as well as λ and plasmid library, allowed to identify three distinct categories.

The main variances were observed after *HindIII* digestion and all the strains with a restriction profile similar to the American isolate are DN-599-like strain, all the strains with a restriction profile similar to the European isolate are MOVAR 33/63-like strain and the strains showing a specific enzymatic restriction pattern are "not classified" [62].

prDNA structure and function

The two stretches of tandem repeats flanking LUR have a general length of 2267 bp with a G+C content of approximately about 71%. The number of prDNAs varies at each end of the genome, but the overall number is relatively constant, about 15 prDNA units per genome (for the 66-P-347 strain) [70].

Every prDNA unit is characterized by the presence of two different kinds of repeated sequences: the first one, of about 196 bp which in turn contains three complete and one incomplete direct repeat, is located at the 3' terminus, while the second one of 25 bp, is repeated twice and situated at the 5' terminus [62,66].

The size of prDNA unit is in general strain-specific; however, small fractions may vary within one strain [58]. Up to eight different sizes of prDNA, ranging from 1450 to 2850 bp, have been observed and seem to be related to the presence of a variable number of 200 bp fragments inside the H-DNA unit which is probably involved in recombination events and for this reason called "hot spot" [64]. This fact should not be surprising since the involvement of G-C rich tandem repeats in the eukaryotic genome, in these kind of processes, is well established [58]. In agreement with Chang and Van Santen (1992) observations, no open reading frame or significant homologies to known proteins were detected through database analysis in prDNA [66]. Different studies allowed to assure that these polyrepetitive sequences are involved in excision and packaging genome process thereby influencing the viral genome size [64] [71] and it was also observed that only one prDNA is enough for cleavage and packaging**.** More precisely, specific sequences called pac-1 and pac-2 located at the 5' and 3' prDNA extremities respectively, are involved in this process. This data is corroborated by previous evidences, in fact, these same signal sequences in herpes simplex virus type 1 (HSV-1) performe cleavage and packaging of viral DNA. Subsequently pac-1 and pac-2 were discovered at the genomic termini of all herpesviruses [66,72]. Not only prDNAs play a key role during rolling circle replication but they seem to be involved in additional functions.

Collins and co-workers showed for the first time that in gamma-2 herpesviruses, intact and multiple prDNAs are essential for the maintenance of episomes in latently infected T-cells. This is probably related to the presence of cis-acting sites in prDNAs which are necessary for latency-associated nuclear antigen (LANA) binding, thereby promoting the attachment to mitotic chromosomes and at the same time the episomal maintenance and itself segregation to progeny cells [73,74].

LUR structure and function

The complete BoHV-4 LUR sequencing was performed through shotgun-cloned viral DNA fragments whose assembly, leaded to a 108.873 bp final consensus sequence, characterized by a G+C content of about 41.4 % [67]. One of the Gammaherpesviruses hallmark, in contrast with Alpha and Betaherpesviruses, is to possess a low CpG dinucleotide frequency with a compensating excess of TpG and CpA content. In BoHV-4 genome, in particular, is recorded a percentage of 1%. Since herpesviruses are lacking of DNA methylation systems, typically belonging to higher eukaryotes, through which they control their gene expression; this feature could reflect the latency phase in dividing cell population and forcing the enter in replication state as host cells divide [62,75].

From gene blocks organization point of view, BoHV-4 is strictly resembling to SaHV-2 than EBV. This evidence is corroborated by different data: the length of BoHV-4 conserved gene blocks is more closely related to the HVS block length than to EBV as well as the space between gene blocks, especially between blocks 3 and 4 and blocks 4 and 5. Lastly, no large internal repeats were found between blocks as in the case of EBV genome [69].

Within the LUR, two regions of multiple direct repeats otherwise known as R1 and R2, were detected. The first one is characterized by several stretches of complete and incomplete direct repeats of 23, 25 and 65 bp in length located at 21858 until 22631 position; R2 in turn, includes 2 different repeat stretches called R2a and R2b. It was observed that also in this case, both the length as well as the sequence and location are peculiar for every BoHV-4 strain.

R2a has 28 perfect and 3 imperfect direct repeats of 22-23 bp whereas R2b has several different repeats ranging from 8 up to 68 bp in length as well as one inverted repeat with a hairpin-loop predicted structure. Interestingly R2 is characterized by a considerable change in G+C content: indeed, whereas R2a contains a high G+C content of 71%, the 750 bp sequence stretch directly upstream of R2 has a lower G+C content corresponding to 30% [67].

Through a further extensive analysis of 60 kb genome stretch, encompassing 45% of both LUR and five prDNA units, a BoHV-4 origin of replication (*Ori*) with positional homology to lytic origin of replication of other gammaherpesviruses, was mapped. More precisely, the Ori is localized in R2b stretch, downstream of the ORF 69 and close to a G+C-rich region, partially overlapping with the Bo11 gene and including the Bo12 one [62,67,76]. To date, the hypothetical involvement of these genes in viral DNA replication has to be ascertained. BoHV-4 sequence revealed the presence of at least 79 open reading frames (ORF); 62 of them are homologues to SaHV-2 and HHV-8 ORFs and for this reason identified from ORF1 to ORF79, while only 17 seem to be unique of BoHV-4 and consequently named from Bo1 until Bo17 (following 5'-3' orientation) [62].

In the LUR central part, 54 genes (ORFs 16 to 69) having the same position in HVS, were identified. Only Bo9 and Bo10 have both a different position and orientation if compared to the 28 and 51 ORFs of SaHV-2 [62]. In all herpesviruses sequenced until now, several differences were observed, like those inherent to the presence and/or position of ORF 16 (v-Bcl-2) as well as the number of individual or subgroup-specific genes between ORF 50 and ORF 69. It was also discovered that BoHV-4 is missing of homology to HVS ORFs 1,2,4,5,11 to 15,28,51,70,72, and 74. Noteworthy, all Rhadinoviruses lack some or all of these genes, except for the ORF 11 which seems to be present in everyone [62,67].

In the previously mentioned ORFs, several homologues genes to those cellular are present and it was already showed that they are involved in several cellular functions, such as cell growth or cell survival, nucleotide metabolism, and in immune escape. These genes which have been acquired from host genome in the past, probably played a crucial role during herpesviruses evolution. Comparing BoHV-4 genome to those of SaHV-2 or other gammaherpesviruses with renowned transforming capacity, seems to be "exiguous"; in fact, BoHV-4 has no genes coding for cytokines or cytokines receptor (contrary to SaHV-2, HHV-8 and EHV-2), for interleukin receptors (contrary to SaHV-2, HHV-8, EHV-2 and MuHV-68) for chemokines or viral macrophage inflammatory protein α/β (while HHV-8 has) [67].

Taking into account all these aspects, BoHV-4 reminiscents Alcelaphine herpesvirus 1 (AIHV-1), more than SaHV-2 or HHV-8 [62] [77].

Cyclin D or complement regulatory proteins are absent, as well as genes for dehydrofolate reductase and thymidilate synthetase, contrary to SaHV-2 and HHV-8 where both are present [67,78].

To date, BoHV-4 has never been associated with lympho-proliferative disorders or with transforming capability generally attributed to other Gammaherpesviruses like HHV-8 or MuHV-68, however there is no definitive evidence and more studies have to be performed, since two ORFs with potential implication in cell survival: v-Bcl-2 like (viral B cell lymphoma) (ORF16) and a v-FLIP (viral FLICE Inhibitory Protein) (ORF17), have been discovered in BoHV-4 genome. These genes are known to have a role in preventing apoptosis, one of the early stage of cellular transformation. Many viruses in fact, encode proteins which inhibit apoptosis thereby promoting persistent infection or prolonging the survival of infected cells with the aim to maximize viral production. GUANG-HUA WANG and co-workers showed that BoHV-4 BORFE2 protein inhibits Fas- and TNFR1-induced apoptosis (tumor necrosis factor receptor 1-induced apoptosis) and, like other gamma-2 herpesviruses, contains two DEDs (Death Effector Domains).

Fas (FLICE Associated Signal) is important for cytotoxic-T cell killing of virus-infected cells while TNF can kill virus infected cells directly.

The exact mechanism through which this inhibition happens is uncertain but recent data suggest two different possibilities. In the first case, BORFE2 interaction with the caspase-8 pro-domain may inhibit the latter to bind FADD, preventing in this way, caspase activation. In the second one, the interaction of BORFE2 with the caspase-8 pro-domain could induce a conformational changing in pro-caspase-8 directly preventing its activation. Studies are currently in progress to distinguish between these two possibilities [79].

However, even if Bellows et all observed that BoHV-4 v-bcl-2 have lost the pro-apoptotic activity counteracting caspase 8 cleavage [80]; Sciortino demonstrated for the first time that apoptosis was the only detectable form of cytopathic effect in several permissive cell lines for BoHV-4, during the late stage of infection. The productive infection of BoHV-4, like in the other γ-2-herpesvirus, seems to be associated with apoptosis and not with a lytic phase, despite the presence of anti-apoptotic genes [81].

BoHV-4, like all herpesviruses, induces in an independent manner, the synthesis of nucleic acids through ORF 21 coding for thymidine kinase (TK) mainly during the early gene expression phase [82].This coding region of 1335 nucleotides

long, has a great homology to those of HVS and is involved in pyrimidine metabolism [83]. Zhang and Van Santen proved that BoHV-4 TK promoter-regulatory region was specifically transactivated by IE2 gene product [84].

ORF 3 and ORF 75, located in the opposite direction at LUR extremities, code for v-FGAM (viral phosphor ribosyl formyl glycinamidine synthetase). These genes upgrade the amount of available nucleotides in the infected cell thereby favoring not only viral replication but also cell proliferation [67].

ORF 29 codes for a terminase involved in DNA cleavage and encapsidation. This gene is present and spliced in all herpesviruses [66].

This ORF is not the only one to be spliced. Compelling evidences showed that other several genes undergo this process like Bo4, Bo5, Bo10, Bo11, ORF 29 (previously mentioned), ORF 50, ORF57.

Bo4 and Bo5 have partial amino acid homology and may be involved in differential splicing. As described by Van Santen, Bo4 and the spliced Bo5 are part of the major immediate-early transcript IE1 [85].

Bo10 is expressed as a late gene during BoHV-4 infection encoding for a 180-kDa envelope glycoprotein. Machiels and co-workers discovered that through alternative splicing it is possible to obtain two different mRNAs from BoHV-4 Bo10 gene, thereby producing virions, which are phenotypically distinct and exhibit specific cell tropism [86].

Bo11 is encoded by a previously described IE2-transactivated, spliced, 1,1 kb late RNA [87]**.**

ORF50 product was shown to be a putative R transactivator, encoded by the spliced IE2 [88].

ORF57 gene product seems to be involved in the spliceosome complex redistribution during HVS infection. This process, in turn could have a relevant role upon the splicing capability of a cell and consequently on the processing of viral intron-containing mRNA [89].

One of the genes that distinguishes BoHV4 from all other viruses, is Bo17.

The Bo17 gene, located at the right end of the LUR, is the only virus gene known to date, that encodes a homologue of the cellular core 2 b-1,6-N-acetylglucosaminyltransferase-mucine type (C2GnT-M) [77]. This viral gene product shows an 81.1% similarity with the human homologous enzyme [90].

It is known that the b-1,6-N-acetylglucosaminyltransferase (b1,6GnT) gene family encodes enzymes playing crucial roles in glycan synthesis and if eventually mutation events happen, lead to pathological processes, such as development, immunodeficiency, and oncogenesis [91].

By virtue of these considerations, it can't be completely ruled out a possible BoHV-4 involvement in the lymphoproliferative or transforming phenomena onset, under certain conditions. Through Gorioynoff Phylogenetic analyses, executed on 34 different BoHV-4 strains, isolated from four different continents and different animal species, Bo17 seems to be the most-recently acquired gene. The data analysis showed that the Bo17 gene was acquired from *Syncerus* lineage rather than *Bos* lineage, about 1.5 million years ago and that, after probable gene fixation in the viral population through natural selection, the virus was transmitted from an African buffalo recent ancestor to the cattle ancestors, about 700,000 years ago [77].

Not only Bo17 expression product has all three enzymatic activities exhibited by cellular C2GnT-M, i.e., core 2, core 4, and I [91] but Lété and co-workers' studies showed that two different mRNAs are encoded by the Bo17 gene, contrary to the cellular enzyme.

The first corresponds to the entire coding region while the second one, results from the splicing of a 138-bp intron encoding critical residues of the enzyme. The two recombinant strains realized in order to investigate the possible functions of these two different mRNAs, didn't reveal any replication differences and no activity is associated to the spliced form as showed by the enzymatic assays [92].

So far, BoHV-4 is the only virus known to encode this gene which is expressed during the early phase and despite it was observed that is dispensable for *in vitro* viral replication, several hypotheses were postulated in order to explain its role in BoHV-4 biology.

It could contribute, *in vivo,* to post-translational modifications of viral structural proteins by the addition of glycans, thereby affecting the tropism of the virion and/or the susceptibility of the virus to antibody neutralization and/or complement lysis and/or cell-mediated cytotoxicity.

These modifications could favor the viral escape from immune response [93]. This should not be surprising since, during co-evolution with their hosts, viruses have developed different mechanisms to hijack, mimic or sabotage host processes to their advantage [94,95].

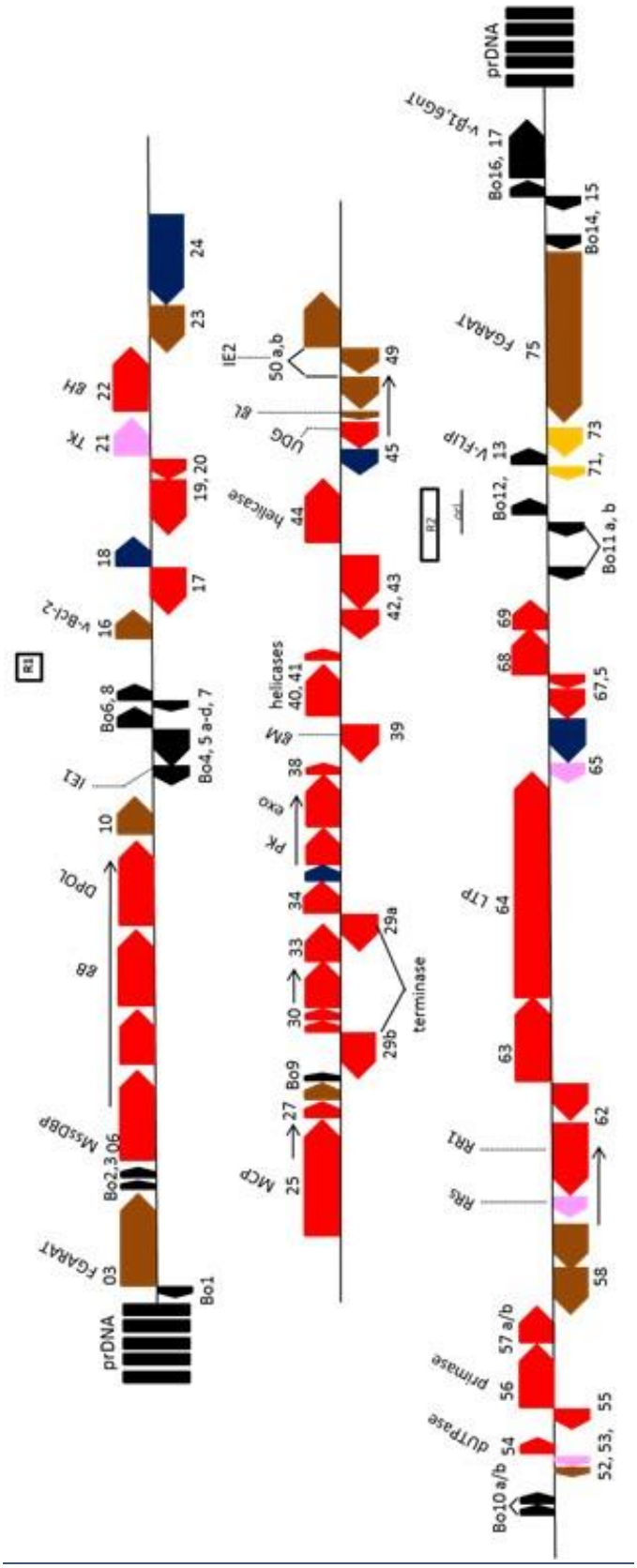


Figure 8. BoHV-4 genomic organization, (not in scale) adapted from [96]. ORFs common to all herpesviruses are represented in red; in blue those to beta and gamma; in pink those to alpha and gamma; in brown those peculiar to gamma. In yellow are the ORFs of gamma 2 herpesviruses and in black are the gene unique for BoHV-4, designed Bo1-17.

Interblock regions

The genomic space between the blocks represents the more variable part of the entire genome. In these area, gene specific to the particular virus are located. It was previously observed that these genes encode for proteins involved in several biological functions as latency, immortalization, lytic-cycle transactivation and other virus-host interactions [69].

In order to discover which kind of genes are actually present in these spaces thereby defining the peculiar BoHV-4 characteristics, a deeply analyze was performed. The 5 regions taken into account were conventionally named as A, B, C, D, E, F. Every of each started from the end of a conserved gene block until the beginning of the next, for a total amount of 23000 nucleotides with a 43% G+C content. To accomplish this goal, several sequencing techniques like the dideoxynucleotide chain termination method or single stranded M13 clones were exploited [57,78].

At the end of the analysis, several potential non-coding regions and 12 ORFs were detected. These latter were designated with the prefix BORF. The C region is the only one where no BORF was found while 2 (BORFA1, BORFA2) are in the A region, 2 (BORFB1, BORFB2) are in B, only 1 in D (BORFD1), 3 in E region (BORFE1, BORFE2, BORFE3) and finally 4 in the F one (BORFF1, BORFF2, BORFF3, BORFF4).

Based on the observed similarity rated, on the same position and orientation exhibited, it was hypothesized that BORFA1, BORFB1, BORFB2, BORFD1, BORFE2 and BORFF4 are homologous to HVS ORF3, ORF10, ORF16, ORF51, ORF71 and ORF73. On the contrary, for the other six BoHV-4 ORFs, so far, no similarity was observed, not only in relation to HVS or other gammaherpesviruses but also for any protein in databases. Based on that, these genes are probably specific and unique for BoHV-4 [78].

The similarity between BoHV-4 and HVS discovered, also in these interblock regions, is another compelling evidence which corroborates the nature of BoHV-4 as a gammaherpesvirus. The only difference with respect to HVS, established until now, is the absence of homology for cell genes [69].

IMMEDIATE EARLY GENE 2 (IE2)

Following the transcriptional process, three distinct classes of proteins are produced and classified as immediate-early (IE), early (E) and late (L) based on their expression time.

Since IE proteins expression does not require a prior viral DNA synthesis, they are generally renowned to be transactivator proteins. In support of this, when cells are treated with cycloheximide, an inhibitor protein, IE genes are the only ones to be transcribed. Moreover, IE proteins are necessary to ensure E and L genes expression whose synthesis is strictly related to viral replication. These data are further corroborated by the fact that the use of DNA polymerase inhibitors repress L genes expression [97].

IE expression in turn, is mediated by the pool of transcriptional factors furnished by cell, and for this reason, immediately available at the infection moment and able to transactivate IE promoters.

Several studies performed on RNA transcribed coming from BoHV-4 infected MDBK (Madin Darby Bovine Kidney) cells, in the presence of cycloheximide, allowed to identify two major IE RNAs and their proteins too. The most abundant BoHV-4 IE RNA, is a spliced 1.7Kb RNA, transcribed from right to left on BoHV-4 restriction map and whose nucleotide sequence contains 60% A+T and 40% G+C [84,85]. Moreover, it does not contain multiple repeat domains which are characteristic of the major IE promoter region of human, murine and simian cytomegalovirus [98]. A 284 amino acids protein of about 33KDa, called IE1, is encoded by IE nucleotide sequence. The presence of zinc finger elements in its N-terminal domain, causes IE1 to be structurally similar to the HSV-1 IE110 protein product and for this reason considered a potential DNA binding protein.

The less abundant IE RNA detected in the infected MDBK cells is a 1.8-kb RNA, transcribed from left to right on BoHV-4 genome. It encodes a 61 KDa protein showing amino acid homology to the EBV transactivating protein R, Rta and also with SaHV-2 Rta [85,99].

Rta/Orf50 is the IE2 gene protein product and it is so-called since it is encoded by the open reading frame 50 (ORF 50). This protein highly conserved among all Gammaherpesviruses including EBV, HVS, EHV-2, MHV-68, and KSHV; plays a pivotal role both into initiate viral lytic replication during reactivation of persistently infected non-permissive cells and during *de novo* infection of permissive cells [88,100].

Herpesviral immediate-early genes transcription is necessary and sufficient to initiate viral replication [101] and Rta in particular, represents the main switch protein involved in virus replication. The BoHV-4 IE2 gene overexpression, provided in *trans* by a plasmid vector, allowed to activate the lytic replication in RD4 (human rhabdomyosarcoma cells), a non-permissive cell line [102].

The Gammaherpesviruses ORF50 transcripts possess similarities with regard to the genomic location, amino acid sequence as well as splicing patterns. There is also a typical architecture characterized by the presence of two exons separated by an intron containing ORF49 gene which in turn has opposite orientation and whose promoter is probably located inside Rta ORF. Splicing of the two exons and excision of the intron results in a single, major Rta transcript but differential splicing can also occurs [103,104].

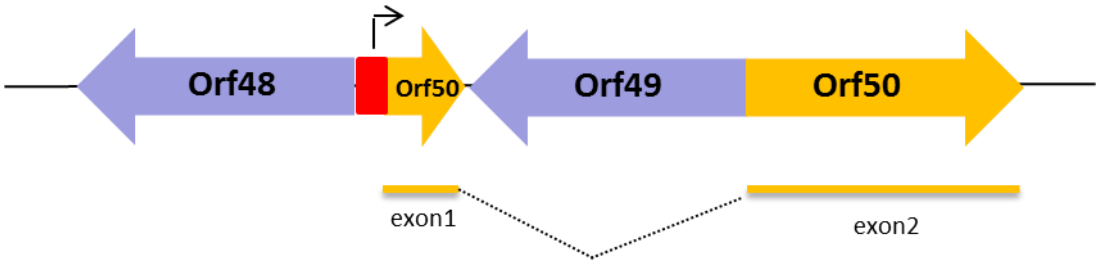


Figure 9. Organization of Gammaherpesviruses-4 Orf 50. Adapted from [88].

Amino acid alignments of the Rta homologues revealed that the most conserved regions are located in the N-terminus, necessary in EBV in order to promote dimerization and DNA binding.

In the C-terminus is located another conserved region, particularly rich in acidic residues, suggesting the presence of transcriptional putative activation domain.

Since similarities in amino acid sequences have been observed in the Gammaherpesviruses Rta homologues, it can't be ruled out the likelihood that they have also similar functions in activating viral as well as cellular promoters [103].

Several studies in different Gammaherpesviruses, revealed that the expression of IE2 gene is able to transactivate not only viral but also host gene promoters [105].

It is not totally clear how Rta/Orf50 can promote protein expression and so, two explanations have been advanced. In the first one, Rta can directly transactivate the target gene promoters by binding specific RRE target sequences (Rta Responsive Element) contrary to the second one, where Rta induces transcription indirectly through cellular factors and/or other viral factors interaction [106].

Noteworthy, it was shown that the same IL-8 chemokine expression is up regulated by BoHV-4 IE2 in BESCs (Bovine Endometrial Stromal Cells) and a specific ORF50/Rta responsive element in IL-8 promoter was detected. Since IL-8 is produced during endometrial infection and BoHV-4 is associated with this pathology, a possible relationship has been investigated [107].

During inflammation, various and numerous pro-inflammatory molecules are secreted. In particular, in endometrial cells, the inflammatory response caused by pathogens, is characterized by a high Tumor Necrosis Factor alpha (TNF-α) production, a cytokine involved in the development of innate and adaptive immune response as well as acute phase reaction. A putative interaction between TNF-α production and BoHV-4 replication was investigated by Jacca et all.

It was observed that TNF-α is able to transactivate IE2 gene expression through the NF-kB signaling pathway, in fact within its promoter the presence of a NF-KB responsive element was discovered [108].

Other cellular or extracellular stimuli within the intrauterine microenvironment can induce IE2 expression. Some bacteria like E.Coli (the most common in uterine diseases) are able to stimulate IE2 promoter activation in a concentration-dependent manner [109].

Moreover, in response to LPS an increased prostaglandin 2 (PGE2) secretion is produced by BESCs. PGE2 reactivates BoHV-4 replication in persistently infected macrophages, acting on IE2 promoter [107,110].

IFN-*γ* is another up-regulated cytokine produced during inflammatory response and contrary to the previously mentioned molecules interacts with IE2 responsive elements, down regulating its expression [111].

BoHV-4 PROTEIN PROFILE

BoHV-4 virion has the typical herpesvirus structure i.e. is characterized by a double stranded linear DNA molecule embedded inside an icosahedral protein capsid which in turn is wrapped by a proteinaceus layer called tegument and finally by the envelope, a lipid bilayer membrane. The different proteins composing the viral particle are not simply structural proteins since they are involved in essential functions, aimed to maximize viral infection.
Especially envelope glycoproteins, they mediate the attachment, penetration, egress and viral spread. Last, but not less important, they induce neutralizing antibodies production and the interactions between the host immune system and the virus-infected cells, as well as between infected cells themselves [41].

The first effort trying to study BoHV-4 protein content was performed by Dubuisson. The purified virion analyzed through a SDS-polyacrylamide gel, revealed the presence of distinct bands corresponding to 29 proteins [112] which were named GVP (gp) or VP respectively, depending on whether they are glycosylated or not, and numbered from 1 to 29, from heaviest to lightest [62].

Further studies showed that ten of them (GVP3, 6, 8, 10, 11, 16, 17, 18, 21 and 29) were envelope glycoprotein, in fact, they were completely solubilized after Triton X-100 treatment and none of them was detected in nucleo-capsid portion [112].
Only recently, a step forward has been accomplished by Lété and colleagues who exploited three different complementary mass spectrometry-based approaches to study the BoHV-4 protein profile. They were able to identified 37 viral proteins, 24 of them, continued to be present after proteinase K treatment of intact virions. Even though their main goal was to analyzed protein content, they were also able to detect glycoprotein thereby defining the glycosylation pattern of some of them [113].
Based on previously experiments carried out by Dubuisson first and then by Lété, to date, five envelope proteins and several major glycoprotein complexes were identified.

gp6/gp10/gp17 (150/120/51 kDa) is the first major complex discovered where gp10-gp17 polypeptides are linked by disulphide bonds while on the contrary, gp6 is not covalently bonded to them [114]. It was observed that gp10-gp17 comes from the proteolytic cleavage of a 135 kDa translation product of the BoHV-4 gB gene.
gB not only is one of the major BoHV-4 virion component but, its primary sequence, highly conserved among all subfamilies members, makes it, one of the most conserved gene among the Herpesviridae family members [115]. Contrary to EBV and MHV-68, gB in BoHV-4 viral particle is always detectable and characterized by a heterodimeric structure [116].
Detailed analysis revealed that the N terminus (NT) of gB is massively glycosylated and characterized by 40 potential O-glycosylation sites [113].
The involvement of this protein during viral entry into host cell was already well established [117] and recently, new insights about gB protein role were shed to light. Franceschi et all. produced soluble gB forms in order to verify if these were able to counteract, in a competitive manner, BoHV-4 binding to host cell receptor thereby hampering viral infection.

No blocking activity was obtained after their observations. Several hypotheses were proposed including the possibility that other viral proteins are involved in this process. Furthermore, they discovered that, not only gB is an essential gene since itself deletion makes BoHV-4 unable to productively replicate, but also that, heterologous VSVg protein complementation didn't work [118]. The choice to exploit VSVg was based on previous compelling proves, indeed, HSV1 gB was successfully complemented and in addition to that, this protein is widely used in pseudo-typing lentiviral vector procedure [119,120].

The second major complex is gp11/VP24 (120kDa/16.5kDa) where the N-glycosylated gp11 is linked to a non-glycosylated polypeptide VP24 in a non-covalent manner [114].

The third envelope glycoprotein is gp8 (135 kDa) which is involved in BoHV-4 interaction with heparane-like molecules present on host cell glycosaminoglycans (GAGs) [117].

This protein was observed by Dubuisson both during SDS-polyacrylamide gel analysis in envelope virion fraction and in the culture medium surnatant of BoHV-4 infected cells. Its presence in the surnatant raised up several questions about its nature and different mechanisms were advanced. Probably gp8 is a phosphatidylinositol anchored protein which is released following phospholipase C cleavage action or gp8 is non-covalently linked to another membrane molecule which on the contrary is firmly anchored to the cell surface and by mass action, it could be released [114].

The last major glycoprotein is represented by gp1 (>300 kDa). This envelope protein proved to be N glycosylated after glycosidases and Tunicamycin treatments [118]. Gp21(26/27 kDa) is another glycoprotein discovered in the sera of infected animals [121]**.**

Based on positional homology, other two glycoproteins gp110 and gp31-35, corresponding respectively to gH and gL of the other herpesviruses, were discovered, in agreement also with Lété analysis [122]**.** The involvement of this heterodimer during viral infection is widely documented and even though it seems to be indispensable to ensure a successfully viral entry; it was observed like in Murid herpesvirus 4 (MuHV-4) lacking gL, both the gH incorporation into virion and the viral infectivity have remained unaffected [123]**.**

Since BoHV-4 as well as MuHV-4, belong to Rhadinovirus genus, a recombinant BoHV-4 gL deleted was created, in order to address the possible effect of this lacking. As for MuHV-4 the BoHV-4 viral particle remained infective but contrary to MuHV-4, growth deficit was observed. This lacking seems to affect cell penetration rather than cell binding. It was observed in fact, that while wild type BoHV-4 viral particle were quickly internalized after cell binding, gL-deficient virions remained stacked at the cell surface for much longer time. These results led to the conclusion that gL deletion alters the normal trafficking of the virion containing endosomes. Another evidence emerged from these studies was that gL lacking didn't affect gB or gp180 but gH N-glycosylation, thereby altering in this manner, itself stability and its recruitment into virions [124].

gp180 is another envelope glycoprotein expressed by Bo10 late gene. This gene contains an intron whose splicing allows to obtain a mRNA encoding for a 273-amino-acid (aa) protein and its presence is still detectable after proteinase K treatment, even if partially digested [49,113].

This protein is massively O-glycosylated and sialylated, characterized by the presence of about 58% of serine or threonine amino acid residues which seems to influence most of its mass [62,125]**.** Also in this case, in order to investigate the Bo10 involvement during BoHV-4 lytic infection, a recombinant BoHV-4 strain deleted for Bo10 was

realized by Machiels et all. They observed that gp180 envelope protein, even if, it is dispensable for BoHV-4 *in vitro* replication, its absence is related to a growth deficit which was not attributed to a defect in cell-cell spread or viral release but rather to the ability to GAG cells binding [49].

Further studied showed that through alternative splicing, it's possible to obtain two different mRNAs from BoHV-4 Bo10 gene, thereby producing virions which are phenotypically distinct and consequently showing a different behavior in relation to their originating cell [86].

From Lété analysis also gM was detected but surprisingly not gN. These two proteins generally are present as a complex in all herpesviruses especially because gN seems to be involved in gM processing, however its absence could be related to the MS (Mass Spectrometry) approach used for the test.

Another unexpected discovery is related to proteomic approaches analysis and proteinase K treatment performed on the intact viral particle. 15 host cellular proteins incorporated into BoHV-4 virion were detected. Actin, Cofilin-1 and Annexin 2 seem to be particularly abundant and their presence has been previously ascertained also in other herpes virions [113].

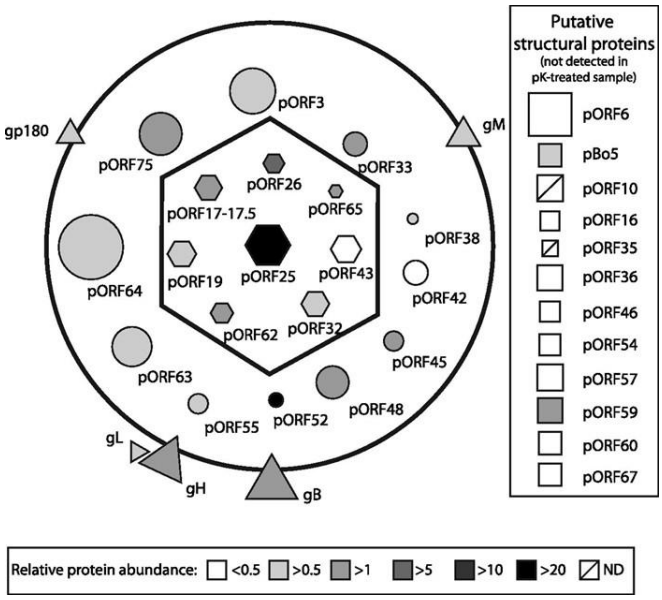


Figure 10. Schematic representation not in scale, of BoHV-4 protein profile. Adapted from [113].

BoHV-4 REPLICATION

BoHV-4 is one of the four bovine herpesviruses known so far, and contrary to the type 1 (BoHV-1), type 2 (BoHV-2) and type 5 (BoHV-5) which belong to the Alphaherpesvirinae, its belonging to Gammaherpesvirinae has been ascertained [9].

During its life cycle is able to establish different kind of infection related to several factors including the susceptibility and permissiveness of the infected cells. The susceptibility concept is related to the presence of cell surface receptors which allow virus attachment and entry. A susceptible cell could be permissive only if allows and supports a productive viral replication.

Non-productive infection

In this scenario, a low viral early and late mRNA levels without the presence of cytopathic effect, in the infected cells, are detected [90].

Productive infection

This biological phase in BoHV-4 is very similar to what previously described for the other Herpesviridae family members, with in addition some peculiarities. To date, it was observed that the adsorption to the host cell membrane, is mediated by the interaction between the major BoHV-4 virion component gB and heparane sulfate-like receptors.

Also the glycoprotein gp8 helps to strengthen the binding to the cell surface, exploiting the same cellular receptors [117] but It is not clear if one or more specific cellular receptor/s for BoHV-4 is/are involved during this step, aimed to stabilize the viral attachment.

Subsequently, viral envelope fuses with the cell membrane thereby releasing the nucleo-capsid into the cytoplasm which, at this point, is ready to reach nuclear pore complexes (NPC) through microtubules pathway. It has been speculated that the remaining tegument proteins contribute to hijack cellular metabolism in order to promote their own replication [62]. The entry of the viral DNA into the nucleus is followed by a transcriptional cascade which exploits the host cellular RNA polymerase II. One of the BoHV-4 hallmark, is the strictly correlation between viral replication and the cell cycle S phase. Several observations corroborate this evidence. The first is related to the kinetic of plaque formation in confluent monolayer compared to freshly seeded cells. The time at which plaques start to appear after infection, is at $10^{th}/12^{th}$ day and $5^{th}/6^{th}$ day respectively [126]. Secondly, in literature several experiments related to HSV-1, another Herpesvirus, support this fact. It was observed that cells infected from early G1 through S phase, were more permissive for viral gene expression contrary to cells infected in late G2 to mitosis phase [127].

In addition to that, Drayman and co-workers proved that, even though the cell cycle stage accounted for 60% in the outcome of viral infection, other factors, like cell velocity and local cell density, correlate with the probability of successful gene expression initiation [128]. Another BoHV-4 hallmark is related to the absence of the Virion Host Shutoff (VHS) proteins, contrary to what was generally observed for herpesviruses. This behavior probably allows the host cell to reach the S phase [126]**.**

During the lytic infection, the protein expression is temporally regulated and three distinct kinetic classes of proteins respectively called immediate-early (IE) or α, early (E) or β, and late (L) or γ proteins are produced [99]. This temporal regulation is accomplished through transactivation of viral transcriptional promoters by products of specific viral genes. IE proteins are among the transactivators [85]. In fact, these proteins are expressed immediately after viral genome release into the nucleus [126]**.** IE1 and IE2 proteins are considered as the two major immediate early proteins and, once produced into the cytoplasm, they are imported into the nucleus in order to control the subsequent events, like slow down their own expression promoting at the same time E and L protein expression [88]**.**

The proteins encoded by the E genes reach their expression peak in four to eight hours after infection thereby regulating the nucleotide metabolism and the events related to the viral DNA replication. More precisely, L proteins expression can occur only after the IE and E protein expression and viral DNA synthesis. Their expression peak can be reach only at that point and not before [99].

Within the γ genes, two different classes could be distinguished: γ1 or partial late and γ2 or real late. The first ones are expressed early during infection and the presence of DNA synthesis inhibitors minimally influence their expression. γ2 on the contrary, are expressed later and their production is completely inhibited by the presence of DNA synthesis inhibitors [129].

L genes code for structural capsid, tegument and envelope proteins which expression occurs inside the cytoplasm. Most of them migrate into the nucleus where they assemble to each other forming new capsids. DNA synthesis and replication take place into the nucleus through a rolling circle mechanism starting from Ori thereby producing a lot of viral concatamers.

These complex structures are characterized by head to tail genomic unit disposition. The neo-synthetized DNA molecules are now ready to be cleaved and packaged as a single genomic unit inside the pre-formed immature capsid. These complexes are now transported to the endoplasmic reticulum (ER) lumen where they are thought to acquire the immature envelope proteins and subsequently to the nucleus periphery. Since it is not yet clear how exactly the capsid leaves the nucleus and acquires the definitive envelope, several models have been proposed and, among these, the envelopment-deenvelopment–reenvelopment model seems to be the more probable [62].

Another BoHV-4 hallmark (feature which defines cytomegaloviruses) is the presence of cytoplasmic inclusions, from 48 to 72 h after infection, characterized by viral particles accumulation at different maturation stages. They consist of an amorphous electron-dense substance, granular fibrils and viral capsids with and without cores [51]. The marked cytopathic effect characterizing BoHV-4 infection, generally occurs after 48-72 hours post infection and is defined by the presence of rounded cells, dispersed in a monolayer [55].

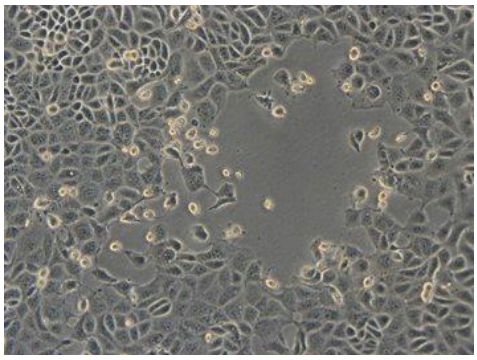


Figure 11. Phase contrast image of the plaques formed by viable BoHV-4 into BEK cells (magnification, ×10).

As observed for all herpesviruses, the productive phase is related with cell destruction and subsequent release of viral progeny. The total lysis is observed after about five days of viral incubation and this time varies according to the type of cells and the multiplicity of infection (M.O.I) used [62]. The lysis plaques produced show an irregular contour and they are generally smaller than those of BoHV-1 [51].

However, another study showed for the first time that, even when a productive infection in permissive cell lines, of a γ-2-herpesvirus occurs, it seems to be associated with apoptosis and not with a lytic phase, despite the presence of anti-apoptotic genes as in the case of BoHV-4 [130]. It was observed that gammaherpesviruses generally prefer to establish a latency infection as soon as they infect the host cell so, the onset of lytic cycle occurs only on rare occasions.

Three transactivating immediate-early proteins, are known to initiate this stage. Two of them are encoded by conserved genes: ORF50 or Rta/IE-2, ORF57 or Mta/IE-1, while the third by a non-conserved gene: Bo4/5 in the specific case for BoHV-4 [48].

Latent infection

One of the common feature shared by all herpesviruses is the ability to enter latency when the conditions defining an organized lytic life cycle are not adequately supported. In this manner they can persist lifelong in their host after a primary or secondary infection [46]. Notably, it was observed that among all Herpesviridae subfamilies, gammaherpesviruses seem to prefer the latency program instead of lytical replicating [48].

However, the mechanisms through which they reach this status differ according to the herpesvirus studied so far, as well as the site of latency, the state of the latent viral genome, the ability to furnish models of latency, the frequency of *in vitro* reactivation and the stimuli able to induce it. It was also observed that the same herpesvirus could assume a different behavior in relation both to the different animal species and cell kind infected [46].

Latency is basically a "dormant" state, during which the viral genome, maintained as an unintegrated episome, expresses only a small number of viral genes and microRNAs aimed at the maintenance of itself and host cells accommodation [131–133].

Infected cells put in place several antiviral responses like apoptosis, immune activation, and cell growth arrest in order to counteract and limit viral spread, while on the other hand, during coevolution with their hosts, viruses have

developed sophisticated strategies in order to hijack some important cellular functions thereby promoting their permanence inside the cell.

In contrast to viral replication, where viral DNA polymerase is employed, reflecting a viral takeover of the cell; during latency state, the cellular DNA polymerases is exploited [47].

Important differences were also found with regard to gene pattern expression required to enter latency and maintain this condition. In fact, it was observed like there is no a common or specific pattern that herpesviruses have to follow since some of them synthesize a specific protein pattern throughout the state, others don't express any protein or even, as in the case of Epstein-Barr virus–infected B cells, three distinct patterns of latent gene expression, depending on the differentiation stage of the cell, are produced.[134].

Regardless of the genes which take part in this phase, in order to assure their own expression, an oriP, Origins of replication for the circular viral genomes, distinct from the Ori used during lytic virus replication, is employed [135].

Several latent membrane proteins of the gammaherpesviruses have been shown to provide constant signaling, which results in growth and activation of the cells, without disturbing the latent state of the resident virus [48]. Even though more has to be discovered about genes involved in latency, it has recently observed that latency-associated nuclear antigen (LANA), encoded by ORF73, is expressed during a latent infection and it is involved both in viral and cellular gene expression modulation by targeting transcriptional regulators or co-regulators such as p53, pRB, CBP and sin3A.

This evidence is also corroborated by Hall and colleagues experiments, where they showed how ORF 73 interaction with the cellular protein p32 (splicing factor SF2/ASF), induces p32 accumulation into the nucleus. This latter protein is probably involved in splicing, nucleocyto-plasmic transport, maintenance of oxidative phosphorylation, transcriptional activation and possible latent-cycle DNA replication.

They also proved that this interaction has a synergic effect both on the heterologous promoters transactivation and its own promoter [136].

Orthologues of this ORF73 gene are present in different rhadinoviruses and BoHV-4 encodes the shortest one [133].

Its presence was also detected in cells harboring viral episomes. More precisely, LANA and viral DNA co-localize at discrete points in interphase nuclei and along mitotic chromosomes thereby suggesting that this multifunctional protein is involved in episomal maintenance by tethering viral genomes to host cell chromosomes [137].

However, its presence is dispensable for *in vitro* viral replication but on the contrary, it prevented latent infection in *in vivo* experiments, as it was proved by Thirion and co-workers through a ORF73 delated recombinant BoHV-4, in a rabbit model [133].

Two peculiar hallmarks characterize the latency state: persistence and reversibility. Various stimuli can turn the latent infection into a lytic one thereby reactivating the full viral gene expression followed by a production of infectious progeny. Phenomenon known as reactivation [138].

The stimuli annoverated so far are various and ranging from stressful conditions, parturition, transport, superinfection induced by another virus, immune-depression, local irritation of the skin, glucocorticoid injection [46].

The mechanisms of reactivation involved in the control of latency are under control of a delicate balance.

Persistent infection

This kind of infection occurs when the permissive cells, surviving to a productive infection, are able to maintain the viral genome for several passages, thereby producing low levels of infectious virus with different effects on infected cells, in relation to the nature of these latter [90].

BoHV-4, like other herpesviruses, is able to establish persistent infections both in its natural host and experimental one and lymphoid organs and mononuclear blood cells are the sites where this is supposed to happen [139,140].Several *in vitro* observations showing the simultaneous coexistence of integrated and episomic viral DNA in persistently infected cells, lead to the idea that BoHV-4 could integrate its genome in those of the host cell. Anyway**,** so far, the existence of this condition was not demonstrated *in vivo* and it is not known if the integration occurs in specific positions or in random one**s** [141].

In *vitro* Host range

The successful replication of a virus is a complex process mainly related to the coevolution of pathogen and host interactions. The viral host range is basically based on the susceptibility and permissiveness concept of the targeted cells. The evidence that gammaherpesviruses, prefer enter to latency instead lytic replication, has led to the misleading idea that these viruses are able to infect a narrow host range [48]. Contrary to that, BoHV-4 breaks this rule, since, is able to infect and replicate in a broad range of host species beyond its natural host, both *in vivo* and *in vitro* [131].

This data is supported by the replication and cytopathic effect detection in a variety of cell lines both of bovine and several other animal species [140].

This BoHV-4 feature, could be attributed to the first virus interaction with host cell heparin-like structures present on the surface of the majority of the vertebrate cells [117]**.**

BoHV-4 replicates both in primary and continue bovine cells cultures. Among bovine primary cell lines those of kidney, testes, lungs, skin, spleen, thyroid, EBTr (embryo bovine tracheal cells), fetal bovine bone marrow and lymphosarcoma calf thymus are annoverated [62]. Several both Kidney and lung continuous cell lines, allow BoHV-4 replication, as MDBK for Madin Darby Bovine Kidney, GBK for Georgia Bovine Kidney and BEK for Bovine Embryonic Kidney Cells, EBL for Embryonic Bovine Lung [62].

BoHV-4 was able to infect also nasal turbinate and tracheal cells, liver, mammary gland, endothelial cells as well as thymocytes, B- and T-lymphocytes, histiocytes, and macrophage-derived cells as showed by Donofrio experiments [102].

It was proved that also bovine endometrial stromal cells are permissive to BoHV-4 infection. In both epithelial and stromal cells, the presence of a strong non-apoptotic CPE is detected in a dose-dependent manner even if, this latter, is more evident in the stromal one [110].

Lin et all, observed that the bovine arterial endothelial (BAE) cell culture is 100–1000 times more sensitive to BoHV-4 than the MDBK cell line, commonly used for BoHV-4 propagation [142].

Histological lesions were detected in the cardio-vascular system tissue and carotid arterial endothelial cells is another primary culture cell line susceptible to BoHV-4 infection [143,144].

Cells of different species were observed to be susceptible to BoHV-4 infection, like for example buffalo, sheep, goat, swine, cat, dog, rabbit, mink, turkey, ferret, horse, mouse, hamster, primary chicken kidneys [55,62,145].

Four monkey cell lines allow BoHV-4 replication: owl monkey kidney (OMK), squirrel monkey kidney, intestines, and lung (SMC), cebus monkey kidney (CMK), and African green monkey kidney (Vero) cells [145].

Although BoHV-4 is not considered a neurotropic virus, it has been detected in peripheral and/or central nervous system tissues during persistent infection [139,146].

BoHV-4 infects differentiated N2a (mouse neuroblastoma cell line) cells and a persistent infection can be established producing the infectious viral particles production which don’t hamper the cellular differentiation property of this cell line [100].

Noteworthy, BoHV-4 replication is allowed also in non-permissive cell lines following BoHV-4 immediate-early protein 2 expression [102]. Recently, several human cell lines, isolated from different organs, were analyzed in order to investigate the sensitivity and permissiveness to BoHV-4 infection.

Gillet and co-workers revealed that human cell lines with a lymphoid and myeloid origins were resistant to the infection, while, epithelial and carcinoma or adenocarcinoma cells were sensitive but poorly permissive. Moreover, their investigations suggest that viral replication occurs in permissive cells while sensitive but not permissive cells undergo to non-permissive persistent infection [131]. The possibility to establish a persistent infection in human cell line was previously proved by Donofrio et all. For their purpose, a recombinant BoHV-4 expressing neomycin resistance gene was realized in order to select the infected rhabdomyosarcoma cells (RD-4). It was observed that, the persistently infected cells grew more slowly if compared with uninfected one and even though no CPE was detected, some infective viral particles were obtained [135].

Based on what has been described, the BoHV-4 ability to replicate *in vitro,* in human cell lines makes BoHV-4 "attractive", since only few primate herpesviruses as herpesvirus simiae (B virus), herpesvirus saimiri, and herpesvirus ateles, are able to replicate in human cells (HEp-2 and human foreskin fibroblasts) [145].

The skill of BoHV-4 to infect human cells has also been investigated to address the potential of BoHV-4 recombinant strains for viro-oncolytic treatment of human cancer cells. These expectations lay the foundations on previous evidences. In a co-culture of A549 (Adenocarcinomic human alveolar basal epithelial line) or OVCAR (Human Ovarian Carcinoma Cell Line) cells mixed with Human embryo kidney 293 cells, BoHV-4 induced a selective apoptosis limited to A549 and OVCAR carcinoma cell lines in a time and dose dependent manner [147].

The capability to infect and replicate in other immortalized cell lines was further observed in GL261 mouse glioblastoma cell line, the F98 rat glioma cell line and the GLI36 human glioma cell line, leading to the idea to exploit BoHV-4 as a vector for gene therapy or oncolytic therapy of brain tumors [148]. In order to accomplish this goal a recombinant BoHV-4 harboring the herpes simplex virus type 1 thymidine kinase (HSV-1-TK) was realized [149].

For its ability to replicate in cultured cells, BoHV-4 is one of the virus among the gammaherpesvirus subfamily that is convenient to study.

In *vivo* Host range

Bovine herpesvirus 4 (BoHV-4) has a worldwide distribution and even though cattle is its natural host, viral isolates have been recovered from other ruminant species like zebu (Bos indicus), American bison (Bison bison), African buffalo (Syncerus caffer), and sheep [131,150–152].
Virological and serological studies performed on 400 sera coming from wild African Buffoaloes, independently of their geographical origin, revealed the presence of anti-BoHV-4 antibodies of around 70%, a prevalence much higher than those detected in cattle, thereby suggesting that probably the African Buffalo could be the original host specie for BoHV-4 [153,154].

Sporadic isolations in non-ruminant species as in the lion, cat, and the owl monkey (Aotus trivirgatus) were reported. In addition to that, although the virus was not isolated in natural condition, other species were observed to be susceptible to BoHV-4 infection and for this reason considered proper experimental animals to study BoHV-4 replication. Experimentally, BoHV-4 is able to infect goats, guinea pigs, and rabbits. Based on these evidences it can be affirmed that BoHV-4 represents an exception to the other gamma herpesviruses whose replication is restricted to their natural host species [147,155].

Among experimental animals, rabbit is considered a good model not only to study BoHV-4 biology [144,156] but also a good model to study BoHV-4 pathogenesis since it successfully supports the infection. Lymphoid organs are considered the site of viral replication and/or persistence.

The viral isolation was obtained from conjunctival swabs and spleen but during latency also in bone marrow, lung, kidney, salivary glands and liver with a low titer [157]. The experimental inoculation was the preferential choice which allowed to investigate the BoHV-4 in vivo distribution. Nasal and conjunctival exudates, peripheral blood leukocytes, milk, semen cells and various organs were the samples collected by calves, pregnant cows and bulls experimentally infected through intranasal, intratraqueal, intravenosa, intra-mammary and intratesticular route.

The same procedure was adopted for the experimental animal model as rabbit, guinea pigs and cats inoculated through intranasal, ocular, intraperitoneal and intravenous route [121,156,158].

Clinical signs and pathogenesis

BoHV-4 has been isolated throughout the world from both asymptomatic and sick cattle.
Several isolates in fact, were detected through tissue scrapings or mucoid exudate swabs from animals showing different clinical syndromes related to different organs and apparatus ranging from conjunctivitis, pneumonia, inflammation of the upper respiratory tract, genital diseases (orchids, vaginitis and metritis), skin lesions, ulcerative mammillitis, mammary pustular dermatitis, mastitis, enteritis until the tumors of the urinary bladder and rumen [56,158–163].
Contrary to the BoHV-1 and BoHV-2 for which, their etiological role is ascertained, BoHV-4 role as a pathogen in ruminants and other species is still ambiguous and this fact is mainly related to some evidences [142,164].

1. In most cases, viral experimental inoculation in different animal model, failed to elicit any clinical disease even if its presence, in animal samples, was detected. This scenario was observed in hamster, rats and chicken after BoHV-4 inoculation [156]. Only few times, this process allowed to reproduce the same clinical picture observed in natural infection. Other times, the experimental inoculation resulted in a mild symptoms condition [156,165].

2. The BoHV-4 isolation from animals with a variety of inflammatory diseases, where other etiological agents were present, suggested the idea of BoHV-4 as a potential secondary pathogen. This scenario is well depicted by an *in vitro* model created by Donofrio and co-workers, laying the foundations on previous compelling data, such as the fact that, like other herpesviruses, BoHV-4 establishes persistent infection mainly in the monocyte/macrophage lineage both of natural and experimental host [166].
   In addition to that, the presence of BoHV-4 in bacterial origin metritis as well as COX2 (cyclooxygenase-2) and PGE2 (Prostaglandin E2) increasing production in response to LPS (lipopolysaccharide) are already documented. Based on this, BoHV-4 may contribute to exasperate a preexisting inflammatory condition [167–170].

3. The presence of persistently infected macrophages is the basis of another questionable doubt i.e., if the BoHV-4 detection from tissue samples is relating to the real viral isolation from the tissue itself or from mononuclear cells harboring BoHV-4 that might be present in the samples [164].

The pathogenesis role of this virus is still not well defined and several hypotheses were proposed in order to explain the transmission and spread modality inside the host. Probably, BoHV-4 can be naturally transmitted *in vivo* both from vertical and horizontal route. In the first case the infection takes place with high probability during parturition through the contact of infected genital tract while, in the second case, a close contact with lesions or contaminated moist surface of respiratory and alimentary tract are supposed to be involved. Since the presence of infective BoHV-4 viral particles in a cell milk fraction were detected also the nursing route can't be rule out among the BoHV-4 transmission routes [162].

The first site of viral replication generally occurs in correspondence of the entry site; then viral multiplication takes place in peripheral blood mononuclear cells exploiting them as a sort of Trojan horse thereby disseminating into the body.

The ability to establish a persistent infection in macrophage/monocyte cell lineage makes them not only a simple means of transport but a possible reservoir ready to be reactivated under proper conditions [140,170]. Diseases due to Rhadinovirus infections are really associated to the latency and inappropriate reactions of the immune system [48]. Spontaneous reactivation can occur during superinfection with other virus or bacteria, in immunologically weak hosts, while in cattle, experimental *in vivo* reactivation was observed through after dexamethasone treatment [170,171]. The lymphoid organs and mononuclear blood cells are not the only sites of viral latency, cause also the nervous system as well as upper respiratory tract, lungs, bone marrow, conjunctives, bilious vesicles were suspected to be involved in the virus persistence [158,172].

Conjunctiva, upper respiratory and genital mucosa represent preferential tracts of replication as well as intestine, larynx, trachea and bronchioles epithelial cells causing ocular, nasal, vaginal discharge, slight catarrhal symptoms and fever respectively [157]. As mentioned previously, also endothelial cells are susceptible to BoHV-4 replication and probably viral particles reach them through persistent infected macrophages [142]. Viral DNA in blood of infected animal is detectable from 10 to 30 days after the infection. Viremia is not always detectable and can re-appear at different times [165].

BoHV-4 as a vector

Although BoHV-4 is classified as a gammaherpesvirus based on his genome sequence, it stands out among others members for biological features that makes it a good candidate as a gene delivery vector and a possible study model to extend the informations about gammaherpesvirus biology.

i. Its genome is less complex than those of other herpesviruses [67]
ii. and its deep knowledge, coming from its complete sequencing, made real the possibility to manipulate it as a Bacterial Artificial Chromosome (BAC) in order to create recombinant viruses carrying exogenous proteins for vaccinal or therapeutical purpose.
iii. In this manner, BoHV-4 allows the stable insertion of foreign genetic material up to at least 10,5 Kb, compensated by the loss of dispensable genes, without any appreciable effect on its replication [155,173].
iv. Contrary to other Gammaherpesviridae members, is able to infect a broad range of cells, coming from different animal species, both *in vivo* and *in vitro.* In cell culture, it propagates easily without interfering with differentiation and in addition, the transgene expression is maintained both in differentiated and undifferentiated cells [174,175].
v. Even though, BoHV-4 is recognized to be a potential secondary pathogen in cows, affected by uterine diseases [170], generally, exhibits limited or no pathogenicity in natural and experimental hosts [174]
vi. Contrary to what was previously observed for Epstein–Barr virus, herpesvirus saimiri, human herpesvirus 8, and murine gammaherpesvirus-68, BoHV-4 did not show any growth transformation capacity despite the presence, in its genome, of some cellular genes homologs expecting to influence the cell growth properties [67,176].
vii. Another remarkable feature is related to the fact that, although BoHV-4 infection has shown to inhibit apoptosis in some cell lines, sometimes in others, this phenomenon is favored, especially in cancer cells [176]. In support of this, glioma cell lines were efficiently killed *in vitro,* in apoptosis manner, when treated with a herpes simplex virus type 1 thymidine kinase suicide gene armed BoHV-4 co-administered with the pro-drug ganciclovir (GCV).

    This data, the striking tropism that BoHV-4 possesses for cancer cells and rat, mouse and human glioma cells, and most importantly, the ability to selectively infect only glioma cells in the absence of pathogenicity in the rat brain after *in vivo* viral infection, corroborated the idea to exploit it as a promising, alternative oncolytic virus for this kind of diseases [149,177].

    It might be involved as a gene delivery vector for the nervous system since its presence, in peripheral and central nervous system, has been observed with high frequency, during persistent infections, even though, BoHV-4 is not renowned to be a neurotropic virus [175].
viii. The ability to support a persistent infection both in natural and experimental hosts, is another noteworthy aspect in a viral vaccine vector, cause in this way, the heterologous antigen could be expressed in the host, for a period long enough to trigger an effective immune response [69]. In particular, the fact that macrophages harbor BoHV-4 persistently, makes sure that these professional antigen processing cells, process and present the antigen thereby amplifying the immune response [170,176]; as observed through the recombinant Bovine

Herpesvirus-4 (BoHV-4)-based vector delivering a (Peste des Petits Ruminants Virus) PPRV-Hemagglutinin expression cassette. Following the BoHV-4-A-PPRV-H-ΔTK inoculation, in immunocompetent mice, both cellular and humoral immune responses with specific T cell, cytotoxic T lymphocyte, and sero-neutralizing antibody against PPRV were elicited [178].

ix. Moreover, a recombinant BoHV-4 delivering several HER-2 (epidermal growth factor receptor 2) transgene isoforms, was employed to break tolerance and elicit a protective, anti-mammary tumor antibody response in HER-2 transgenic BALB-neu T mice [179].

Always inherent to mammary cancer field, starting from the great results mentioned above, a new BoHV-4 recombinant virus, expressing the mouse xCT gene, was recently realized.

xCT, is a cysteine-glutamate antiporter protein, overexpressed in mammary Cancer Stem Cells (CSCs), which plays a pivotal role in their redox-balance maintaining and self-renewal thereby making them resistant to the traditional/conventional chemotherapeutic drugs.

The so generated recombinant virus, delivering mxCT expression cassette, was able to induce the generation and expansion of effector T lymphocytes as well as to promote an efficient humoral response. The Abs produced, not only recognized the full length protein, but were able to detect every loop and the protein in its native conformation. This binding ability resulted in the activation of ADCC (antibody dependent cell mediated cytotoxicity), thereby proving the Ab therapeutic potential. The Abs produced, following BoHV-4 mxCT vaccination, directly affect CSCs and impair xCT function *in vitro*, as shown by reduction in tumor-spheres number and dimension, inhibition in cell cycle progression and intracellular ROS accumulation. These surprising results were not limited only to *in vitro* experiments since *in vivo* assays surpassed the experimental expectations. The BoHV-4-mxCT vaccination proved to prevent lung metastases and to decrease mammary cancer growth and metastases dissemination of preexisting tumoral masses [180].

x. BoHV-4 acts as a useful adjuvant and delivery system since its presence in the host doesn't elicit the production of serum neutralizing antibodies; a inherent shortcoming that is often related to the development of viral vector for a vaccine purpose [176]. Therefore, since BoHV-4 naturally behaves as a replication incompetent viral vector and although it doesn't require to be attenuated, a further attenuation, in terms of replication, could be obtained by disrupting the late gene encoding the 1.7-kb poly-adenylated RNA (L1.7) [181].

For this and for all the reasons mentioned above, BoHV-4 is considered as a safe, potent and large-capacity vaccine vector.

xi. Several studies showed the possibility to exploit BoHV-4 as a tool to express and assess unknown antigens of other category A agents, avoiding to directly manipulate them, as was observed for Monkeypox and Ebola viral proteins. In both cases, the recombinant BoHV-4 viruses, successfully expressed the transgenes and a 100% protection was obtained in STAT1(-/-) mice when inoculated with recombinant BoHV-4 delivering Monkeypox M1R glycoprotein, and a generation of high titer of antibodies was detected in goats inoculated with recombinant BoHV-4 delivering immune-dominant antigen coming from Ebola virus [182,183].

BoHV-4 as a Bacterial Artificial Chromosome and recombineering process

The potential use of BoHV-4 as a gene delivery vector, in the vaccine and gene therapy field, was allowed by the complete sequencing of its genome and itself manipulation as a bacterial artificial chromosome (BAC). Because of the large size of its genome, the classical homologous recombination in eukaryotic cells was impossible to perform, and so, BAC cloning and prokaryotic recombination technology were adopted [155].

BAC exhibits several suitable qualities such as the ability to accommodate large insert size of DNA, ranging from 100 until 300 Kb, the permission to stably clone methylated eukaryotic DNA, high transformation efficiency and the ability to yield high-quality DNA during DNA extraction. All these features make BAC an attractive resource and an exciting alternative to conventional vector system in order to study genome structure and function [184].

The recombination-mediated genetic engineering, commonly known as recombineering, is a recently developed powerful approach involved in BAC modifications. Since recombineering exploits homologous recombination, it allows insertion, point mutation or deletion of any sequence precisely, thereby overcoming the drawbacks of traditional cloning and mutagenesis methods [185].

Contrary to the traditional homologous recombination methods, with recombineering, any position on a BAC can be potentially targeted and the required homology size (~ 40 bp) is short enough to be easily synthesized *in vitro* [186].

In order to limit the pathogenic risk, an "apathogenic" BoHV-4 (so designated BoHV-4-A), isolated from the cell milk fraction coming from a healthy cow, was used to generate a BoHV-4 BAC [181].

To accomplish that, a special recombineering E.Coli strain, called SW102, is used. This DH10B derivative strain carries the functional bacteriophage lambda (λ) Red recombination system and a precise deletion of the GalK gene thereby allowing the two selection/counter-selection in the subsequent steps [187].

Exo, Bet and *Gam*, are the three λ Red genes, which take part during the recombineering process: *exo* encodes for a 5' to 3' dsDNA exonuclease producing overhanging extremities in the double-stranded DNA targeting cassettes, *bet* encodes for a protein which mediates the annealing of the overhanging extremities to the complementary DNA present in the BAC cassette, while *gam* encodes for a RecBCD exonuclease inhibitor preventing the target DNA fragment from degradation. Their expression is strictly controlled by the heat sensible promoter, which doesn't work at 32°C thanks to the presence of the temperature-sensitive repressor, cI857. Based on that, in order to promote their expression but at the same time in order to avoid random over-recombination events, the temperature of the bacterial culture has to be shifted at 42°C only for 15'. The λ Red genes are flanked by both Biotin Operon and Galactose Operon systems which allow to detect only positive colonies harboring the recombinant clones during the metabolic selection [187].

The BAC cassette is composed by the presence of some BoHV-4 ORFs, among which Bo2, Bo3 and a partially deleted Thymidine Kinase (TK) sequence, flanking the Kana and GalK ORFs which, in turn, are essential to properly select the putative recombined clones. The BAC cassette includes also an origin of replication, chloramphenicol resistance gene, an EGFP (Enhanced Green Fluorescent Protein) reporter gene, *redF* and *repE* genes involved in plasmid replication, *parE* and *parC* genes encoding for a topoisomerase, *parA* and *parB* genes regulating the replication rate. Last, but not less important, is the presence of two *LoxP* sites flanking the BAC cassette [188].

After BoHV-4 BAC is transformed into this E.Coli strain, a linear recombination cassette with two short homology arms of ~ 40 bp, in this specific case the thymidine Kinase sequences, each flanking the GalK gene marker, is generated by PCR. The cassette is then transformed into the BAC within the recombination-competent bacteria, and subsequent λ Red genes induction leads to the precise integration of GalK cassette into the BAC, in a process called Targeting [189].

During this process the selection is performed on minimal plates using galactose as the sole carbon source, supplemented with biotin and leucine to make up for the SW102 shortcomings and chloramphenicol for BAC maintenance [190].

The BAC GalK cassette is then replaced with a second recombination cassette containing the desired modification flanked by the same short homology arms as in the previous step. The resulting GalK− bacteria, coming from the retargeting process, can be readily selected using plates containing a galactose analogue, 2-deoxy-galactose (DOG) which phosphorilated by GalK enzyme leads to the buildup of a toxic compound, 2-deoxy-galactose-1phosphate [190,191].

The main purpose of this cloning technology is to reconstitute a recombinant virus, in this precise context, a recombinant BoHV-4, able to express the protein of interest from the BAC plasmid.

In order to accomplish this, following the selection/counter-selection of the putative recombinant clone, the BAC extracted DNA is checked on agarose gel and subsequently transfected in the eukaryotic permissive BEK finCre cells (Bovine embryo kidney cells) which, exploiting the *Cre-loxP* recombinase system, excide the BAC cassette, allowing to obtain the expected recombinant BoHV-4 [155].

BoHV-4 and uterine diseases

To date, in regards to the clinical aspects of BoHV-4 infection, it is still difficult to say if this virus is actually involved in certain bovine pathologies. However, it must be taken into account the high seroprevalence observed against BoHV-4, mainly in Belgium but also in the rest of the world. In addition to that, several findings incriminate BoHV-4 in the onset of bovine genital diseases and, for this reason, the pathogenic role of BoHV-4 as a co-factor, in a potential vicious circle, has been hypothesized.

Postpartum uterine diseases in cattle and immune response

Bos Taurus, especially dairy cattle, are prone to uterine infection and diseases, thereby compromising uterine function [192].

The characterization of uterine diseases is mainly related to various symptoms and clinical signs detected in the affected cows. On these bases, it is possible to classify pelvic inflammatory diseases as: *puerperal metritis, clinical metritis, clinical endometritis, subclinical endometritis and pyometra*.

Puerperal metritis and clinical metritis, regards the entire depth of the uterine wall and are associated to an enlarged uterus, a purulent watery red-brown uterine discharge, that often has a fetid odor. A number of systemic signs such as the increased temperature, limited milk yield, depression, dullness and lack of appetite characterize these pathologies. When these latter systemical symptoms are not detected, the disease is classified as clinical metritis. Metritis are depicted as severe diseases if compared with endometritis, which on the contrary, is a localized infection regarding only the uterus lining and the underlying glandular tissues.

Purulent uterine exudation in the vulva 21 days or more after calving or mucopurulent discharge are associated to a clinical endometritis status [193,194].

Histological analysis performed on samples collected from these animals showed the epithelial surface destruction, vascular congestion, stromal edema accumulation of lymphocytes and plasma cells [195]. The absence both of clinical endometritis signs and purulent uterine discharge allow to classify these animals as having subclinical endometritis.

Even if relatively rare, pyometra is defined by the accumulation of purulent material within the uterine lumen in the presence of a persistent corpus luteum and a closed cervix [193,194]. Several statistical data have shown that reproductive genital tract inflammation in dairy cattle is common especially after calving; in fact, the 40% of animals present metritis while endometritis is detectable in the 20% until more than 3 weeks [107,192] and about 30% of cows have chronic uterine inflammation without clinical signs [194].

These conditions heavily affect reproductive performance since they alter uterine and ovarian functionality, thereby reducing pregnancy and decreasing milk production. More precisely, it was observed like the presence of metritis and/or endometritis status is related to uterine regression and embryonic survival disorder. In these animals, changes in the hypothalamic-pituitary axis activation were detected thereby resulting in variations of estradiol, progesterone and LH (Luteinizing Hormone) secretion.

As a consequence of this, the slower postpartum ovarian follicular growth as well as the lower capacity to ovulate are related to the reduction in the conception rate [194,196].

It's clear that all these effects are incompatible with the main reproduction management target of dairy cattle which is to have cows become pregnant in an efficient manner and at a profitable interval after parturition [197]. Since bovine uterine diseases represent the major economic problem for the efficient performance of dairy herds, it is appropriate to understand the etiology and pathogenesis in order to prevent their onset or improve their treatment.
Bovine uterine diseases are widely related to bacterial infections as proved by the broad-spectrum of bacteria detected from uterine lumen isolates. Among them, *Escherichia coli, Arcanobacterium pyogenes, Prevotella melaninogenicus, Fusobacterium necrophorum* and *Fusobacterium nucleatum* are the main pathogenic bacteria found [198,199]**.**

E. coli in general and, a specific endometrial pathogenic E. coli (EnPEC) which seems so to be more adherent and invasive to endometrial cells, increases the endometrium susceptibility to the subsequent infection with A. pyogenes F. necrophorum and P. melaninogenicus.
They play their pathogenic role in different ways, in fact, E. coli releases the bacterial-wall lipopolysaccharides (LPS), A. pyogenes produces the cholesterol-dependent cytotoxin pyolysin and a growth factor for F. necrophorum which in turn produces leukotoxin while P. melaninogenicus produces a substance that inhibits phagocytosis. From this scenario it is clear that they act synergistically in order to enhance the uterine disease severity [198,200].
The qualitative and quantitative uterine bacterial infection is mainly related to the delicate balance between bacterial contamination and the animal defense mechanisms.
Vertebrates have evolved several immune defense systems in order to eliminate infective pathogens from the body, both through innate and acquired immunity.

The maintenance of the female genital tract sterility, is usually guaranteed by the presence of several functional anatomical barriers such as vulva, vestibule and cervix which in turn, is characterized by the presence of mucosa-lined collagenous rings. Furthermore, both the presence of vaginal mucus and circular and longitudinal layers of uterine musculature impede the ascent of microorganisms through reproductive tract [195].

During parturition the cervix dilatation provides to bacteria the opportunity to ascend the genital tract from the environment thereby establishing uterine bacterial infection. This unwanted situation seems to be favored also by progesterone secretion which in turn suppresses immune defenses.

Uterine involution, endometrium regeneration, ovarian cyclic activity restoration are the necessary events in order to reduce or prevent the onset of these pathologies [200,201].
Endometrial epithelial layer, the first site where the infection occurs, plays a key role in the immune response through the pattern recognition receptors (PRRs) expression, like the toll-like receptors (TLRs), that specifically recognize conserved pathogen-associated molecular patterns (PAMPs), such as bacterial lipids, nucleic acids (both from bacteria and viruses) or other structural components synthesized by micro-organisms. All 10 members of this family are expressed throughout the cow endometrium. More precisely, TLR2, TLR3, TLR4, TLR6 and TLR9 are expressed in the caruncular and intercaruncular endometrium both before and after parturition. TLRs 1 to 7 and TLR9 expression was detected in the endometrial epithelial cells whereas TLRs 1 to 4,6,7,9 and 10 in the stromal cells ones.
The presence of TLRs, also in the endometrial stromal layer, is an evidence of their involvement in supporting the epithelial layer function, in equal degree, if not more. In support of the above, in fact, stromal cells are much more

abundant than epithelial ones and are adjacent to the vasculature, so their cytokines and chemokines production might have more impact than epithelial cell inflammatory mediators [202].

Mucin 1 (MUC1), an epithelial cell glycosylated transmembrane protein, may contribute in the microbial endometrium defence. The inflamed district, lead to the production of other antibacterial molecules like complement, calgranulins, antimicrobial peptides (AMPs), defensins family components such as lingual antimicrobial peptide (LAP), tracheal antimicrobial peptide (TAP) and bovine neutrophil β-defensins (BNBD4, DEFB5) all expressed by endometrial epithelial cells.

Following PAMPs- mediated TLRs activation, signal transduction pathways for mitogen-activated protein kinase (MAPK) and the nuclear factor-kappa B (NFKB) transcription factors start, thereby resulting in the pro-inflammatory cytokines including Tumor Necrosis factor- alpha (TNF-α), interleukins (IL-1, IL-6) and chemokines (IL-8) synthesis and production.

Also nitric oxide synthase (NOS), prostaglandin-endoperoxide synthase 2 (PTGS2 or COX2), prostaglandin $F_{2\alpha}$ and E_2 (PGF2α and PGE2) are produced as a consequence of PAMPs detection. The released cytokines are also involved in pyrexia and acute phase protein hepatic secretion. The molecules, previously mentioned, act all together in order to improve immune cell mobilization: neutrophils, lymphocytes, eosinophils, mast cells and macrophages are in fact chemotactically attracted from the bloodstream into the uterine lumen [192,194,200,201,203–206].

Vicious circle

Bovine uterine diseases are widely related to bacterial infections, while the viral involvement in the metritis and endometritis development is poorly investigated mainly because viral serology or isolation is less convenient to perform.

However, several analyses detected BoHV-4 in many samples coming from cows affected by pelvic inflammatory diseases; based on that, a probable correlation between BoHV-4 infection with uterine diseases and chronic infertility in dairy cattle has been hypothesized [207].

The first isolation of BoHV-4 from a case of bovine metritis was reported in U.S.A. in 1973 [159] and other several findings coming from different geographic areas in the world, from cattle with different reproductive disorders, are reported in literature, such as Italy [208], India [209], Belgium [207], United States [210], Spain [211], Serbia [110,212], and Argentina [213].

As, initially, the role of BoHV-4 was not clear, several cows were used for in *vivo* viral inoculation in order to evaluate the possible correlation between viral infection and female genital tract diseases, abortion and infertility [214,215].

Since these pathologies are mostly attributed to the presence of bacteria and since, sometimes, BoHV-4 was detected in the presence of other viruses or bacteria, in animal completely healthy or showing a different clinical symptomatology, a remarkable question has been raised up about the exactly involvement of BoHV-4; more precisely, if it takes part directly in the onset of uterine diseases or if it acts as a secondary pathogen in the later infection stages, thereby increasing uterine inflammation started by other microorganisms [216].

Donofrio et al. (2007) shed more light on the BoHV-4 etiological role. Based on compelling experimental *in vitro* evidences, inherent to BoHV-4 tropism for stromal and epithelial cells and consequent cytopathology, they suggested a possible vicious circle taking place during uterine pathology. This model is supported by other experimental data as for example the fact that BoHV-4, like other Herpesviridae family members, is able to establish a persistent infection in bovine macrophages that in turn can act as a prompt BoHV-4 reservoir [110,170].

During parturition, infection of the uterus can take place from environmental bacteria. Among them, E. coli is the most prevalent and paves the way to subsequent infections. Following LPS binding to the TLR4/CD14/MD2 receptor complex, expressed on the endometrial epithelial cells, results in the activation of downstream kinases, leading to the expression and production of a broad range of pro-inflammatory mediators in the inflamed damaged tissue [204]. In particular, the COX2 expression and PGE2 production, in association with the LPS presence, induce the replication of harbored BoHV-4 in persistently infected macrophages which can easily reach the inflammation sites through the bloodstream [170].

This is probably the mainly pathway that allows BoHV-4 to reach the uterus stromal layer, a fertile cellular substrate where the virus can replicate.

The general activator PGE2 supports BoHV-4 lytic replication, inducing IE2 gene expression, in a concentration-dependent manner, leading to virus spread which, at this point can infect the adjacent stromal cells. TNF-α is another key molecule, produced by LPS-induced macrophages, which promotes BoHV-4 replication, binding to the TNF-α receptor 1 on the surface of BoHV-4 infected stromal cells. Also in this case, viral replication is mediated through IE2 gene promoter activation [108].

It was proved that IE2 gene product, ORF50/Rta, is involved in the transactivation of both viral and host cell genes such as those coding for the chemokine IL-8, whose production, in infected Bovine Endometrial Stromal Cells (BESCs), increases in a time- and dose-dependent manner.
This is possible, since a specific responsive region for ORF50/Rta, in the IL-8 gene promoter was detected, thereby showing the striking relationship existing between chemokine IL-8 and BoHV-4.
Based on this data and on the fact that IL-8 plays a pivotal role attracting granulocytes such as macrophages and neutrophils in the infection site, it is reasonable to think that the IL-8 involvement may represent a virulence mechanism through which viral replication can recruit more susceptible host cells.
The secretion of this chemokine, could compromise an already complex clinical condition, shifting the inflammation from a transitory and acute phase (metritis) toward a chronic status (endometritis) [107].
If host cellular genes, like IL-8, promote viral replication, other, such as IFN-*γ* hamper this process. IFN-*γ* not only is notoriously known to have antiviral, anti-proliferative and immunomodulatory properties but it has proven to be protective against several intracellular pathogens and, perhaps more important, is the fact that its increased level production was detected in BoHV-4 infected animals [217].

In support of its protective role, Jacca and co-workers showed the direct involvement of IFN-*γ* in counteracting BoHV-4 replication. The T lymphocytes and NK (natural killer) cells resident in the inflamed endometrial stromal layer produce IFN-*γ* which in turn exerts its function binding to specific position in the IE2 promoter thereby reducing viral replication. This model, which highlights the role of BoHV-4 as a potential secondary pathogen in uterine diseases, paved the way

to new insights and new possible ruminations like the fact that, with high likelihood, IFN-*γ* is able to control BoHV-4 replication in normoergic animals leading to the uterine acute infection resolution, within 3 weeks after calving. This scenario has an opposite outcome in the animals with an impaired IFN-*γ* axis response [111].

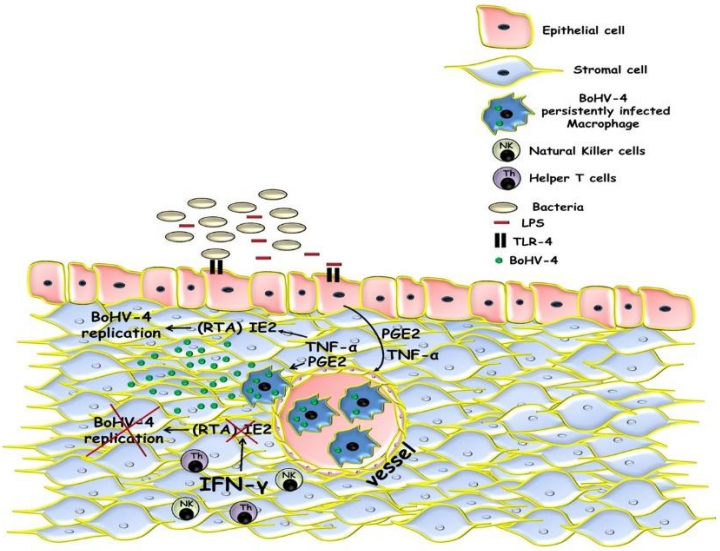


Figure 12. Schematic representation about the role of cytokines in BoHV-4 infected BESCs. Adapted from [111].

MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs), along with disintegrin and metalloproteinases (ADAMs), ADAM with a thrombospondin-like motifs (ADAMTS), bacterial serralysins and astacins proteases belong to the large multidomain zinc (II)-dependent endopeptidases family, generally referred to as metzincins [218,219]. This large family is renowned to be involved in connective tissue remodeling processes as observed by their ability to degrade both *in vivo* and *in vitro* extracellular matrix protein components like interstitial and basement membrane collagens, proteoglycans, fibronectin and laminin [220].

The first MMP was discovered by Gross and Lapiere in 1962, through experiments aimed at explaining collagen remodeling in the metamorphosis of a frog tadpole [221]. These matrixins, the other name by which MMPs are commonly known, are present across all kingdoms of life; in fact, after the discovery of MMP-1 (interstitial collagenase 1) in 1962, the inactive form of this enzyme was purified from human skin in 1968 and subsequently isolated also in invertebrates (sea urchins, Caenorhabditis elegans, soybean, and Arabidopsis thaliana) and plants [222,223].

Classification

MMP family members have in common roughly 40% of their primary structures and so, to date, based on their structural elements, homology, substrate specificities and cleavage mechanisms, more than 20 different types of MMPs have been discovered and classified into 7 groups [224].

MMPs are further divided into soluble and membrane-anchored proteins depending on their physical localization [225].

Collagenases

MMP-1, -8, -13 belong to the collagenase group and function by degrading α-helices of interstitial collagen of type I, II, III. Contrary to other MMPs, they are able to cleave collagen helices in the native state at neutral pH through a non-denaturing mechanism [223]. Their substrate specificity is not limited to collagen as they also digest gelatin, casein, aggrecan, laminin, versican, perlecan, fibronectin, and tenascin [226,227].

Gelatinases

MMP2 and MMP-9 are within this group, and they cleave denaturated type V, VII, X, XIV collagen and intact type IV collagen in basal membranes. They are also involved in fibronectin, elastin and aggrecan cleavage. Noteworthy, they seem to exert their function detecting specific recognition pattern, such as repeated sequence with Pro-XX-Hy-(Ser/Thr) where X is any amino acid residue and Hy is a hydrophobic amino acid residue, or again, motifs rich in arginine residues [223].

Stromelysins

MMP-3 and MMP-10 work on a rather wide range of substrates, including non-collagenous extracellular matrix proteins. MMP-3 in particular, presents an elevated proteolytic activity if compared to MMP-10. It is also involved in the activation of other MMPs [222,228].

Matrilysins

Matrilysins lack the hemopexin-like domain found in other MMPs. This absence mitigates the capability to cleave triple-helical collagen but does not influence hydrolytic activity related to gelatin, casein or synthetic substrates [220]. MMP-7 and MMP-26 process ECM (extracellular matrix) components and in particular, MMP-7 acts on cell surface molecules like pro-α-defensin, Fas-ligand, pro-tumor necrosis factor-α (TNF-α) and E-cadherin [228].

Membrane-associated MMPs

Within this group, MMP-14, MMP-15, MMP-16 and 24 are type I transmembrane proteins while MMP-17 and 25 are glycosylphosphatidylinositol (GPI) anchored proteins. They are responsible not only of ECM remodeling, degrading mainly collagen, but also activation of other MMPs [228].

Macrophage elastase

Like many metalloproteinases, MMP-12 is able to degrade extracellular matrix components such as elastin but also fibronectin, laminin, collagen, basal membrane, enactin, chondroitin sulphate. It is mainly expressed in macrophages and its activity allows them to penetrate the basal membrane thereby promoting the remodeling process in inflamed tissue [223].

Other MMPs

MMP-19, MMP-20, MMP-21, MMP-23, MMP-27 and MMP-28 are expressed in many cell and tissue types such as liver (MMP-19) odontoblasts and periodontal tissue (MMP-20), keratinocytes (MMP-28) as well as reproductive cells (MMP-23) [228].

The matrix metalloproteinase family		
MMP designation*	**Structural class**	**Common name(s)**
MMP-1	Simple hemopexin domain	Collagenase-1, interstitial collagenase, fibroblast collagenase, tissue collagenase
MMP-2	Gelatin-binding	Gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase
MMP-3	Simple hemopexin domain	Stromelysin-1, transin-1, proteoglycanase, procollagenase-activating protein
MMP-7	Minimal domain	Matrilysin, matrin, PUMP1, small uterine metalloproteinase
MMP-8	Simple hemopexin domain	Collagenase-2, neutrophil collagenase, PMN collagenase, granulocyte collagenase
MMP-9	Gelatin-binding	Gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase
MMP-10	Simple hemopexin domain	Stromelysin-2, transin-2
MMP-11	Furin-activated and secreted	Stromelysin-3
MMP-12	Simple hemopexin domain	Metalloelastase, macrophage elastase, macrophage metalloelastase
MMP-13	Simple hemopexin domain	Collagenase-3
MMP-14	Transmembrane	MT1-MMP, MT-MMP1
MMP-15	Transmembrane	MT2-MMP, MT-MMP2
MMP-16	Transmembrane	MT3-MMP, MT-MMP3
MMP-17	GPI-linked	MT4-MMP, MT-MMP4
MMP-18	Simple hemopexin domain	Collagenase-4 (*Xenopus*; no human homologue known)
MMP-19	Simple hemopexin domain	RASI-1, MMP-18‡
MMP-20	Simple hemopexin domain	Enamelysin
MMP-21§	Vitronectin-like insert	Homologue of *Xenopus* XMMP
MMP-22	Simple hemopexin domain	CMMP (chicken; no human homologue known)
MMP-23	Type II transmembrane‖	Cysteine array MMP (CA-MMP), femalysin, MIFR, MMP-21/MMP-22¶
MMP-24	Transmembrane	MT5-MMP, MT-MMP5
MMP-25	GPI-linked	MT6-MMP, MT-MMP6, leukolysin
MMP-26	Minimal domain	Endometase, matrilysin-2
MMP-27#	Simple hemopexin domain	
MMP-28	Furin-activated and secreted	Epilysin
No designation	Simple hemopexin domain	Mcol-A (Mouse)
No designation	Simple hemopexin domain	Mcol-B (Mouse)
No designation	Gelatin-binding	75-kDa gelatinase (chicken)

Figure 13. Classification of MMPs. Adapted from [229].

Structure

X-ray crystallography and nuclear magnetic resonance (NMR) allowed definition of the basic MMP structure which is generally characterized by a signal peptide, a N-terminal propeptide, a catalytic domain, a hinge region and a haemopexin domain (Hpx) [230].

The signal peptide of about 20 amino acids allows MMPs to reach their final destination: either secretory or the plasma membrane insertion pathway.

The N-terminal propeptide of about 80 amino acids, is defined by three α chains and their connecting loops. The cysteine, the most important functional amino acid in these enzymes, and present in the highly conserved sequence (Pro-Arg-Gly-Cys-X-Pro-Asp, where X represents any amino acid), constitutes the so-called "cysteine switch". It interacts with the catalytic zinc ion through the thiol group, thereby keeping the proMMPs inactive. This hampers both the access and the binding of the water molecule to the zinc cofactor. Both of these events are essential for catalysis.

The catalytic domain of about 170 amino acids, consists of five β-sheets, three α-helices and their connective loops. The zinc-binding motif: His-Glu-His-XX-XXXX-Gly-His (where X represents any amino acid) defines this domain. It is associated with a methionine residue, leading to the formation of a unique structure known as Met-turn, whose function is to support the structure around the catalytic zinc. This domain, in fact, contains two zinc ions: the first is present in the active site and is directly involved in the catalytic processes, while the second, has a structural function through coordination with three calcium ions. The catalytic zinc is further coordinated and properly stabilized by the presence of three histidine residues in the zinc-binding motif. Additionally, the glutamic acid proximal to the first histidine is essential for the catalysis process [228,230]**.** The catalytic domain is covalently connected to the C-terminal domain by a proline-rich hinge region. The length of this **flexible linker peptide** ranges from 10 up to 75 amino acids, and has no determinable structure. The 195 residues which constitute the C-terminal **haemopexin-like domain** mediate the interaction with substrates, thereby defining the enzyme specificity. With the exception of MMP-7, all vertebrate and human MMPs are expressed with a C-terminal haemopexin-like domain [220].

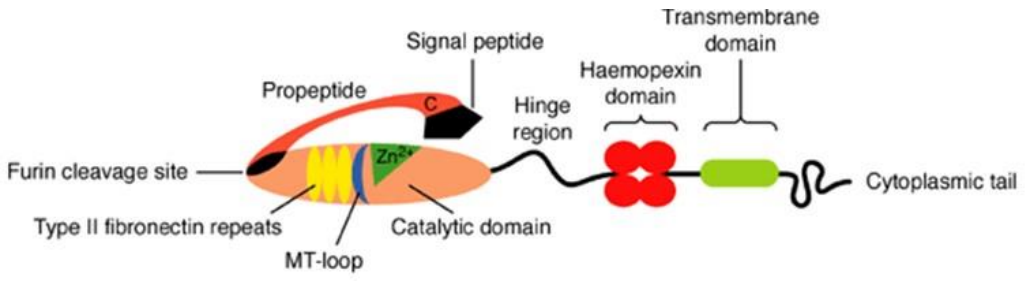


Figure 14. Variable structural domains of MMPs. All MMPs possess a signal peptide that targets the MMPs for secretion, a pro-peptide domain (containing a conserved Cys residue) and a catalytic domain. Adapted from [231].

Regulatory Mechanisms of MMPs

The MMP family members are produced by a variety of connective tissue and pro-inflammatory cells such as fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils and lymphocytes. Some inactive MMPs are secreted and others are bound to the extracellular matrix components, as the case of MMP-2, which is linked to elastin. MMP-3 is linked to the basal membrane and occasionally to collagen fibrils, while MMP-13 is linked to proteoglycans, collagen and elastin. Some are stocked, MMP-8 and MMP-9, in specific cell granules, while MMP-1, MMP-2 and MMP-3 are constitutively produced by cytokines and inflammatory cell factors [223,232].

Although one of the hallmarks of MMP genes is that they are "inducible" [222], their proteolytic activity is strictly regulated in many ways, before and following induced expression. Regulation of their expression occurs at the transcriptional, post-transcriptional and post-translational level. The latter is achieved through cognate activators, inhibitors and their cell surface localization as mature enzymes. These precise regulatory mechanisms result in a low basal level of these enzymes in normal physiological status [232–234].

Transcriptional Mechanism/MMPs gene expression

A wide variety of stimuli is found to be involved in MMP spatial and temporal expression control, ranging from cytokines, growth factors, interleukins, interferons, hormones and cellular transformation [222]. Some of them trigger MMPs expression, as in the case of IL-1β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), Epidermal growth factor (EGF), TNF-α, Placental growth factor (PGF), estrogen and progesterone. Other stimuli, such as TGF-β, retinoic acid and glucocorticoids act in the opposite manner, down-regulating their expression [222,235,236].

It was proved that, after short exposure to stimuli, MMPs are often co-expressed or co-repressed thereby leading to the idea that their promoters represent downstream targets within signaling pathways of early response genes which are promptly induced without the need of new protein synthesis [237].

Structural and functional analyses of several MMPs promoter regions provided new insights inherent to the operating and expression mechanisms. The activator proteins (AP)-1 and -2 sites, the polyomavirus enhancer-A binding protein-3 (PEA3) site, the nuclear factor Kappa B (NF-KB) site and the signal transducer and activator of transcription (STAT) site are some of the transcription-binding sites playing a pivotal role in the regulation of MMP expression [238].

Noteworthy, among them is the AP-1 response element. This element is harbored in the proximal promoter region, approximately 70 bp upstream of the transcriptional activation site, and appears to be the major mediator in the MMP promoter transcription regulation. Additionally, even the AP-1 complex composition itself, as well as the juxtaposition of transcription factor binding sites, may determine the specificity among different genes [233,237].

Recently, it has been proved that cell-matrix and cell-cell interaction as in the case of EMMPRIN (Extracellular Matrix Metalloproteinase Inducer), could also take part in MMP gene expression. This cell glycoprotein, also known as basigin or CD147, belongs to the immunoglobulin superfamily and stimulates the production of several MMPs [239].

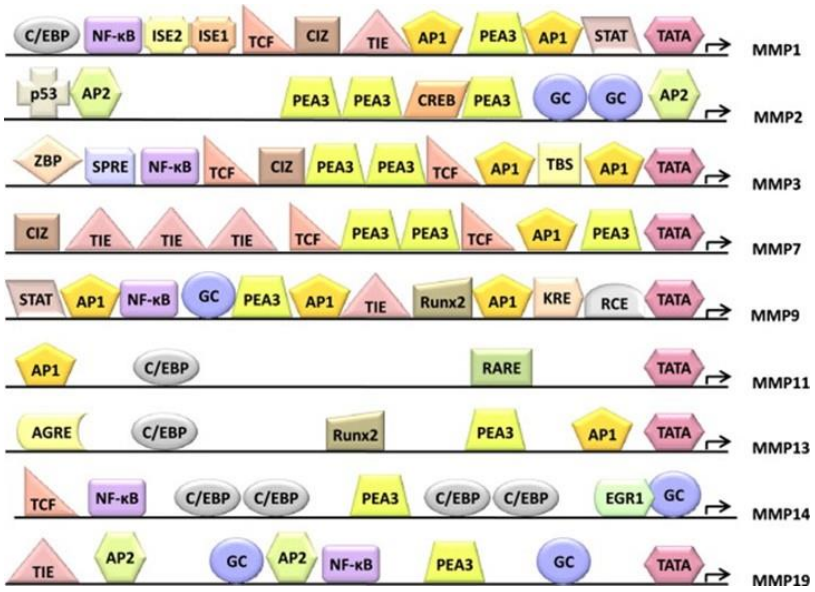


Figure 15. Schematic representation, not in scale, of the regulatory elements in the promoter regions of human MMP genes. Adapted by [238].

MMP gene expression could also be modulated by epigenetic mechanisms such as DNA methylation or histone acetylation. Even though, it is well renowned that CpG methylation in the promoter region favors the transcriptional repression, surprisingly, the opposite has been observed for MMP9 and MMP2 expression. The promoter region methylation in fact, triggers their expression. In addition to this, the presence of specific sequences, in the 5' or 3' untranslated regions (UTRs) could promote the linking of UTR-binding proteins thereby stabilizing or destabilizing the mRNAs. Alternative mRNA splicing rather than membrane shedding and alternative polyadenylation represent another way to generate MMPs in active forms [232]. Finally, MMP expression could be regulated post-transcriptionally by the presence of microRNA (miRNAs). Nevertheless, so far, only one study has demonstrated this latter mechanism [238,240].

Pro-enzyme activation

All MMP members are firstly synthesized as pre-pro-enzymes where the signal peptide is removed during translation thereby generating the pro-enzyme. This latent form, better renowned as zymogen, in order to achieve functionality, need to be activated *in situ* in a stepwise process [230]. The foreamentioned *cysteine switch,* proposed by Van Wart's group, explains the first step of zymogen activation. The thiol group of the conserved cysteine in the pro-peptide domain, interacting with a Zn^{2+} ion in the enzyme's active site, keeps MMPs in the inactive status. Activation involves dissociation of this bound and the following replacement of the cysteine ligand by water molecule that, at this point,

can interact with the peptide bonds of MMP substrates [241]. The second step requires the involvement of other proteases in order to completely remove the pro-peptide domain, thereby converting the partially active enzyme into its fully functional active mature form [230].

Activation pathways vary among MMPs in relation to their spatial position. *In vivo,* most MMPs are activated in the pericellular space by tissue and plasma serine proteinases (plasmin, trypsin, kallikrein), opportunistic bacterial proteinases or other MMPs, already activated. These latter can cleave peptide bonds within MMP prodomains [222]. MMP 11, 23, 28 and MT-MMPs are activated intracellularly, prior to secretion by the *trans*-Golgi associated furin proteases [223,233]**.**

The Cysteine-Zinc bond can be perturbed *in vitro* by several physiochemical agents such as heat, low pH, thiol-modifying agents (4-aminophenylmercuric acetate), mercury chloride, heavy metal compounds, sulfhydrylalkylating agents as N-ethylmaleimide (NEM), oxidized glutathione, SDS (sodium dodecyl sulfate), oxygen radicals and chaotropic agents, all of which trigger MMP activation [223,235,241].

Tissue inhibitors

Specific and non-specific inhibitors are involved in MMPs regulation. More precisely, TIMPs (tissue inhibitors of matrix metalloproteinases) are the major specific endogenous regulators while α_1-proteinase and α_2-macroglobulin are the non-specific one [233].
MMPs activity in the fluid phase is mainly regulated by the α_2-macroglobulin, which is the most abundant plasma protein. α_2-macroglobulin entraps irreversibly the proteinase thereby promoting the clearance through the receptor-mediated endocytosis [230].
The all 4 TIMPs members (TIMP1, TIMP2, TIMP3, TIMP4) discovered so far in vertebrates, share a conserved structure composed by an N- and C- terminal domain encompassing three conserved disulfide bonds [228].

The N-terminal domain binds to the MMP active site in a 1:1 ratio thereby forming binary non-covalent complexes, which hamper the substrate access. It was observed that TIMP-1 and TIMP-2 C-terminal domain, binds to the hemopexin-like domain of proMMP-9 and pro-MMP-2 respectively [218]. TIMP 1-3 are glycoproteins whereas TIMP 2 and 4 are unglycosylated; moreover, TIMP 1, 2 and 4 are secreted as soluble proteins while TIMP 3 is anchored to the ECM. The TIMPs expression is controlled by the same cytokines and growth factors, which are involved in MMPs expression although often in different ways. Despite their MMP inhibitory function, they are involved in other function such as promoting growth or cell death, as well as suppress mitogenic signals or directly act as mitogens in several cell types [223,232].

Protein subdomain, coming from procollagen C-terminal proteinase enhancer protein (PCPE) proteolytic cleavage, not only has structural similarity to the TIMPs N-terminal domain but is also able to inhibit MMP activity and for this reason, recognized as a new MMP inhibitor class [232]. In addition to this, several exogenous compounds such as carboxylic acid derivatives, heterocyclic structures, hydroxamate, biphenyl and tetracycline analogs can be exploited *in vitro* in order to inhibit MMPs activity [218].

MMPs functions

Since from their first discovery by Gross and Lapiere, their biological function has been traditionally associated to the ECM components degradation and turnover in order to promote embryonic growth and tissue morphogenesis.

With the passing of the years, big advances have been made; in fact, recent evidences and more detailed studies brought to light the unexpected MMP complexity often revealing surprising mechanisms of action.

The misleading and reductive idea to think MMPs to be exclusively involved in matrix microenvironment remodeling as simple degrading enzymes was surpassed.

MMPs can cleave also circulating, cell surface and pericellular proteins, large insoluble ECM components and ECM associated molecules thereby liberating bioactive proteins like cytokines, growth factors and chemokines as well as contributing to the generation of protein species with often new, completely different biological functions [223,232,242].

The ECM is much more than a simple passive structural support, in fact, is a network of structural and signaling molecules thereby providing dynamic support to cells and tissue as well as harboring embedded informations.
For this reason, the liberation of these "cryptic" molecules, not only leads to ECM architectural change but also to the cellular behavior regulation in several ways [242,243]. Cell-matrix, cell-cell interactions, the release, activation, or inactivation of autocrine or paracrine signaling molecules as well as the potential activation or inactivation of cell surface receptors, are some of the possible pathways involved [232].
Compelling evidences proved that their function can't be ascribed only to ECM remodeling during physiological process but also to pathological ones [230].

MMPs and TIMPS are involved in a delicate balance between proteolytic and anti-proteolytic activity to confine them in time and space. When this balance is perturbed in some way, a wide range of pathological phenotypes are observed ranging from tissue destruction in chronic inflammatory conditions, such as rheumatoid arthritis and chronic obstructive pulmonary disease (COPD), liver cirrhosis, gastritis ulcer disease, vascular disease as well as neurological disorders and cancer metastasis [223,225,227].

It is reasonable the existence of MMP multiplicity with distinct and sometimes overlapping functions as well as several regulation levels. All these check points might represent a strategic mechanism in order to maintain and safeguard the physiological equilibrium.

MMPs in cancer and infectious disease

In tumor biology context, the new vision of MMPs as cell signaling regulators extended their involvement in several steps of cancer progression, completely revolutionizing the initial idea that they were involved only in tumor metastasis.

Thanks to the ability to digest ECM and basement membrane, MMPs provide an access for tumor cells to the vascular and lymphatic systems, which in turn allow tumour growth and constitute an escape route for further dissemination.

However, it is mainly thanks to their ability to cleave growth-factor-binding proteins, growth-factor precursors, receptor tyrosine kinases, cell-adhesion molecules and other proteinases that they are able to deeply modify both the cell microenvironment and cell behavior, thereby promoting all steps of carcinogenesis process [229,244,245].

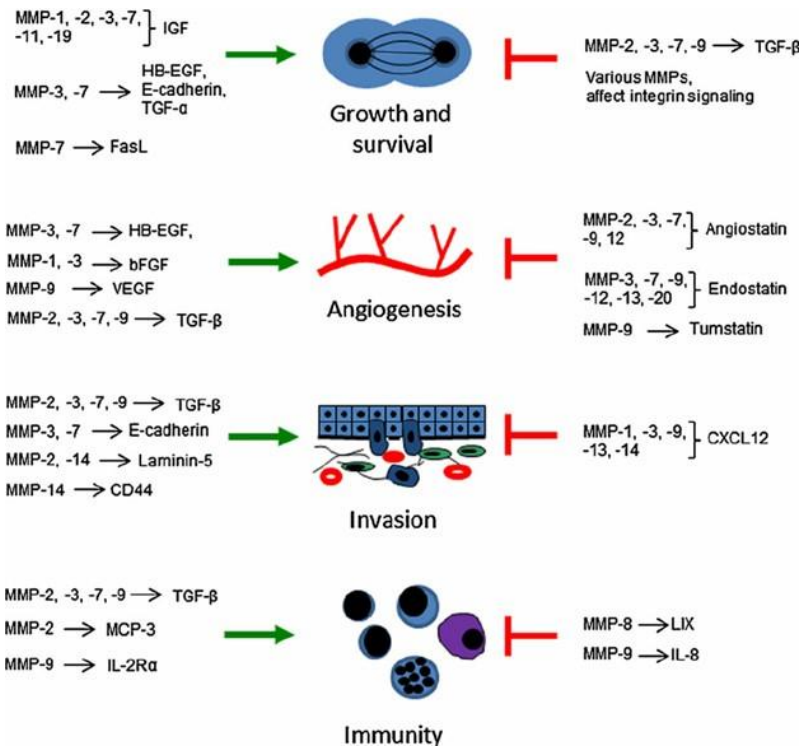


Figure 16. Schematic representation of the different roles of MMPs in cancer development. Adapted from [246].

It was observed that the cleavage of laminin-5 and collagen type IV leads to the exposure of "cryptic" sites which promote migration while the cleavage of insulin-like growth-factor-binding protein (IGF-BP) and perlecan releases IGFs and fibroblast growth factors (FGFs), respectively.

To date, more than 40 cell-surface proteins are known to be cleaved and among growth factors, Transforming growth factor-α (TGF-α), TNF-α, Delta, FGF-R, stem cell factor (SCF), DCC (deleted in colon cancer) and ephrin are numbered [247].

Also growth-factor receptors are processed by MMPs or ADAMs, like in the case of FGF receptor 1 (cleaved by MMP-2), HER2/neu (also known as ERBB2) and HER4 (also known as ERBB4) belonging to the epidermal-growth-factor receptor (EGFR) family as well as the hepatocyte-growth-factor receptor c-MET.

For each of these, the released extracellular domain acts as decoy receptor for ligands. Cell-adhesion molecules are also MMP substrates and the cleavage of E-cadherin and CD44 promotes the release of extracellular domains fragments thereby increasing the invasive behavior [229].

Interestingly, although initially, tumor cells were considered for a long time to be the main MMPs source, it was subsequently discovered that, many of the MMPs are produced by neighboring stromal cells rather than the tumor cells themselves. For example, contrary to MMP-7 which is produced by cancer cells, MMP-2 and-9 are primarily produced by stromal cells. Cancer cells might stimulate tumour stromal cells to synthesize MMPs in a paracrine manner through secretion of interleukins, interferons, EMMPRIN and growth factors. The glycosylated cell surface transmembrane protein, EMMPRIN, associated to the tumor cell, stimulates MMP synthesis in neighboring fibroblasts. MMP-1, MMP-2 and MMP-3 production can be stimulated by EMMPRIN which, however, has no effect on their physiological inhibitors TIMP-1 or TIMP-2, thereby altering the collagenolytic balance towards MMP production and inactivation [229,248].

MMP activity is exploited by tumor cells in order to escape host immune system. MMP-9 can suppress the T lymphocytes proliferation as well as MMP-11, which reduces the tumor cells sensitivity to natural killer cells. Contrary to this detrimental effect, it was observed that, MMPs may act also in a favorable way by stimulating the host innate and adaptive immune responses. The MMP-8 lacking favors tumour susceptibility probably because its proteolytic processing activity on inflammatory mediators fails, thereby hampering the host antitumor defense [244].

This latter aspect represents a sort of paradox in MMP behavior which strongly highlights the delicate balance underling their function and it is also particularly evident during infectious diseases. When host organism in fact, undergoes a viral or bacterial infection, the first step is the recruitment of immune system cells in order to eradicate the pathogen, to reduce the inflammatory status and to properly restore the damaged ECM [249]. MMPs play a key role during inflammatory process cause degrading ECM components and modulating chemokine and cytokine gradients, they pave the way to leukocytes to inflammatory site. Proteolytic processing of chemokines and chemokine receptors, releasing of chemotactic fragments or accessory proteins are some of the mechanisms through which MMPs promote leukocyte migration [250]. The release of the TNF-α active form from its membrane-anchored precursor, is performed by MMP-1,-2,-3,-7,-9 and -12 likewise to TNF-α converting enzyme.

Moreover, IL-1β is another potent pro-inflammatory cytokine which needs to be activated. MMP-2, -3 and -9 can do that as well as convert the IL-1β activated form into the inactive one, suggesting their involvement both in positive and negative regulation. MMP-9 and MMP-8 action on the N-terminal domain of the mature CXC chemokine CXCL8 (IL-8) and LIX lead to a final products much potent than the initial full-length molecules. Also in this case, MMPs proteolytic activity can lead to the formation of inactive, truncated or even antagonistic forms [250].

Another MMP important function is represented by the ability to activate defensins and antibiotic peptides, which disrupt the pathogen membrane thereby allowing bacteria killing. Even though, all these evidences highlight the important MMP contribution to ensure a normal immune response and infection resolution, it is equally true that an

MMP excessive activity, following a balance dysregulation, caused by an increased MMP or decreased TIMP secretion respectively, results in immunopathology condition. This alteration could be enhanced by pathogen proteolytic enzymes activity. Human proMMP-1, -8, and -9 were found to be strongly activated by bacterial proteinases such as those secreted by *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Porphyromonas gingivalis* which hijacking host enzymes are able to disseminate throughout all host organism thereby reaching immune-privileged sites poorly accessible to host immune cells [249,251].

MMPs in uterine disease

In addition to the above, MMPs play a crucial role in many reproductive processes such as menstruation, ovulation, embryo implantation and uterine involution. In *vitro*-culture systems as well as samples coming from the intact uterus allowed to follow both their expression patterns and their enzyme activity throughout the uterine cycle.

In reproductive organs, connective tissue cells residing in the stroma layer such as resident fibroblasts, endothelial cells in newly forming vessels and infiltrating cells, like macrophages and neutrophils, are the main MMP production sources [236]. The only exception is represented by MMP7 that is secreted by cells with epithelial origin [252].

Several molecules ranging from reproductive hormones, growth factors and cytokines taking part in reproductive processes, finely regulate their secretion. It has recently been discovered that in many mammalian species such as humans, rodents and bovines, EMMPRIN (extracellular matrix metalloproteinase inducer) molecules mainly present in the epithelial layer, influence MMP secretion.

The exact mechanism by which this happens is still not well clear but it' s probable that progesterone production acts on EMMPRIN expression which in turn affects those of MMPs in the stromal level [253]. In the particular context of bovine uterus, several aspects have to be taken into consideration, especially after parturition which represents a critical moment for cows. Very frequently, after calving, cows enter a period of negative energy balance (NEB) that alters the global gene expression. Microarray analysis revealed that most of the affected pathways were inherent to those of immunological, inflammation and connective tissue processes. The main up-regulated genes in fact, were those coding for chemokines, proinflammatory cytokines and their receptors, calgranulins (S100A8, S100A9, S100A12), vascular adhesion molecules as well as genes associated to interferons. Among tissue remodeling genes, MMPs were the main found to be up-regulated [254].

MMPs, like for example MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 perform an extensive matrix remodeling during late gestation and especially in post-partum uterus which, following placenta expulsion, has to remove cellular debris and acquire the normal tissue architecture in order to restore its size and shape [255]. Contrary to normal cows, where as soon as this happens, MMP expression is restored to basal level, in NEB affected cows MMP-1, MMP-3, MMP-9, and MMP-13 are all up-regulated [256]. MMP-1, MMP-3, MMP-9 and MMP-13 showed a strictly correlation to each other but not to those of MMP-9 expression. MMP-1 and MMP-3 in human uterus are produced by stromal cells while MMP-9 by migratory immune cells [255].

Since MMP-1, MMP-3 and MMP-13 expression raised in a coordinately manner, it leads [257] to think to a putative common regulatory mechanism in the uterine environment. Based on previous data, where in human uterine fibroblast, MMP-1 and MMP-3 secretion was found to be promoted by IL1 and TNF-α action, these latter molecules are proposed as potential regulators underlying this mechanism [255,258].

MMP expression is also influenced by those of AHSG (alpha-2-HS-glycoprotein), a natural inhibitor of insulin receptor signaling which in turn is highly up-regulated in NEB cows. AHSG circulating concentration, as well as being related to insulin resistance and hepatic fat accumulation, promotes the binding of several molecules to the plasma membrane

where they are located. In this way, they anchor MMPs thereby activating them and protecting them from autolytic cleavage [254].

MMPs expression inherent to NEB condition impairs uterine repair process thereby promoting a chronic state of inflammation. In this situation, where the mucosal layer, the first defense lane is damaged, animals are more susceptible to bacterial infection. *E.coli* in fact, is detected 90% of the time in bovine uteri and the LPS presence, significantly alters a complex clinical picture. Also in this case, different cellular genes coding for inflammatory molecules are up-regulated, including those related to MMPs such as MMP-1 and MMP-13.

Noteworthy, MMPs evolved the ability to degrade not only ECM components but also non-matrix proteins too, thereby activating potential "cryptic" molecules for inflammatory process [256]. In support of this, MMP-1 and MMP-13 are collagenases while MMP-3 is a stromelysins that is involved in ECM digestion but not in collagen and it also favors pro-MMP activation [230].

It is clear that uterine infection associated to NEB status may delay or impair the uterine repair process in postpartum endometrium through MMP alteration levels.

Although MMPs were found to be involved during inflammation in order to provide a proper defense and tissue repair process and though inflammation is essential in order to dampen and hamper pathogen action, the duration and magnitude of the immune response has to be regulated to avoid persistent tissue inflammation [257].

RNA-seq

The transcription of a gene subset into corresponding RNA molecules defines the cell identity and their biological activities regulation. Collectively, the entire repertoire of RNA molecules, ranging from coding to noncoding RNA (ncRNA) species, constitutes the transcriptome, which presents a very high complexity degree [259].

In fact, besides the presence of the canonical proteins coding mRNA and the most known ncRNAs, employing in basic cellular functions, such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNA (snRNAs), and small nucleolar RNAs (snoRNAs), new ncRNA classes have been discovered [260]. Among them, for example, microRNA (miRNA) and piwi-interacting RNA (piRNA), or as well as long noncoding RNAs (lncRNAs) are counted [261].

All these RNA molecules are involved in several gene expression levels in physiology and development, including chromatin remodeling, epigenetic memory, transcription regulation, RNA splicing, editing, translation and turnover [262–264].

RNA expression pattern can be referred to an entire organism or a specific cell type subset in a well-defined developmental stage or physiological condition. Based on this, since various factors such as cancer, genetic and infectious diseases might influence their quality and quantity levels, understanding the transcriptome is a crucial goal in order to decipher the genome functional elements, reveal the molecular constituents of cells and tissues as well as understand how disease progress works [265].

Starting from this, quantitative and qualitative transcriptome analysis were developed during the last past decade to study human genome, its variability, and the effects of variants on health and disease [266]. Transcriptomic techniques provide the ability to catalogue all transcripts species, detect gene transcript structure in terms of starts and ends sites, splicing patterns, post-transcriptional modifications, gene fusion, mutations/SNPs (single-nucleotide polymorphism) and changes in gene expression over time, but also differences in gene expression in different groups, treatments or under different conditions [265,267].

Northern blots and quantitative polymerase chain reaction (qPCR) were the first techniques used to evaluate gene expression but the ability to measure only single transcripts was their main limitation. The advent of high-throughput technology such as microarrays, allowed genome-wide quantification of gene expression, better renowned as transcriptomics [259]. However these hybridization-based approaches have a restricted ability to exhaustively catalogue and quantify the different RNA molecules expressed from genomes over wide ranges of levels [268].

Not only, but the need to know a priori the sequence to be analyzed, the high background levels related to problematic cross-hybridization artifacts in the analysis of highly similar sequences, the limited ability to precisely quantify lowly expressed and very highly expressed genes and the limited dynamic range of detection, inherent to both background and signals saturation, are the other inherent shortcomings related to the use of these techniques [259,265].

Thanks to the great strides made in the high-throughput next-generation DNA sequencing (NGS) technology field, the genome complexity was revealed.

NGS technologies such as 454 Life Sciences (a Roche company), Illumina Genome Analyzer (initially developed by Solexa) and Applied Biosciences SOLID system (now Life Technologies) are the three main platforms commercially available [269,270].

RNA-seq (RNA sequencing), the latest innovation based on NGS techniques, became the gold standard method to map and quantify RNA species thereby overcoming the limitations previously mentioned and providing new insights in regards to the complex and dynamic nature of the transcriptome [270].

Specifically, NGSs are a potent and cost-efficient tool allowing the genomic DNA sequencing, not only in its entirety, but also focusing on coding exons, specific targeted genes or amplicons only. The broad RNA species characterizing the transcriptome, the DNA methylation pattern (MeDip-Seq) as well as which specific proteins are bounded to DNA or RNA molecule (Chip-Seq) can be explored thereby bringing new knowledge to the transcriptomic and epigenetic fields [271].

One of the strengths, conversely to hybridization-based methods, is that RNA-seq doesn't require a reference genome to detect transcripts, so it might be exploited in the case of unknown, incomplete or altered sequence, thereby making it an attractive mean for non-model organisms study. It allows also to analyze all the expressed transcripts, including those of the small RNAs, as short regulatory RNAs, too short to be adequately detected by microarrays probes. Since the precise location of transcription boundaries as well as how exactly two exons are connected to each other, can be determined, this approach could be applied for complex transcriptomes analysis. Moreover, RNA-seq is characterized by lower background noise since the putative sequence is mapped in a unique position in the genome and considering the upper limit, the quantification of a large dynamic range of expression levels is allowed. The increased specificity and sensitivity compared to microarrays, the high reproducibility among biological replicates, the reliability, the low starting RNA quantity needed for the analysis, make RNA-seq a real revolutionary powerful technique [265,272,273].

Even if, many different NGS platforms are available, the traditional RNA-seq experiment follows a specific standardized workflow, basically based on: **sample isolation, sequencing library preparation, cluster generation, sequencing and finally, but not less important, data analysis**. However, this procedure is characterized by a certain variability degree, mainly related to the experimental design thereby representing a crucial step in order to accomplish the prefixed goal [259].

Biological and technological replicate number, the library choice, coverage and data artifacts generation are some of the parameters which might jeopardize the final result, and for this and to avoid losing money, they have to be carefully evaluated during the experimental set up.

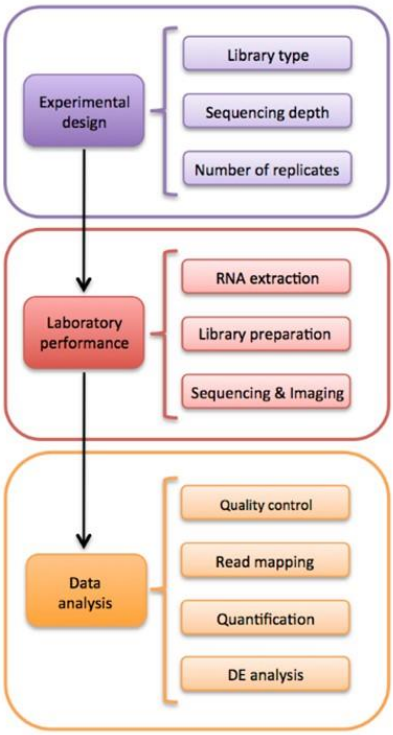


Figure 17. Overview of a RNA-seq experiment setup. Adapted from [274].

Sample isolation

Isolation of RNA and selection

As mentioned above, the aim of work could be centered on the genomic or transcriptomic profile study and in this case, the starting material choice changes. In the first case, the DNA isolation has to be performed while in the second one those of the RNA. Since all NGS protocols have been developed to analyze double-stranded DNA in the form of isolated genomic DNA, reverse-transcribed cDNA or immunoprecipitated chromatin; the starting extracted RNA requires a step more, i.e., it must be converted into cDNA in order to be properly sequenced [275].

Although many methods are available to extract RNA from any particular source, obtain the highest purity RNA, is the best assurance for the subsequent steps and to guarantee reliable and accurate data. For this reason, investigators need to carefully choose their methods of tissue and cell isolation.

Phenol-Chloroform (e.g. TRIZol) and the silica-gel column procedure (e.g. Qiagen) are the most commonly used methods to extract RNA that subsequently has to be evaluated from a quality point of view [276]. Since it was observed that during classical RNA extraction using Trizol reagent, small nucleic acids such as miRNA, precipitate less efficiently than long nucleic acids, thereby leading to their loss, the use of MirVana kit is preferable [277].

Agilent Bioanalyzer is the device generally employed to asses RNA quality, reporting its value through a specific RNA Integrity Number (RIN) ranging between 1 and 10 where 10 represents the highest quality. It is desirable to get high

quality RNA since an RNA having a low RIN value (less than six) might counteract the sequencing results thereby obtaining misleading data. However, this is not always possible, since the source of departure could be potentially any, such as cells, tissues but also autopsy/biopsy samples and paraffin embedded tissues where the likelihood to have degraded RNA is very high [259].

Library preparation

Selection

The RNA-seq library preparation represents not only the second step but mainly the crucial one. This step in fact, reflects how much cDNA sequence data are obtained from the initial RNA source.

Another aspect that is important to take into account is related to the extracted RNA composition. In fact, a large amount is given by non-coding RNA and the ribosomal RNA, which alone represents 90% of the total of a cell, while only 1-2% is related to messenger RNA (mRNA), the real focus [278].

rRNA removal is a critical step, since its presence affects the overall depth of sequence coverage thereby reducing the less-abundant RNAs detection [259]. In eukaryotes different strategies allow to do that. The first one is based on mRNA enrichment using poly(A) selection which in turn is performed by mixing RNA with poly(T) oligomers covalently attached to a substrate, typically magnetic beads.

For biological samples, for which is impossible to obtain a considerable amount and a good enough mRNA integrity to produce poly(A) RNA-seq libraries, the ribosomal depletion is applied [278,279].

Based on the above, the poly(A) libraries are realized when the main goal is to detect only coding RNA while the ribosomal depletion represents the first choice when non-coding RNAs have to be selected [259].

Recently, new efforts have been accomplished, in order to allow the researchers to selectively enrich regions of interest thereby isolating specific RNA species from the starting RNA pool. To do this, PCR-based approaches, hybrid capture, in-solution capture, and molecular inversion probes, are the main methods used [280].

In addition to this, if the RNA target is represented by small RNA species, several kits are commercially available in order to enhance their selection. The low quantity, the short length and the lack of polyadenylation in fact make their separation difficult and these kits provide a great help basically employing size exclusion gels as well as size selection magnetic beads [259].

Conversely, for bacteria, which don't have polyadenylated mRNA, the ribosomal depletion or enzymatic degradation approach such as duplex specific nuclease treatment are the only possible strategies to pursue [279,281].

From recent comparative studies it has emerged that the exonuclease treatment is less efficient if compared to the subtractive hybridization, probably due to the presence of stable secondary structures that block exonuclease advancement [282].

Fragmentation and adaptors ligation

The final fragments size is a crucial factor to take into consideration in order to optimize flow cell binding and sequencing primer. Several parameters could influence the lenght variability such as those related to the selected RNA species, to the used NGS platform as well as to the experimental purpose [270]**.**

During this step, in order to build a proper library, several manipulation stages are performed which can inadvertently introduce bias like for example the RNA fragmentation or RNA retro-transcription. Contrary to small RNAs (smaller than 200 bp) which can be directly retro-transcribed in cDNAs, the other larger RNAs have to be necessarily fragmented, into 200-500 bp molecules, to be suitable for NGS platforms sequencing. Different approaches can be involved ranging from chemical, mechanical, physical and enzymatic. Hydrolysis or nebulization are mainly used for RNA fragmentation while DNase I treatment or sonication for cDNA. For RNA fragmentation, the RNase III enzymatic digestion (used by Life Technologies in the Total RNA Seq kit) and chemical zinc-induced hydrolysis (used by Illumina) were compared, thereby showing a different manner of action.

RNase III cleaves double-stranded RNA selectively recognizing specific sequences and structures in contrast to zinc-mediated RNA cleavage. It was also observed how the use of RNase III leads to a lower homogeneity and a subsequent reduced autocorrelation between neighboring nucleotide across the transcriptome. Other inherent shortcomings were discovered after RNase III treatment such as the lower abundant of two non-coding RNA classes and how the transcript computational reassembly is compromised, leading to a lower number of reassembled transcripts, characterized by 5’ and 3’ ends less defined. Based on these evidences, the zinc-induced hydrolysis has to be preferred [282]. Once fragmentation has occurred, an additional resizing step is necessary in order to obtain a proper library size which in turn is related both to the sequencing platform involved and to the analysis purpose.

Illumina system, for example, allows to obtain good results with fragments up to 1500 base pairs (bp) in length. However, if an exome sequencing is desired, it is recommended to create sequencing libraries with inserts no longer than 200- 250 bp, simply because, the average size of a human exon is 200 bp [283].

The small RNA fragments are now converted into cDNAs molecules by reverse transcriptase, exploiting random primers. cDNA is more stable than RNA and can be easily amplified and modified; however, sequencing errors and artifacts which might interfere with transcripts characterization and quantification, can be introduced during this step by reverse transcriptase [279,284].

Immediately after, additional several steps are commonly executed and the end-repair process is one of these. The cDNA fragments in fact, may have the 5'and -3' overhang ends, which must be converted into blunt ends. To do this, DNA polymerase I or Mung Bean Nuclease approaches can be adopted [285].

At this point the 5' prime ends phosphorylation and the 3' ends A-tailing is performed in order to enhance adapters ligation [286].

These short sequences have different functions: I) they are necessary to anchor and immobilize the fragments to the support on which the sequencing reaction will take place; II) allow the sequencing machine to recognize the fragments and III) allow to sequence different samples at the same time, since different samples can use different adaptors.

There are at least three different types of adapters and therefore three different ways of preparing the sequencing library: linear adapters, circular adapters and bubble adapters. There are of course also different types of anchorage support [283].

Note of worthy, it was observed that, the optimal adapter:fragment ratio to use is ~10:1, in order to avoid adapter dimers formation, which in turn might be difficult to separate and thus dominate the next PCR amplification step [286].

Cluster generation

Immobilization of cDNA molecule template

This step allows libraries conversion into clonal clusters on a flow cell. The cDNA molecules, also named sequencing templates, are loaded onto a specialized chip or flow cell surface where amplification and sequencing will take place.

Thousands of short synthetic DNA oligonucleotides (P7, P5) present along the bottom of flow cell surface, not only allow to recognize and bind the cDNA, fragments presenting a complementary sequence, but are also used as primers for the subsequent amplification [287].

The so hybridized cDNA fragments are denatured and the single DNA strand is then used as a template for the second strand synthesis by 3' extension through a high-fidelity DNA polymerase to prevent the potential errors incorporation [287]. The amplification is an essential step, especially when the starting material is poor. The recent sequencers in fact, require several billion input molecules. One of the major drawbacks encountered during this phase is related to the loss of specific regions of the template DNA while sometimes, it may happen that other regions are more efficiently amplified [275].

The original template is now denaturated leaving the copy immobilized on the flow cell.

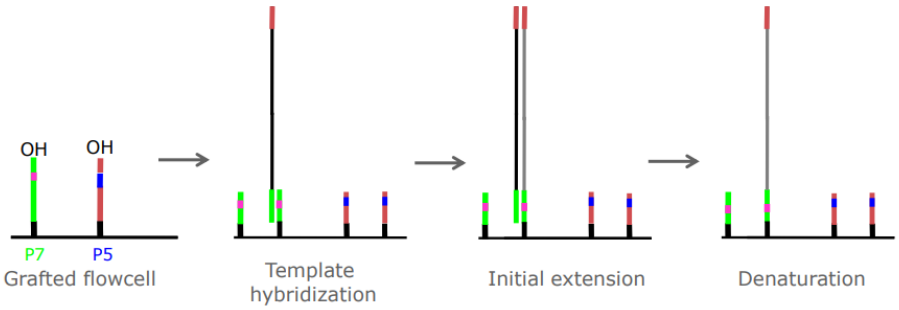


Figure 18. Schematic overview of Cluster Generation: Hybridize Fragment and Extend. Adapted from [288,289].

Isothermal Bridge Amplification

The immobilized DNA templates are ready to be amplified through an isothermal bridge amplification. The single strand template loops over to hybridize to the neighboring oligonucleotides. DNA polymerase synthetizes the complementary strand leading to a dsDNA bridge which in turn undergoes denaturation thereby creating two ssDNA strands. These two strands loop over and hybridize the adjacent oligonucleotides and are amplified again to create another dsDNA bridge. This Solid-phase amplification process is repeated until ~ 2000 of identical strands are achieved [289].

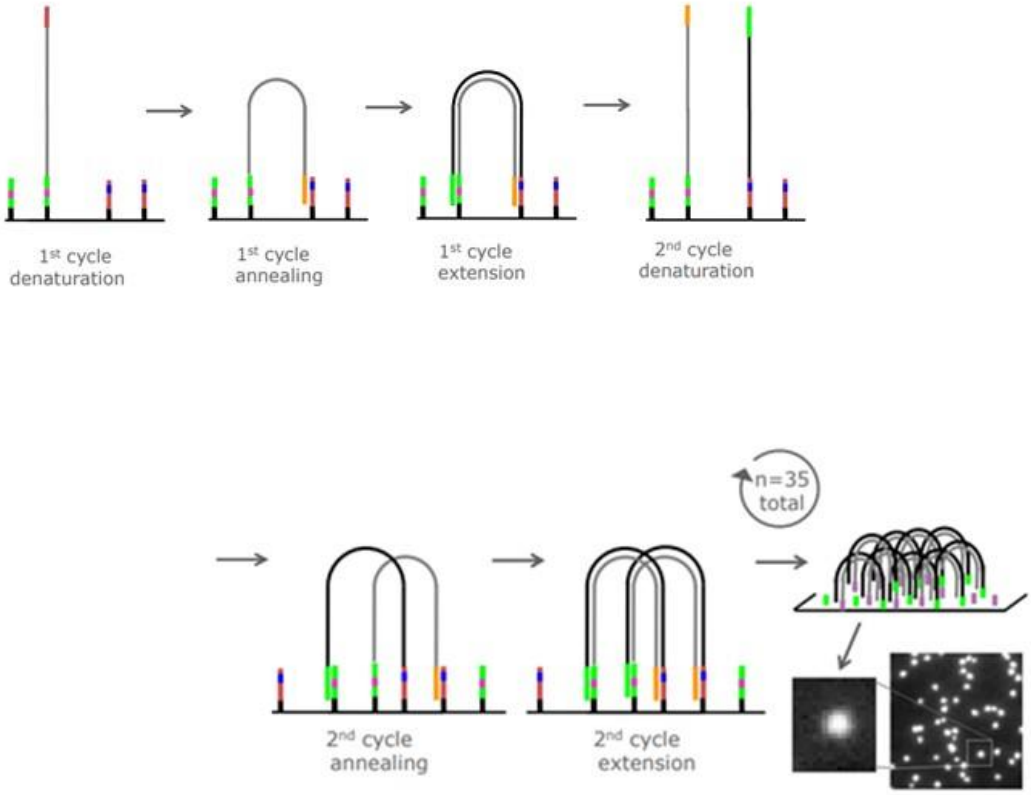


Figure 19. Schematic overview of Cluster Generation: Bridge amplification. Adapted from [289].

The ds-cDNA cluster molecules are now ready to be sequenced.

Sequencing by synthesis

At the end of cluster amplification, the sequencing by synthesis (SBS) step takes place, exploiting four fluorescently labeled reversible-terminator nucleotides. As soon as the sequencing primers (SP) attach to the DNA template, the DNA polymerase starts to add a single modified dNTP where the presence of a terminator blocks further polymerization. In this way, only a single base per round can be added to each growing DNA copy strand [259].

The terminator presents also a fluorescent label that promote the detection by a proper camera. Since only a single fluorescent color characterizes each dNTP, it means that each of the four bases must be added in a separate cycle of DNA synthesis and imaging.

Following the addition of the four dNTPs to the templates, the images are recorded and the terminators are then enzymatically cleaved thereby preparing each strand for the next incorporation by DNA polymerase. This chemistry is called "reversible terminators" and the short DNA sequence so sequenced, is called read. The computer, connected to the camera, determines which base was added recognizing the fluorescent tag wavelength and records it. The sequencing can be executed starting from one end of the interest fragment (sequencing named with single-end reads) or starting from both ends and proceeding in opposite directions (sequencing named with paired-end reads). This last procedure, is more sensitive and accurate than single-end reads, since it makes alignment operations much easier, allowing the DNA deletions, duplications or insertions detection.

This is enhanced by the fact that, in paired-end reads sequencing, the reads obtained from each end (conventionally called R1 and R2) are neither complementary nor superimposable, thereby not covering the entire length of the sequencing library fragment (except in Illumina MiSeq system).

The length of the sequence between R1 and R2, called inner mate, remains unknown, but it can be easily deduced since, during library preparation, the read length is established [288].

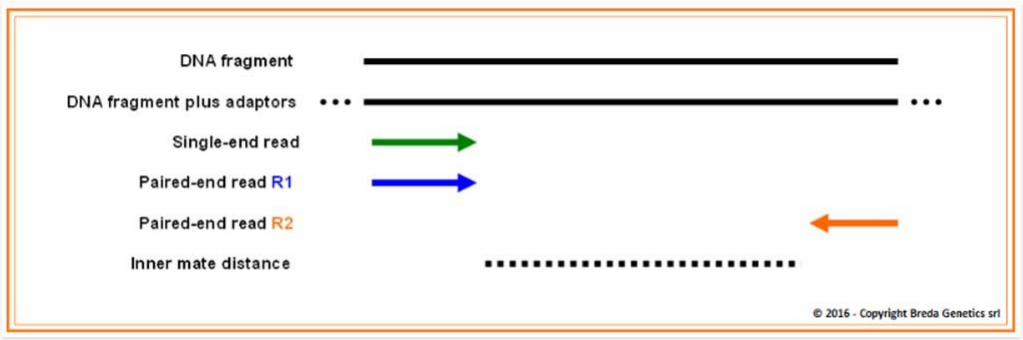


Figure 20. Sequencing can be performed starting from one end of the fragment (called single-end reads) or from both ends and proceeding in opposite directions (called paired-end reads). Adapted from [288].

This method has several strengths points; in fact, the bias incorporation is minimized by the fact that the dNTPs are present as single, separate molecule, thereby reducing the natural competition and, in addition to that, the result obtained is highly accurate base-by-base sequencing eliminating sequence-context specific errors [287,290].

In addition, since the molecules of interest were previously clonally amplified, this technique allows evaluating the relative RNA expression levels of genes [259].

Data analysis

The conventional data analysis workflow includes the generation of a FASTQ-format files containing the sequenced reads from the NGS platform; the alignment of these reads on an annotated reference genome and finally the quantification of the differential expression between samples. More in detail, the RNA-seq approach allows to discover which signaling pathways are involved, which potential transcriptional programs are induced or suppressed as well as which specific biological processes are involved in a specific disease or state [291].

AIM OF WORK

Bovine uterine infections represent a real burden for the cattle industry management and cause significant economic losses every year. Metritis and endometritis are among the most frequent bovine uterine diseases, especially after parturition, causing chronic infertility by altering the normal uterine and ovarian function.

The prevalent pathogens associated with these diseases are bacteria, which seem to increase the susceptibility of the host to subsequent infections with other bacteria or viruses, among which Bovine herpesvirus 4 is the most frequently found.

Several evidences, such as the striking tropism of BoHV-4 toward BESCs as well as the ability of this virus to persistently infect the bovine macrophage/monocyte cell line, support the hypothesis that co-infection may be involved in a possible vicious circle

During infection, the innate immune system provides a prompt response to pathogens and damage through activation of several cellular pathways. Prostaglandin E (PGE), one of the first secreted molecule in response to infection with pathogens containing LPS, has been shown to activate BoHV-4 lytic replication in persistently infected macrophages reaching the inflammation site through bloodstream.

Because a correlation has been observed between increased viral replication and PGE production as a result of bacterial infection, the BoHV-4 role seems to be limited to that of secondary pathogen.

Thus far, it has not been possible to prove a direct pathogenetic correlation between BoHV-4 and bovine uterine diseases. This is mostly because experimental inoculation with BoHV-4 reproduces the clinical symptoms only in very few cases. Additionally, the virus has been isolated both from healthy animals and from animals with varying levels of clinical and subclinical pathologies.

Some of the questions that need to be clarified are which host cell biosynthetic and metabolic pathways are affected by BoHV-4 infection; which extracellular stimuli associated with the intrauterine environment influence the BoHV-4 replicative cycle and finally how host immune system responds to virus infection.

To answer these questions, we aimed to find a putative direct link between BoHV-4 infection and bovine uterine diseases. To do this, a BoHV-4 infected BESC transcriptome analysis was performed using RNA sequencing technology (RNA-seq). Thanks to this advanced technology, the gene expression profile of BoHV-4 infected BESCs was revealed. In contrast to the uninfected control, many genes appeared to be differently regulated in BESCs following infection with BoHV-4.

The genes for MMP-1 and IL-8 were among these differentially expressed genes, and appear to be significant for pathogenesis.

By virtue of the multiple physiological roles performed by MMP-1, in particular those related to ECM remodeling as well as wound repair, it was decided to further investigate its role in the particular context of bovine uterine pathologies.

EXPERIMENTAL SECTION

INTRODUCTION

Reduced reproductive efficiency, decreased milk production, altered uterine and ovarian functions are just some of the drawbacks related to bovine uterine diseases thereby deeply affecting the dairy herds economic management.

Metritis and endometritis, in fact, are the main postpartum clinical conditions since uterine lumen sterility, maintained throughout the pregnancy duration, fails during calving as a consequence of cervix dilatation, promoting in this way, environmental bacteria entry and causing infection in 90% of cows [194,200,201,292]. These data were supported by the findings that a broad-spectrum of bacteria such as *Escherichia coli, Arcanobacterium pyogenes, Prevotella, Fusobacterium necrophorum* and *F.Nucleatum*, were isolated from uterine lumen [196,199]. Even though in a first moment, based on these findings, bovine uterine diseases were mainly associated to bacterial infections while viral causes rarely investigated; several subsequent analyses revealed the viral involvement as a cause of bovine abortions. Among them, Bovine viral diarrhea (BVDV), Bovine herpesvirus 1 (BoHV-1) as well as BoHV-4 are numbered [293,294].

In particular, the BoHV-4 isolation, coming from many sample of post-partum cows affected by pelvic inflammatory diseases [207] and metritis, all around the world, leaded to the idea of a putative correlation between BoHV-4 infection and both pelvic inflammatory diseases and chronic infertility in dairy cattle [159,210–212].

In addition, further *in vitro* and *in vivo* experiments corroborated the existence of a real vicious circle, between BoHV-4 and the endometrogenic bacteria, underlying bovine uterine diseases, also showing several differences in the pathogenetic processes, generally related to pathogen tropism.

If, for the environmental bacteria, in fact, the uterus epithelial layer represents the first site of infection, for viruses, on the contrary, the stromal layer is the primary one, which they can easily reach through bloodstream in chronically infected animals [170].

Although several evidences revealed that BoHV-4 replication, is related to post-entry events, as proved by viral reconstitution both in BESC and epithelial cells after viral nude DNA electroporation; the exact molecular mechanisms driving this process, are still unclear [109,110].
To date, this efficient replication within host cells was attributed to the Immediate Early 2 (IE2) gene activation and, in the particular context of co-infection, where BoHV-4 and E.Coli are both present; many extracellular stimuli seem to directly or indirectly influence viral replication through IE2 stimulation.

Following LPS binding to the TLR4, expressed on the endometrial epithelial cell surface, a broad range of pro-inflammatory molecules, in the inflamed damaged tissue, are produced [109,110,204].

The so secreted COX2 and PGE2 in association with LPS, induce the replication of harbored BoHV-4 in persistently macrophages which can reach the stromal layer through bloodstream thereby generating new replicating viral particles [170].

Another molecule, discovered to positively induce IE2 gene promoter activation, is TNF-α, whose production is promoted by LPS-induced macrophages [108]. Contrary to what above, if all these molecules positively contribute to

BoHV-4 replication, other act in the opposite way, dampening viral replication, such as IFN-γ, whose production is generally up regulated during inflammatory processes [111,295].

It is notoriously known that, infected cells put in place several antiviral responses like apoptosis, immune activation, and cell growth arrest in order to counteract and limit viral spread, while on the other hand, during coevolution with their hosts, herpesviruses have developed sophisticated strategies to hijack some important cellular functions thereby promoting their permanence inside the cell, viral replication and spread [38,47].
Consistent with this, it was proved that IE2 gene product, ORF50/Rta, is involved in the transactivation of both viral and host cell genes such as those coding for the chemokine IL-8, whose production, in infected Bovine Endometrial Stromal Cells (BESCs), increases in a time- and dose-dependent manner [107].

Despite these evidences and the fact that a correlation between BoHV-4 and bovine uterine diseases exists, many efforts are underway in order to deeply understand the molecular mechanisms of the physio-pathological response underlying the pathogen-host interaction.

Since BoHV-4 has been isolated from completely asymptomatic and even with different symptoms, it is natural to wonder which metabolic pathways of the host cell and molecular responses are involved in the outcome of infection in BoHV-4 persistently infected animals.

In order to shed light on these aspects, a transcriptome analysis of BoHV-4 infected BESCs compared to uninfected one, was performed. For this aim, the innovative efficient RNA seq tool was used, since great strides, in the high-throughput next-generation DNA sequencing (NGS) technology field, have been accomplished. In this manner, a greater accuracy and coverage were guaranteed, comparing this technique to that of microarray which was extensively exploited in the previous era.

Greater clarity has been made regarding genes differentially expressed following BoHV-4 infection and, thanks to enrichment functional analysis, their involvement in several cellular pathways was revealed. In particular, among up-regulated genes, IL-8 was present thereby confirming previous executed *in vitro* experiments. Another detected relevant gene was MMP-1, whose up-regulation in different host cellular pathways, was not surprising since this collagenase plays a key role both in endometrial ECM remodeling and balance regulation between normal and hyper activated immune response. This latter aspect, in particular, takes on a significant connotation during the course of infections as for example uterine diseases, thereby positively or negatively influencing their outcome. MMP-1 over expression can promote cytokines and chemokines activation, exasperating in this way, uterine conditions during metritis and endometritis. Since all these factors can promote BoHV-4 replication as well as reactivation, attracting persistently infected macrophages, many efforts have been made, to better understand the etiology and pathogenesis with the aim to develop preventive or therapeutic treatments.

MATERIAL AND METHODS

Cell Lines

Madin Darby bovine kidney (MDBK; ATCC: CCL-22), bovine embryo kidney (BEK; BS CL-94; from Dr. M. Ferrari, Istituto Zooprofilattico Sperimentale, Brescia, Italy) and BESCs (Bovine Endometrial Stromal Cells) cell lines were cultured with complete growth medium Dulbecco modified essential medium (DMEM; Sigma) containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), and 2.5 µg/ml of amphotericin B and incubated at 37°C, 5% CO_2 in a humidified incubator.

Endometrial Stromal Cells Isolation and primary cell culture preparation

In order to properly set experimental BoHV-4 infection parameters, a pure bovine endometrial stromal cells population (BESCs) was isolated and a primary cell colture prepared. Bovine uteri from post-puberal non-pregnant BoHV-4 serum-negative animals with no evidence of genital disease were collected at a local abattoir immediately after slaughter and kept on ice (no more than 3 h) until further processing in the laboratory. The ovarian morphology observation allowed to correctly establish the physiological stage of the reproductive cycle of every genital tract. For the experimental purpose, i.e., endometrial cell isolation and primary culture preparation, only genital tracts showing an ovarian stage I corpus luteum were selected, and more precisely, only the horn ipsilateral of the corpus luteum was used [296].

Briefly, uteri were initially dissected and washed with a 70% alcohol solution and subsequently with sterile PBS (Phosphate Buffered Saline) solution, supplemented with 100 IU/ml of penicillin (Sigma), 100 µg/ml of streptomycin (Sigma), and 2.5 µg/ml of amphotericin B (Sigma). The intercaruncular areas of the endometrium were cut into thin strips and placed into sterile Hank balanced salt solution (HBSS; Sigma), supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 2.5 µg/ml of amphotericin B. 25 ml of a sterile filtered solution, composed by 1 mg/ml of collagenase II (Sigma) diluted in HBSS solution supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 2.5 µg/ml of amphotericin B, were used for tissue digestion. Following 1 h of incubation in a shaking water bath at 37°C, a 40 µm mesh (Fisher) was used to filter cell suspension thereby removing the undigested material. The filtered material so obtained was resuspended in washing medium consisting of phenol-red free HBSS, 10% fetal bovine serum (FBS) (Sigma), 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 2.5µg/ml of amphotericin B. The suspension was then centrifuged at 1400 rpm for 7 min, the pelleted cells were washed twice in washing medium, and finally resuspended in Eagle minimal essential medium (Eagle-MEM) (Sigma) containing 10% FBS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 2.5 µg/ml of amphotericin B. The cells were plated in 75-cm^2 flasks, and 18 h after plating the medium was replaced with a fresh one thereby promoting a selective attachment of the putative BESCs cells. An immunocytochemistry assay was then performed to assess the stromal cell purity and a reverse transcription (RT)-PCR for the CD45 pan-leukocyte marker to confirm the absence of immune cells was carried out [111,204]. To investigate the BESCs cells innate immunological properties preservation, a functional bioluminescence assay based on LPS treatment of a bovine IL-8/luciferase gene promoter reporter construct-transfected BESCs was performed [107]. The

culture media was changed every 48 h until the cells reached confluence. All cultures were maintained at 37°C with 5% CO_2 in air in a humidified incubator.

Cell Immunostaining and counterstaining protocol

2.5 x 10^5 BESCs, at second passage, were seeded in a six-well plate and incubated at 37°C with 5% CO_2 in air in a humidified incubator. When BESCs reached the sub-confluence, the culture medium was removed and replaced with a suitable volume of acetone/methanol solution (1:1 proportion) in order to completely cover the cell monolayer and fix the cells for 20' at room temperature (RT). After two quick washes with PBS solution, the fixed cells were blocked for 1 h at RT with 10% FBS diluted in PBS plus 1% BSA.

Subsequently, cells were washed with PBS and incubated with the primary antibody diluted in PBS plus 1% BSA. As primary antibodies, we used anti-alpha vimentin mouse monoclonal antibody (diluted 1:200; sc-32322; Santa Cruz Biotechnology Inc.), anti-cytokeratin (CK) 14 rabbit polyclonal antibody (diluted 1:500; PRB-155P; Covance), and anti-CK18 mouse monoclonal anti-bovine antibody (diluted 1:200; KS-B17.2; Sigma). After 1h of incubation at RT, the antibody was then removed and the cells extensively washed with PBS three times for 3' each.

Cells were subsequently incubated with the secondary antibody which was diluted 1:500 in PBS plus 1% BSA. As a secondary antibody we used goat anti-mouse IgG AlexaFluor 488 conjugated (A11029, Life Technologies), goat anti-mouse AlexaFluor 594 conjugated (A11032, Life Technologies) and goat anti-rabbit AlexaFluor 594 conjugated (A11037, Life Technologies).

After 1 h of incubation at RT in the dark, the secondary Ab was removed and three washes in PBS solution were performed. All the antibodies, used in this experimental section, were previously validated for bovine specimens [297].

Cells were then counterstained in the dark for 10 minutes with 4',6-diamidino-2-phenylindole (DAPI). Following a final washing with PBS, images were obtained with a dedicated camera under fluorescence microscope.

For the infected cells, after a fixing step with 4% paraformaldehyde solution for 20' at RT, two quick washes with PBS solution were performed and cells were then counterstained in the dark for 10' with DAPI. Following a final washing with PBS, images were obtained with a dedicated camera under fluorescence microscope.

MTT assay

Is a simple and accurate method to quantify cell proliferation and viability. Viable cells with active metabolism convert MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound in an insoluble formazan product. Thus, color formation serves as a useful and convenient marker of the only viable cells. The measured absorbance at OD 620 nm is proportional to the number of viable cells, cell proliferation, cell viability and cytotoxicity.

MTT stock solution: 5mg/ml MTT (Promega) in PBS. This solution is filtered through a 0.2 µm filter and stored at 2-8°C

Lysing solution: 10% SDS (sodium dodecyl sulfate), 0.01 M HCl in dH_2O

1. Add MTT working solution into wells with plated cells. For example, 10 µl for each well of 96-well plate.
2. Incubate at 37°C for 6 hours
3. After 6 hours add 110 µl of lysing solution
4. Cover the plate PARAFILM®M (VWR®) and incubate at 37°C Over Night (O/N)
5. Read the plate at OD 620 nm

Viruses

BoHV-4-EFGPΔTK and BoHV-4-U (isolated from cow with uterine disease) were the two viruses involved for this experimental section [188,298]. They were propagated infecting bovine embryo kidney (BEK) cells confluent monolayers (from Dr. M. Ferrari, Istituto Zooprofilattico, Brescia, Italy) and Madin-Darby bovine kidney (MDBK) cells (CCL-22; ATCC), using in both cases a multiplicity of infection (M.O.I) of 0,5. Minimal essential medium (MEM; Sigma) containing 2% FBS was used to maintain infected cells for 2h and then replaced with fresh MEM containing 10% FBS. 72h post infection (P.I.) when approximately the 90% of cell monolayer showed cytopathic effect (CPE), the virus was obtained by freezing and thawing cells three times. Virions were pelleted with 30% of sucrose and then resuspended in cold MEM without FBS as previously described [299]. TCID50 (50% tissue culture infectious dose) was determined using MDBK cells with limiting dilutions.

BESCs infection with BoHV-4

For this experiment, three BESC batches at third passage, coming from three individual cows, were used in order to have a proper biological replicate number. Each batch was then divided into two different T75cm^2 flasks thereby obtaining 3x10^6 cells per flask. One flask was used as uninfected control while the other one was infected with BoHV-4-U for 12 h at 1 M.O.I (infected sample). After infection, the RNA coming from all 6 BESC T75cm^2 flasks (3 infected and 3 uninfected controls) was isolated. When BoHV-4-EFGPΔK was used for BESC infection, the EGFP fluorescence was monitored during time, thereby allowing the infection efficiency evaluation. Tali Image-Based Cytometer (Life Technology Invitrogen) was the instrument used in order to make a quantitative analysis of EGFP/RFP expression, apoptosis, cell cycle and cell viability.

RNA extraction and cDNA library preparation and sequencing

Transcriptome analysis was performed with the total isolated RNA coming from the 6 BESC pellets (3 infected and 3 uninfected each containing 3x10^6 cells), using TRIzol (Invitrogen, Carlsbad, CA) and purified by NucleoSpin® miRNA kit (Macherey-Nagel, Germany). More precisely the TRIzol lysis with small and large RNA in one fraction (total RNA) protocol was employed for this procedure. Agilent 2100 Bioanalyzer (Santa Clara, CA) was used to estimate RNA concentration (ranging from 292 to 380 ng/µL per sample) and quality (RNA Intergrity Number, also called RIN, RNA

integrity number ranging from 6 to 8 per sample). The isolated RNAs were stored at -80°C until use in order to preserve them. TruSeq®RNA Sample Preparation v2 Illumina kit was used to generate the libraries. In order to that, 2 µg of total RNAs per sample were used and a poly(A) enrichment step was executed in according to manufacturer's instructions. Six samples were used for cluster generation and subsequent sequencing on a single lane of Illumina Hiseq 2000 (San Diego, CA). 100-base paired-end reads were generated with an average of 63.539.480 reads per sample (ranging from 35.973.861 to 85.182.721).

Protocol of RNA extraction:

1. Wash the flask with cold PBS and detach the cells with a scraper.
2. Pellet cells at 1500 rpm for 3 minutes at 0°C.
3. Discard the supernatant and leave ~50 µl
4. Add 500 µl of Trizol Reagent (Invitrogen) and vortex well immediately.
5. Add 100 µl of Chloroform (Carlo Erba Reagenti) and vortex well for 15 seconds.
6. Centrifuge at 14000 rpm for 5 minutes and recover the aqueous solution in a new Eppendorf.
7. Precipitate the RNA adding an equal volume of isopropanol and leave to incubate on ice for 10 minutes.
8. Centrifuge at 14000 rpm for 10 minutes at 0°C, discard the supernatant and wash the pellet in 70 % cold ethanol in DieEthilPyrocarbonate (DEPC) water. DEPC is commonly used in molecular biology laboratories to inactivate RNAs eventually present in water and on instruments.
9. Spin for 1 minute at 14000 rpm at 0°C, eliminate ethanol and dry well the pellet.
10. Resuspend RNA in DEPC water

Reverse transcription PCR

Infected, un-infected and transfected BESCs were used for total RNA isolation by TRIzol (Invitrogen). 3 µg of the total isolated RNA were reverse transcribed using Ready to GO, T-Primed first-Strand Kit (Amersham Bioscience), following manufacturer's instructions. Bovine matrix metalloproteinase 1 (MMP-1), BoHV-4 glycoprotein gp80, BoHV-4 glycoprotein gB [300], BoHV-4 L1.7 late gene [181], and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were amplified using primers listed in Table 1. In addition, GAPDH gene was used as internal control.

PCR protocol set up

PCR amplification was carried out in a final volume of 50 µL, composed by 10 mM Tris-hydrochloride pH 8.3, 0.2 mM deoxynucleotide triphosphates, 3mM $MgCl_2$, 50 mM KCl, 5% dimethyl sulfoxide (DMSO) and 0.25 µM of each primer. Each reaction was executed with one hundred nanograms of cDNA sample, which were amplified over 35 cycles. Each

cycle consisted of 1 min at 94°C for denaturation, 1 min at 55°C for primer annealing, 30 sec at 72°C for chain elongation with 1U of Taq DNA Polymerase (Fermentas) and 5 min at 72°C for the final extension. The amplicons so obtained were subsequently examined in a 2% agarose gel, exploiting ethidium bromide signal. Three triplicates were performed for every sample.

Table 1. List of PCR primers used. Adapted from [301].

PRIMER NAME		SEQUENCE 5' -> 3'	PRODUCT SIZE (bp)
GAPDH	sense	GGC CTG AAC CAC GAG AAG TAT AA	103
GAPDH	anti-sense	CCC TCC ACG ATG CCA AAG T	
IE2	sense	ACA AAC ACA CAG ACC AGT CA	386
IE2	anti-sense	GTT TCA CAA CAG ATT GAG CA	
MMP-1	sense	TGT GGA GAC GGT GAA GAA ATA CCT	102
MMP-1	anti-sense	AGC TTT TCA GTT ATG AGG CCA CC	
gp80	sense	CCA CCA ACC AGA CCA CAA AT	372
gp80	sense	ATC TGT CTA TAG CAA CAT CAA ATC C	
gB	sense	CCC CCC GCG ATC GCA TGT ATT ATA AGA CTA TCT TAT TCT TCG CT	2185
gB	anti-sense	CCC CCC ACG CGT AAG GTC TGC CAT CAT TTC AGA GAG ATC TTT	
L1.7	sense	CCT TTA CAA CTG TTC TTA TGG TGC C	237
L1.7	anti-sense	CCG TTT GGT GGG AAT GTT GGT ATG	

Real-Time PCR

Real time PCR was used to evaluate MMP-1 and IE2 gene expression exploiting StepOne Real Time PCR System (Applied Biosystem), Maxima SYBR Green qPCR Master Mix (2x; Rox solution provided) and starting from 50 ng of reverse transcribed total RNA. Since BoHV-4 infection does not affect GAPDH expression, this was used as internal control. In the Table 1 are listed the primers used for PCR reaction whose conditions were: 5 min at 95°C for the initial denaturation, 1 min at 95°C, 30 sec at 55°C and 30 sec at 72°C. Quantitative real-time PCR data were analyzed with the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems, StepOne software v 2.3) and gene expression levels, normalized to the GAPDH cDNA amount, were expressed as relative quantities (RQ). qRT-PCR values of each gene were obtained from triplicates samples. The comparison was made among infected and uninfected samples (Applied Biosystems, StepOne software v 2.3). Data representation was the mean ± SD and they were considered as significant only when $p \leq 0.05$ (calculated by Student's t-test).

Construct Generation

The p2xCMVeIE2 construct was generated as described before [302]. The pMMP-1-Luc was obtained by sub-cloning the 1089 bp MMP-1 promoter sequence, cut with MluI and XhoI from pEX-A2 (Eurofins) and inserted into pGL3 basic vector (Promega) digested with the same restriction enzymes.

BESCs transient transfection

In this study, the co-transfections with reporter or/and effector plasmids of confluent BESCs (4x 10^4) plated in 24-well plates were performed using LTX transfection reagent (Invitrogen). Briefly, 3 µg of DNA were mixed with 7.5 µg LTX (ratio 1:2.5 DNA- LTX) in 500 µL of Dulbecco's modified essential medium (DMEM) high glucose (Euroclone) without serum and antibiotics. After 15 min of contact at RT, in order to promote lipocomplex formation, 2000 µL of DMEM, without FBS and antibiotics, were added. 100 µL of the transfection solution were then transferred to the cell well and left to act for 6 hours at 37°C with 5% CO_2, in a humidified incubator. The transfection mixture was then replaced with 1mL of fresh medium EMEM, containing 10% FBS, 100 IU/ml of penicillin, 100 µg/mL of streptomycin and 0.25 µg/mL of amphotericin B and incubated for 24 hours at 37°C with 5% CO_2.

Luciferase Report Assay

For the Luciferase reporter assay, a Dual Luciferase Reporter Assay System Kit (Promega) was used. Following transfection, cells were washed with PBS solution and lysed with 100 µL of passive lysis buffer with a freeze-thawing step at -80°C. Then, 50 µl of cell lysate was coupled with 35 µL of luciferase assay reagent (LAR) and enzyme activity was evaluated with a Victor3 Multilabel Counter (PerkinElmer), following manufacturer's instructions.

Western Blot Analysis

In this study, infected and uninfected control were obtained from 3x10^6 BESCs plated in 75 cm^2 flasks, treated with 1 M.O.I of BoHV-4 in 5 ml of complete medium and 5 ml of complete medium without virus, respectively. To eliminate any serum proteins trace and in order to obtain only cell-secreted proteins in the surnatant medium; cells were gently washed with 10 ml of serum-free F-12 medium (Sigma) at 3h post infection. With a centrifugation at 3500 rpm for 5 minutes at 4°C, infected and uninfected BESCs conditioned serum-free F-12 medium was clarified and cellular debris were removed.

The clarified surnatant was subsequently 40 times concentrated through Amicon Centrifugal Filters (Millipore). 5, 10 and 20 µl aliquots of the concentrated BESCs conditioned serum-free F-12 medium were separated by 12% SDS-PAGE and transferred to nylon membranes (Millipore), through the electroblotting step. Membranes were firstly incubated

with mouse anti bovine MMP-1 antibody (3B6, Thermo Scientific) diluted 1:1000 and then with a secondary antibody probed with horse radish peroxidase-labelled Anti-Mouse immunoglobulin G (A0545, Sigma), diluted 1:10.000 to be visualized by enhanced chemiluminescence (ECL Kit, Pierce).

Bioinformatic analysis

Illumina raw sequences were trimmed using Trimmomatic v0.32. Minimum base quality 20 (Phredscale) over a four-bases sliding window was required. The analysis allowed only trimmed sequences above 36 nucleotides in length, which in turn, were mapped against "Bos_taurus.UMD3.1.68" reference genome using Spliced Transcripts Alignment to a Reference (STAR) aligner, thereby obtaining a standard BAM alignment file. "Samtools" was used to sort and index BAM alignment files. "R" was exploited in read count for gene relative abundance, differential expression analysis and statistical analysis on RNA-seq data.

More in detail, the edgeR package was used to identify differentially expressed (DE) genes and gplots package to plot data. Further analysis were performed only for genes with at least one count per million over at least 3 samples, to avoid low expressed genes. A statistically relevant genes list was obtained by a statistical generalized linear model for a differential expression study between the two conditions. Only genes with fold change ≥2 and false discovery rate (FDR) <0.01 were considered as differentially expressed (DE).

Transcriptomic data are available in Sequence Reads Archive, accession numbers SRS1250917, SRS1250884, SRS1250924. RNA-seq data results were analyzed and interpreted with "DAVID bioinformatics resources", a useful and functional annotation tool exploited to obtain informations about gene functions [303].

This tool also allows the direct annotation of Bos Taurus ENSEMBLE gene IDs to DAVID IDs and to group enriched term in processes with the method "gene-gene/term-term association". However, only up or down-regulated genes were analyzed. For the enrichment analysis and for functionally relevant gene ontology (GO) terms or pathways identification a threshold FDR of 10% was set (Table 2). 598 DE genes were identified, among these 558 were associated with DAVID IDs and 173 were represented in enriched terms. A further verification was executed to best understand and study grouping of terms in processes and to do that, the Enrichment map in Cytoscape was used [304,305].

Genes, discovered to belong to the same process, were employed for network production and subsequently visualized in Cytoscape. To accomplish this, STRING v10 was used [306]. An edge-weighted force-directed layout method on the combined score attribute was used for laying out the graphs.

Animal Transcription Factor Database (TFDB) was used to identify all the transcription factors coding genes among all selected genes [307]. Heat maps and hierarchical clustering were produced using MT4 MeV (Multi Experiment Viewer) [308].

Table 2. Significant processes. Adapted from [301].

Process	Term	Count	%	Fold Enrichment	FDR
Intracellular signaling cascade					
All DE genes					
	GO:0043549~regulation of kinase activity	12	2.15	4.21	0.20
	GO:0051174~regulation of phosphorus metabolic process	15	2.69	3.38	0.22
	GO:0019220~regulation of phosphate metabolic process	15	2.69	3.38	0.22
	GO:0051338~regulation of transferase activity	12	2.15	4.07	0.27
	GO:0042325~regulation of phosphorylation	14	2.51	3.30	0.50
	GO:0045859~regulation of protein kinase activity	11	1.97	4.07	0.57
	GO:0043086~negative regulation of catalytic activity	9	1.61	4.86	0.78
	GO:0044092~negative regulation of molecular function	9	1.61	3.89	3.37
	GO:0007242~intracellular signaling cascade	24	4.30	1.98	3.46
	GO:0051348~negative regulation of transferase activity	6	1.08	6.32	3.79
	GO:0033673~negative regulation of kinase activity	6	1.08	6.32	3.79
	GO:0007243~protein kinase cascade	9	1.61	3.69	4.63

Process	Term	Count	%	Fold Enrichment	FDR
Cell cycle					
All DE genes					
	GO:0022402~cell cycle process	12	2.15	2.72	7.29
Up-regulated only genes					
	GO:0022402~cell cycle process	10	2.56	4.22	0.88
	GO:0000087~M phase of mitotic cell cycle	7	1.79	6.44	1.12
	GO:0051301~cell division	8	2.05	5.31	1.13
	GO:0005856~cytoskeleton	15	3.85	2.60	1.44
	GO:0044430~cytoskeletal part	12	3.08	2.96	2.08
	GO:0007049~cell cycle	11	2.82	3.22	3.20
	GO:0000279~M phase	7	1.79	4.82	4.90
	GO:0000278~mitotic cell cycle	7	1.79	4.73	5.35
	GO:0000280~nuclear division	6	1.54	5.80	5.52
	GO:0007067~mitosis	6	1.54	5.80	5.52
Regulation of apoptosis					
All DE genes					
	GO:0042981~regulation of apoptosis	19	3.41	2.64	0.47
	GO:0043067~regulation of programmed cell death	19	3.41	2.62	0.53
	GO:0010941~regulation of cell death	19	3.41	2.61	0.55
Microtubule cytoskeleton					
All DE genes					
	GO:0015630~microtubule cytoskeleton	15	2.69	3.08	0.39
Positive regulation of transcription					
Up-regulated only genes					
	GO:0045941~positive regulation of transcription	9	2.31	3.35	7.85

Process	Term	Count	%	Fold Enrichment	FDR
Positive regulation of cellular biosynthetic process					
Up-regulated only genes					
	GO:0031328~positive regulation of cellular biosynthetic process	11	2.82	3.27	2.86
	GO:0010604~positive regulation of macromolecule metabolic process	12	3.08	3.01	3.10
	GO:0009891~positive regulation of biosynthetic process	11	2.82	3.23	3.11
	GO:0051789~response to protein stimulus	5	1.28	9.09	3.24
	GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	10	2.56	3.34	4.49
	GO:0051173~positive regulation of nitrogen compound metabolic process	10	2.56	3.24	5.46
Glycoproteins and extracellular proteins					
All DE genes					
	GO:0005576~extracellular region	35	6.27	1.77	0.93
	GO:0044421~extracellular region part	20	3.58	2.12	2.93
Membrane fraction					
All DE genes					
	GO:0005626~insoluble fraction	14	2.51	2.99	0.90
	GO:0005624~membrane fraction	13	2.33	2.88	2.05
	GO:0000267~cell fraction	14	2.51	2.67	2.48
Oxidation reduction					
Down-regulated only genes					
	GO:0055114~oxidation reduction	14	8.33	2.42	6.32

Process	Term	Count	%	Fold Enrichment	FDR
Lipid metabolism					
All DE genes					
	GO:0008203~cholesterol metabolic process	13	2.33	12.41	3.9E-07
	GO:0016125~sterol metabolic process	13	2.33	11.35	1.2E-06
	GO:0006695~cholesterol biosynthetic process	9	1.61	20.53	6.1E-06
	GO:0016126~sterol biosynthetic process	9	1.61	18.47	1.7E-05
	GO:0006694~steroid biosynthetic process	10	1.79	10.26	5.7E-04
	GO:0008202~steroid metabolic process	13	2.33	6.35	1.2E-03
	bta00100:Steroid biosynthesis	7	1.25	13.29	8.4E-03
	GO:0008299~isoprenoid biosynthetic process	6	1.08	14.49	0.06
	GO:0008610~lipid biosynthetic process	15	2.69	3.64	0.10
	bta00900:Terpenoid backbone biosynthesis	5	0.90	11.53	0.79
	GO:0006720~isoprenoid metabolic process	6	1.08	8.49	0.96
Pathways in angiogenesis					
Up-regulated only genes					
	bta05200:Pathways in cancer	14	3.59	2.65	1.99
Metal ion binding					
Up-regulated only genes					
	GO:0046872~metal ion binding	46	11.79	1.41	8.93

RESULTS

Setup of balanced *in vitro* BoHV-4-U infection conditions

Considering the striking tropism of BoHV-4 for BESCs and the key role, which these cells perform at uterine endometrial level from a physiological and immunological point of view, a transcriptomic analysis of a pure bovine endometrial stromal cells population (BESCs), infected with BoHV-4, was performed. The first experimental step was the obtaining of a pure BESC population deprived of leukocytes contamination. BESCs, obtained from pathogen free animals and used for these experiments, were firstly validated for the absence of immune cells through RT-PCR for the CD45 pan-leukocyte marker while immune staining for vimentin (stromal marker) allowed to guarantee their stromal nature (Figure 22) [111,204].

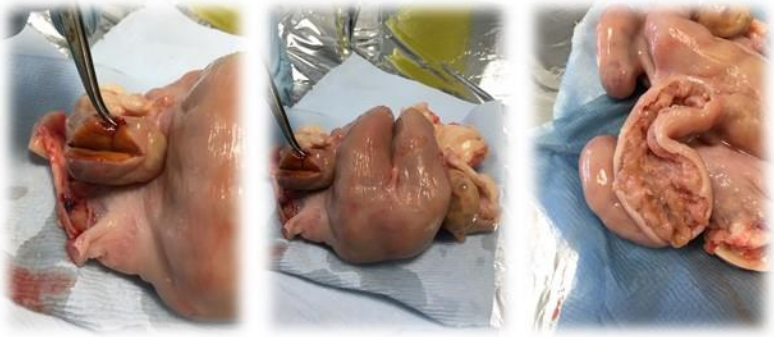


Figure 21. Pure bovine endometrial stromal cells population (BESCs) isolation and a primary cell culture preparation. Bovine uteri from post-pubertal non-pregnant BoHV-4 serum-negative animals with no evidence of genital disease were collected at a local abattoir. Only genital tracts showing an ovarian stage I corpus luteum were selected, and the horn ipsilateral of the corpus luteum was used.

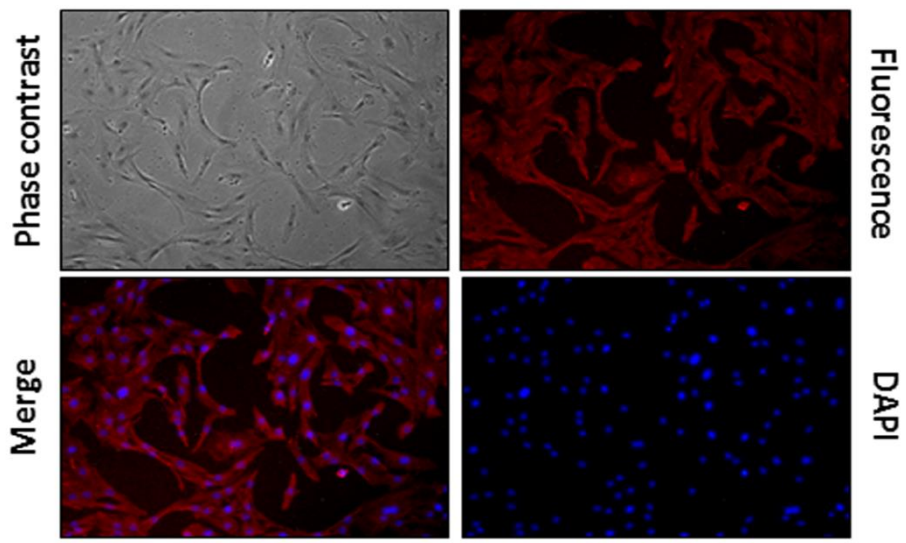


Figure 22. Phase-contrast and fluorescent images (magnification x20) of BESCs expressing the stromal marker, vimentin (red rhodamine staining). Counterstained nuclei with DAPI were merged with red fluorescent image (merge). Adapted from [111].

Before proceeding, preliminary experiments were performed, considering various parameters, in order to establish the optimal infection conditions that would have allowed to obtain an excellent quality RNA, to be used for the analysis of the BoHV-4 infected BESC transcriptome.

For this reason, BoHV-4-BESC contact duration and viral amount were the parameters carefully evaluated in order to set up the proper *ex vivo* infection conditions. Based on viral properties and the high BESC permissiveness to BoHV-4 infection, it was observed in fact that, an exceedingly high viral dosage and/or prolonged contact time are usually related to an unsuccessful or not significant patho-physiologically transcriptional changes detection as well as an increase cellular death [108–110,170]. Considering the above, high viral dosage and prolonged contact time are not recommended for *ex-vivo* studies. In addition, also mild infection conditions are not suitable, mainly because, they would damper the detection of appreciable changes in the transcriptome analysis.

In order to select the proper viral load, BESCs were infected at different M.O.I (0.1, 0.5 and 1 M.O.I) keeping the contact time fixed at 12h. For this aim, a specific BoHV-4 isolate, BoHV-4-U, coming from a persistently infected BoHV-4-positive cow, having a diagnosed post-partum metritis, was used [188].

12 hours post infection (P.I.), a MTT assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide], using uninfected cells as control, was performed in order to monitor the cell metabolic activity. Not only, but in order to better evaluate cellular conditions and to estimate infection efficiency, a parallel infection using a recombinant BoHV-4 expressing GFP (BoHV-4-EGFPΔTK) [175], was executed following the same infection conditions (contact time and viral load) mentioned above for BoHV-4-U infection.

Noteworthy, the use of BoHV-4-EGFPΔTK was restricted only to the preliminary test phase, mainly because, the deletion of early viral genes, such as that of thymidine kinase [175], could influence the host cellular genes expression.

Data obtained by inverted fluorescence microscopy (Figure 23 A) and image-based cytometry (Figure 23 B) at 12 h P.I. corroborated those obtained under the same exposure time conditions through the MTT assay.

As revealed by green fluorescence detection after 12 h P.I., a nearly 100% efficiency infection was obtained using 1 M.O.I of BoHV-4-EGFPΔTK. At the same way, the use of BoHV-4-U at any M.O.I did not compromise neither cell survival nor metabolic activity, compared to the uninfected control BESCs (Figure 23 C). In addition, also DAPI counterstaining allowed to observe how the fluorescent and therefore infected cells were actually viable. (Figure 23 A).

To further verify if 1 M.O.I and12 h of infection represented the best compromise between BESCs survival and infection efficiency thereby allowing the entire gene cascade expression, implicated in the BoHV-4 replicative cycle, a further preliminary experiment was performed.

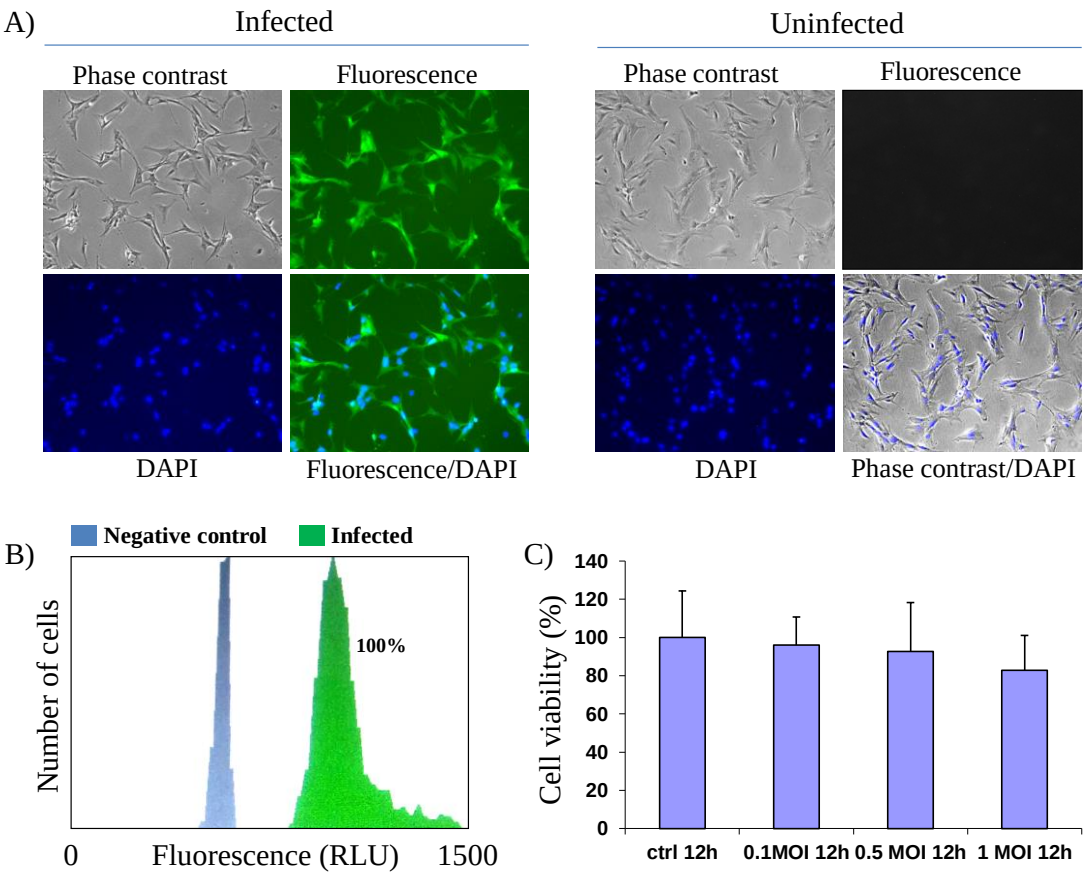


Figure 23. BoHV-4 infected BESCs A) Phase contrast and fluorescence images (10X) of BoHV-4-EGFPΔTK infected and uninfected BESCs counterstained with DAPI. B) Image-based cytometry measurement of BoHV-4-EGFPΔTK percentage infected BESCs C) BESCs cell viability after infection with different BoHV-4-U doses (0.1, 0.5 and 1 M.O.I) at 12 h post infection. Adapted from [301].

After BESCs infection with BoHV-4-U strain, immediate early and late BoHV-4 gene expression levels were analyzed using real-time reverse transcription (RT) and RT-PCR.

IE2 expression level was primarily estimated since it is an immediate early gene homologous to Epstein-Barr virus RTA gene (replication and transcriptional activator) [88], that is able to induce viral lytic replication both during the novo infections and in virus reactivation into latently infected non-permissive cells. For its own properties, IE2 (RTA) is expressed during the entire viral replication [88,102].

Its ability to induce replicative cycle initiation is strongly impaired in IE2 (ORF50/RTA) BoHV-4 mutants and it can be restored only when the open reading frame is provided in trans [309].

Being an early gene, its expression occurs after only two hours from infection as demonstrated by both RT-Real Time PCR (Figure 24 A) and RT-PCR analysis (Figure 24 B) reaching the plateau between 10 and 12 h P.I.. This time range is probably related to an advanced stage of late gene expression such as glycoprotein B (gB), glycoprotein 80 (gp80) and 1.7 major late transcripts as confirmed by semi-quantitative RT-PCR analysis (Figure 24 C).

Based on what observed from these preliminary experimental tests, 1 M.O.I and 12 h P.I. were chosen as optimally balanced infection conditions for the subsequent steps, i.e., RNA isolation and RNA-seq analysis.

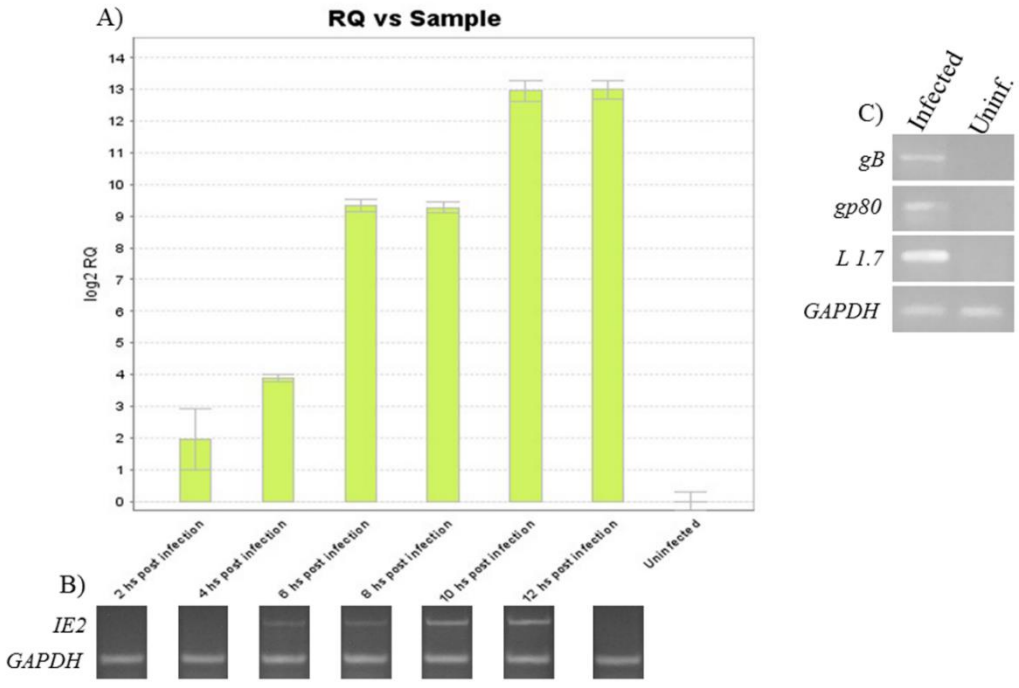


Figure 24. Viral gene expression in BoHV-4 infected BESCs A) Quantitative Real-Time PCR analysis (mean ± SD of 3 independent determinations) of BoHV-4 immediate early IE2 gene expression levels; the asterisk (*) indicates significant differences (P≤0.05) respect to the uninfected control. **B)** Semi-quantitative reverse transcription-PCR. **C)** Semi-quantitative reverse transcription PCR analysis of the indicated late BoHV-4 transcripts in BoHV-4-infected and uninfected BESCs. Bovine GAPDH was used as a house-keeping, internal reference gene in all experiments performed. Adapted from [301].

RNA-seq output-quality and reference assembly

Among the counts obtained by RNA-seq analysis, an average of 22.7 million was mapped against the "Bos_taurus.UMD3.1.68" reference genome, leading to the detection of 13.387 expressed genes with expression value up to 21.197 counts per million (CPM) and 13.081 genes with expression levels of 24.695 CPM, for non-infected and BoHV-4-U infected BESCs, respectively.

Furthermore, 507 and 201 genes were expressed only in uninfected and BoHV-4-U infected cells, respectively, while 12.880 mRNA sequences were found to be common to the two samples (Figure 25 A).

A significant expression difference was discovered in 598 genes, with a FDR (false discovery rate) p-value correction ≤ 0.01 and with a fold change value ≥ 2 level. More precisely, among these 598 differentially expressed genes, 417 genes

were found to be up regulated (fold change values from 2.00 to 1530) while 181 down regulated (fold change values from -2.00 to -7.8) (Figure 25 B, C).

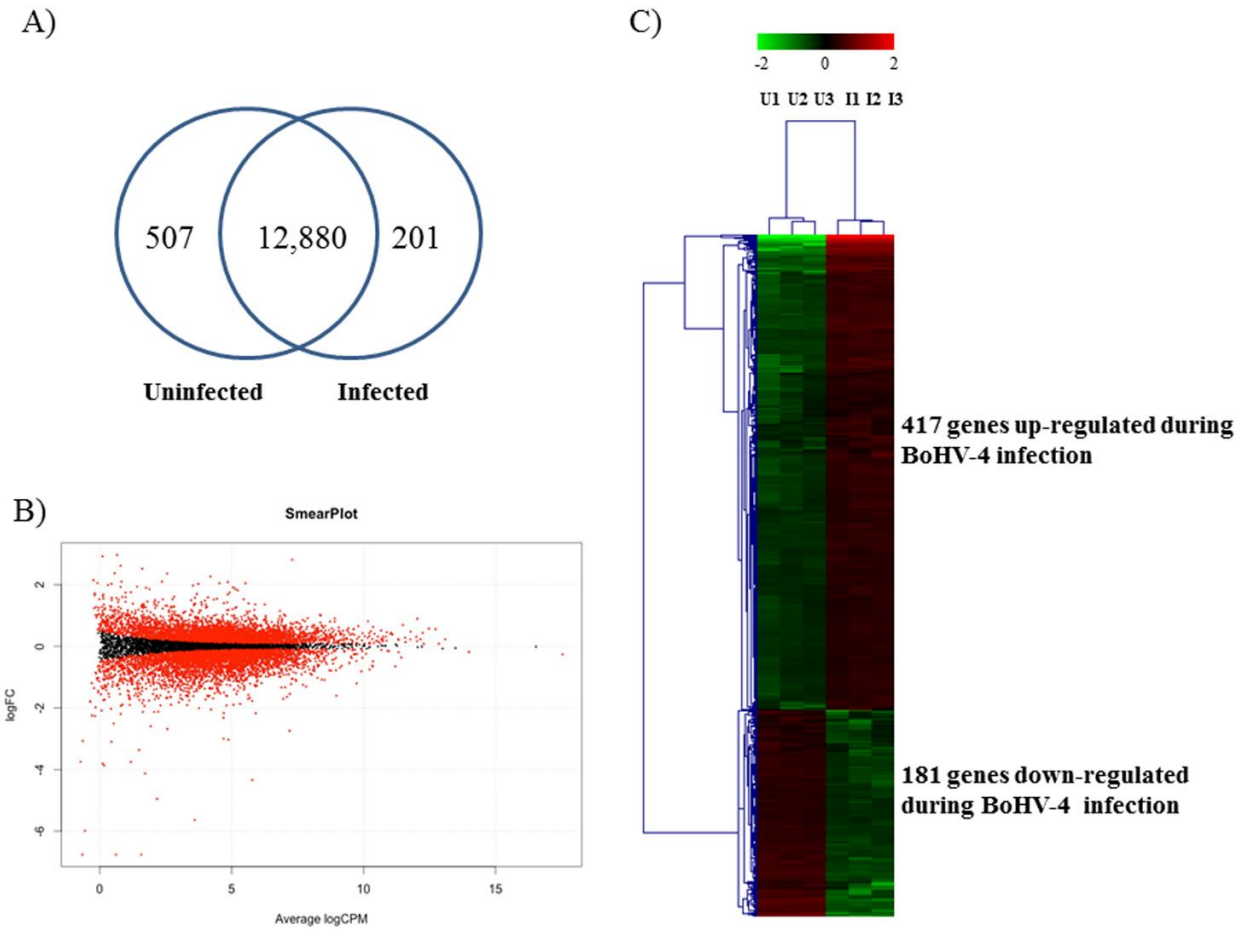


Figure 25. BoHV-4-infected and uninfected BESCs comparative transcriptome profiling A) Venn diagram representation of genes detectable in at least two biological replicates (at least 5 counts/gene) in uninfected and infected cells and in BESCs regardless BoHV-4 infection. **B)** Scatter plot showing the relationship between log fold-change and average log counts per-million. Black dots represent genes expressed in a non–significantly differential manner in infected and uninfected cells, while for the DE genes with a FDR ≤0.01 in the two states, red dots were used. **C)** Hierarchical clustering of the 598 differentially expressed genes (FC≥2, FDR≤0.01) in the three biological replicates; before visualization the genes were median-normalized. Up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

Predicted functional landscape of DE Genes in BoHV-4-U-Infected versus non infected cells

Genes, coding for several protein kinases, cytokines, transcription and growth factors, appeared to be differentially expressed (DE genes).

In functional annotation analysis, many enriched terms were identified and the most important were: “Intracellular signaling cascade”, “Positive regulation of transcription”, “Glycoproteins” and “Extracellular proteins”.
In addition to that, also “Cell cycle”, “Regulation of apoptosis”, “Microtubule/cytoskeleton”, “Positive regulation of cellular biosynthetic process”, “Membrane fraction”, “Oxidation reduction”, “Lipid metabolism”, “Pathways in angiogenesis” and “Metal ion binding” emerged from enrichment analysis. (Table 2).

Within DE genes list, “Glycoproteins” (43 genes) and “Extracellular proteins” (35 gene) were the most abundant from a quantitative point of view since these two functional classes, together, represent more than 10% of the total DE genes identified with DAVID.
Through this analysis, other genes involved in the inflammatory response such as IL-8, growth factors and cytokines like GDNF, KITLG, LIF, NDP, NMB, STC1, CXCL14 and IL1RN (or Interleukin-1 receptor antagonist) were strongly positively or negatively modulated.
Noteworthy is the fact that, the data observed here through transcriptome analysis, related to IL-8 gene, are perfectly in line with what previously observed, thereby corroborating and validating previous experimental results
IL-8, in fact, as previously demonstrated by Donofrio et al., is the main BoHV-4 induced cytokine in infected bovine endometrial stromal cells and its expression level it’s closely related to the endometrial inflammatory status [302]**.** In BoHV-4-U infected cells, G protein coupled receptors CALCRL, GPR133, GPR68, NMB, P2RY14, SSTR2 as well as other surface receptors and proteins involved in signal transduction pathway, like FST, RSPO3, SFRP1 and ITGA2 and ITGAV integrins were differentially expressed. Furthermore, also many enzymatically active proteins, such as ARSA, Bt.99682 (GPX3), GCNT3, LOXL4, P4HA3, PNPLA2, PTGS2 and some peptidases like ANPEP, C4A, CPQ and PAMR1, the matrix metalloproteinases such as MMP-1, MMP23B and MMP-3, protease inhibitors like ITIH5, PI15, PIK31P, SERPINF1, SERPING1 were included into this functional class. In addition, within DE genes, other membrane proteins are included (Figure 26, Membrane fraction).
In particular, matrix metalloproteinase (including MMP-1), integrins and IL-8 seem to be the center of an interconnected node into the “Glycoproteins and Extracellular Proteins” interaction network (Figure 27).

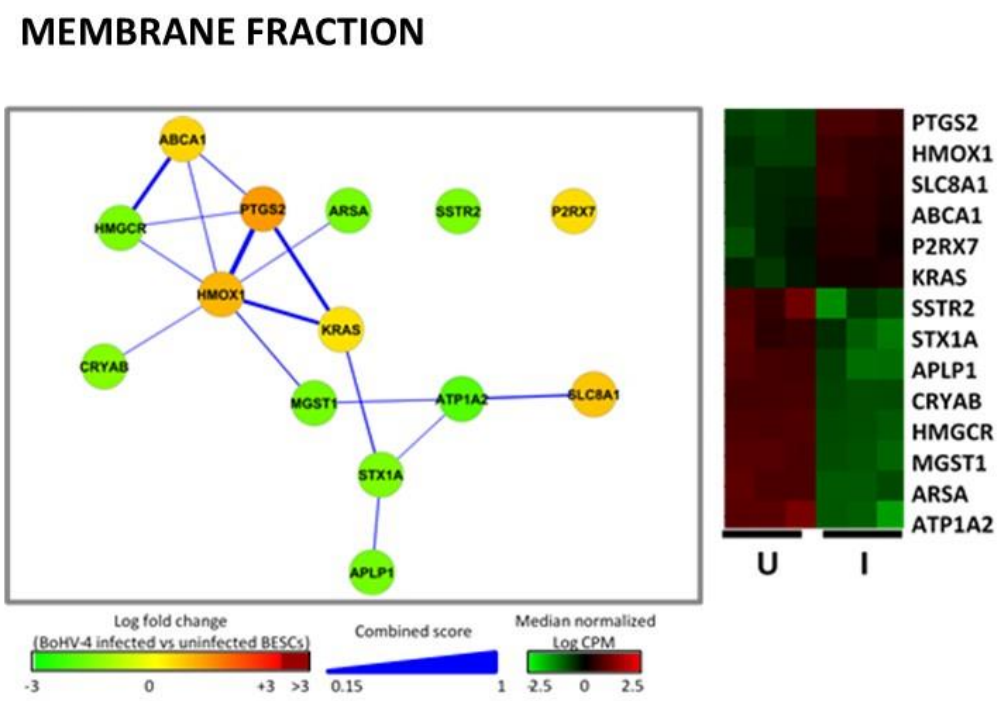


Figure 26. BESCs gene products classified as "Membrane fraction" differentially modulated by BoHV-4 infection. Differentially expressed gene products functionally classified as "membrane fraction" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to individual interactions score/strength. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

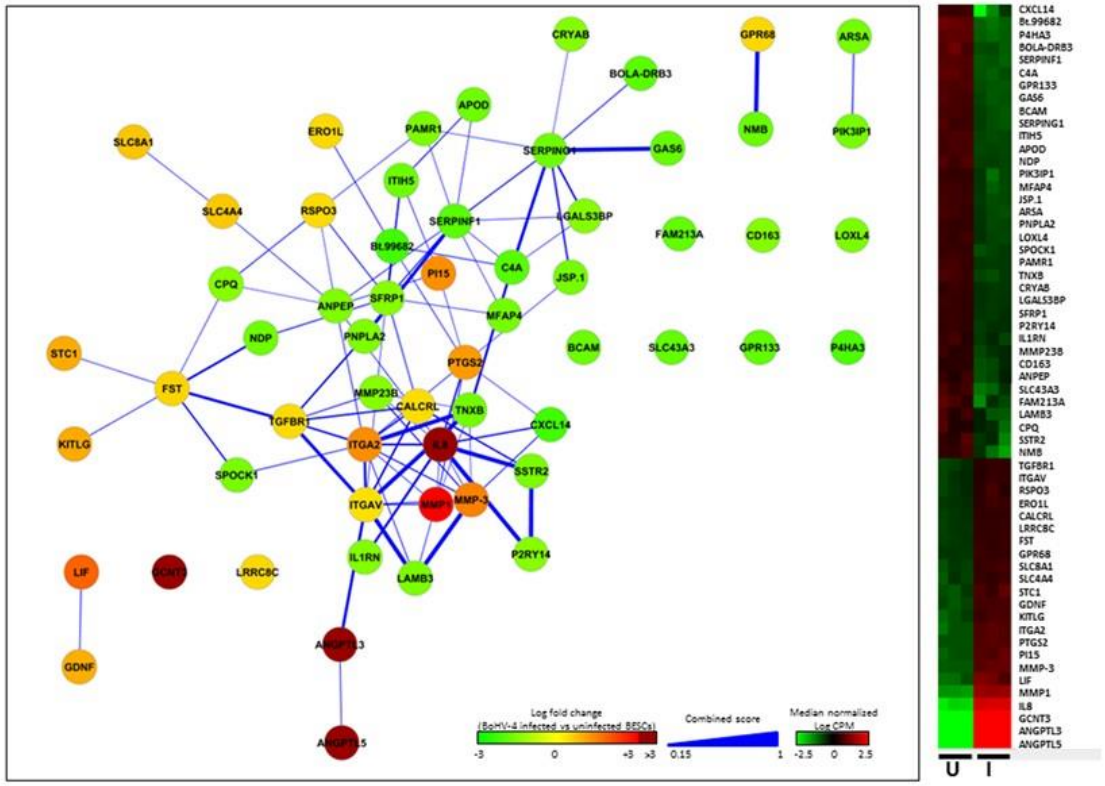


Figure 27. BESCs gene products classified as "Glycoproteins and Extracellular proteins" differentially modulated by BoHV-4 infection. The differentially expressed gene products functionally classified as "glycoproteins and extracellular proteins" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to the score/strength of individual interactions. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

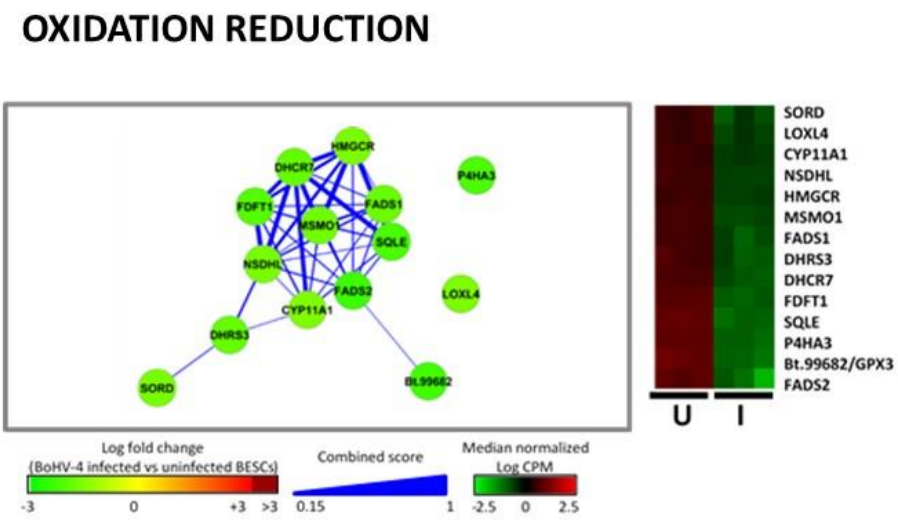


Figure 28. BESCs gene products classified as "Oxidation reduction" differentially modulated by BoHV-4 infection. The differentially expressed gene products functionally classified as "oxidation reduction" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to the score/strength of individual interactions. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

From the transcriptome analysis of the infected BESCs, also the "Oxidation reduction" process was found to be altered, in particular when down regulated genes were considered. Among them, glutathione peroxidase, dehydrogenases DHRS3, NSDHL and SORD, lysyl oxidase LOXL4, hydroxylase P4HA3 and many lipid and sterol biosynthesis responsible enzymes, like CYP11A1, HMGCR, DHCR7, FADS1, FADS2, NSDHL, SQLE, MSMO1 and FDFT1 (Figure 28 *Oxidation reduction*) were included.

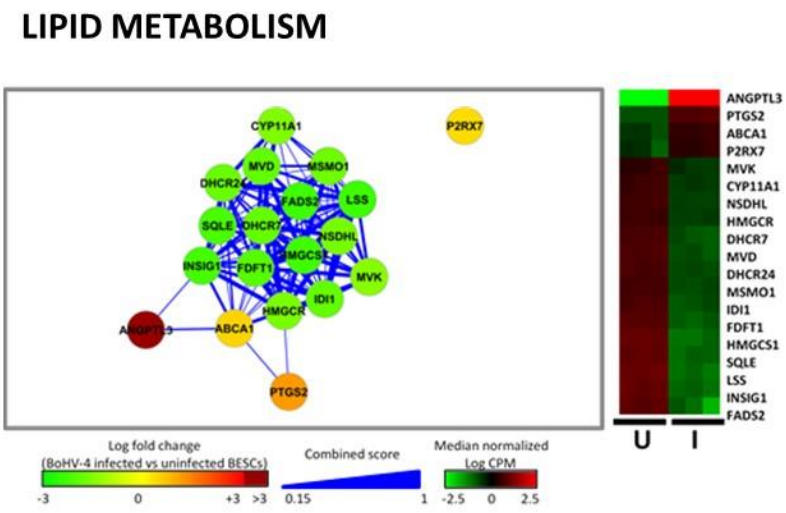


Figure 29. BESCs gene products classified as "Lipid metabolism" differentially modulated by BoHV-4 infection. The differentially expressed gene products functionally classified as "lipid metabolism" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to the score/strength of individual interactions. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

The "*Lipid Metabolism*" was also classified as a significantly enriched functional class (Figure 29, *Lipid metabolism*). It includes, in addition to the above mentioned genes, some down-regulated lipid/sterol biosynthesis genes, like HMGCS1, IDI1, LSS, MVD and MVK, cholesterol synthesis repressor (INSIG1), plus some oppositely regulated genes which encode prostaglandin synthase PTGS2, purinergic receptor P2RX7, angiopoietin-like protein ANGPTL3/5 and ATP-binding cassette transporter ABCA1.

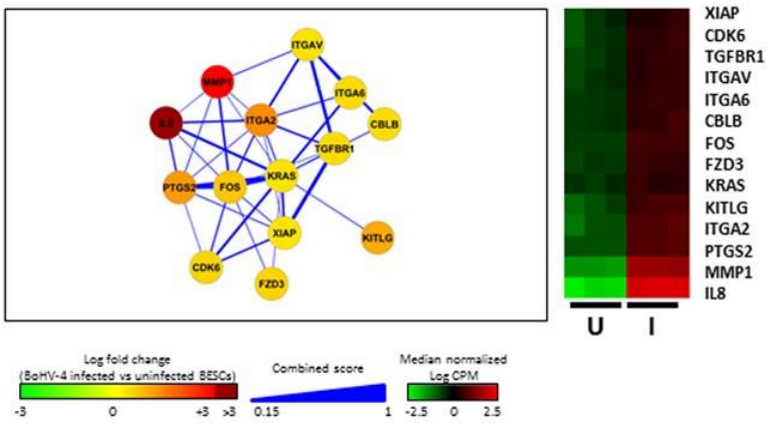


Figure 30. BESCs gene products classified as "Pathways in angiogenesis" differentially modulated by BoHV-4 infection. Differentially expressed gene products functionally classified as "Pathways in angiogenesis" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

Conversely to the "*Lipid Metabolism*" class, a significant enrichment was found when only up-regulated genes were considered for the "*Pathways in angiogenesis*" and "*Metal ion binding*" classes. More in detail, the first class includes IL-8 and MMP-1 genes, integrin-coding genes ITGA2, ITGA6 and ITGAV, genes involved in proliferation regulation like FOS gene, KRAS and the prostaglandin synthase gene PTGS2 (Figure 30), whose up-regulation is indirectly but strongly related to prostaglandin E2 secretion, a BoHV-4 infection linked molecule [110]. All these data further corroborate the reliability of our *in vitro* infection system as well as those of RNA-seq tool.

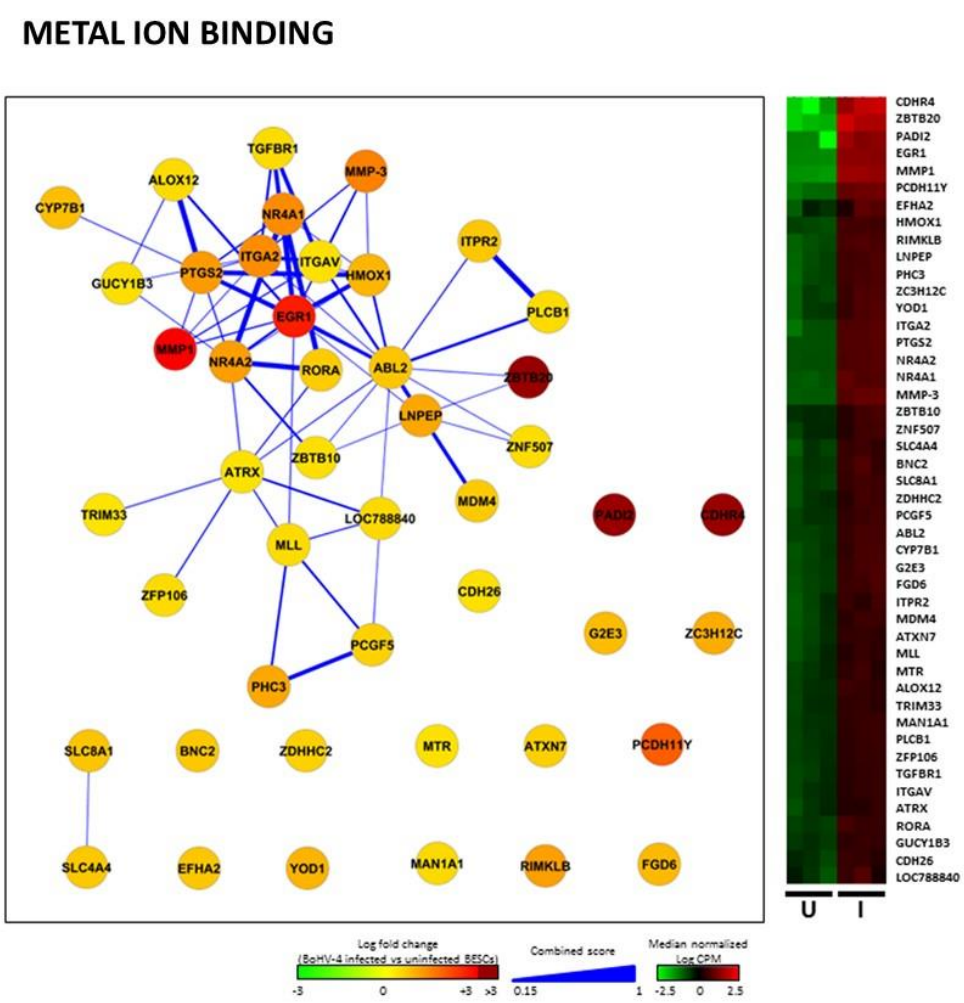


Figure 31. BESCs gene products classified as "Metal ion binding" differentially modulated by BoHV-4 infection. Differentially expressed gene products functionally classified as "Metal ion binding" are shown on the left as a network generated with STRING v. 10 (Bos taurus ENSEMBLE gene IDs). The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

The "*Metal ion binding*" Gene Ontology (GO) term comprehends 46 DE genes. It is not only the most populated and significantly enriched process discovered through functional analysis (Figure 31), but it also includes half of the genes (five out of twelve resulting by DAVID IDs) induced more than five-fold in infected BESCs. CDHR4 (cadherin-related family member 4 precursor), PADI2 (protein-arginine deiminase) transcription factors EGR1 and ZBTB20 and matrix metalloproteinase-1 (MMP-1), which in turn was 8-fold induced in BoHV-4 infected cells, are only few of the genes that is possible to find among these genes.

Moreover, other transcription factors such as BNC2, NR4A1, NR4A2, RORA, ZBTB10, ZNF507 and the integrins ITGA2 and ITGAV are included in this functional class.

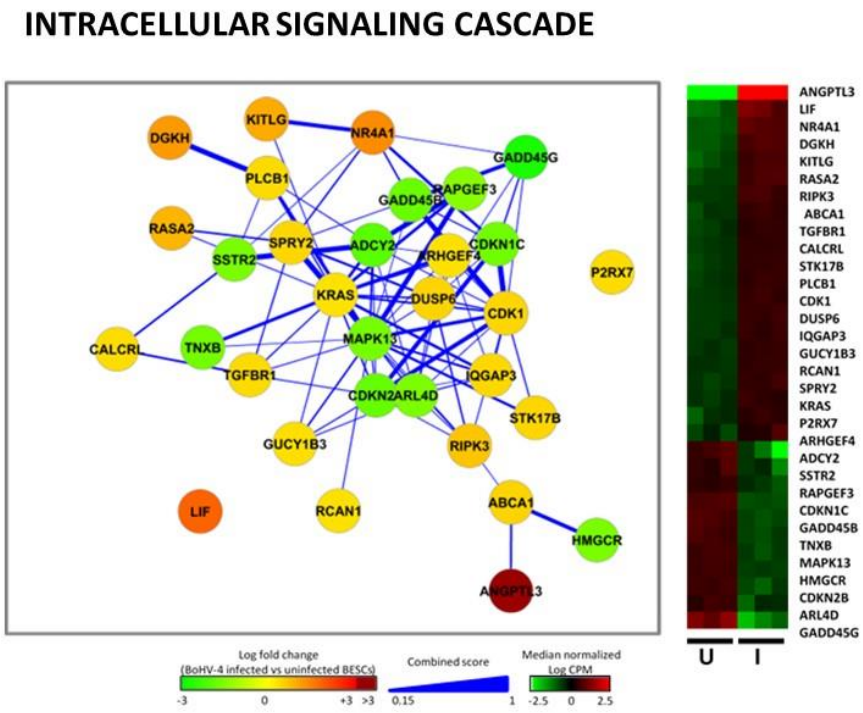


Figure 32. BESCs gene products classified as "Intracellular signaling cascade" differentially modulated by BoHV-4 infection. Differentially expressed gene products functionally classified as "intracellular signaling cascade" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to the score/strength of individual interactions. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

"*Intracellular signaling cascade*", conversely to the previous functional class, includes either up and down-regulated genes. In particular, it includes the previous mentioned ANGPTL3 (angiopoietin growth factor 3), one down-regulated (MAPK13) and five up-regulated kinases (CDK1, DUSP6, RIPK3, STK17B and TGFBR1), two down-regulated cyclin-dependent kinase inhibitors (CDKN1C and CDKN2B) and the up-regulated LIF and KITLG growth factors (also included in the *"Extracellular Proteins"* class) (Figure 32, *Intracellular signaling cascade*).

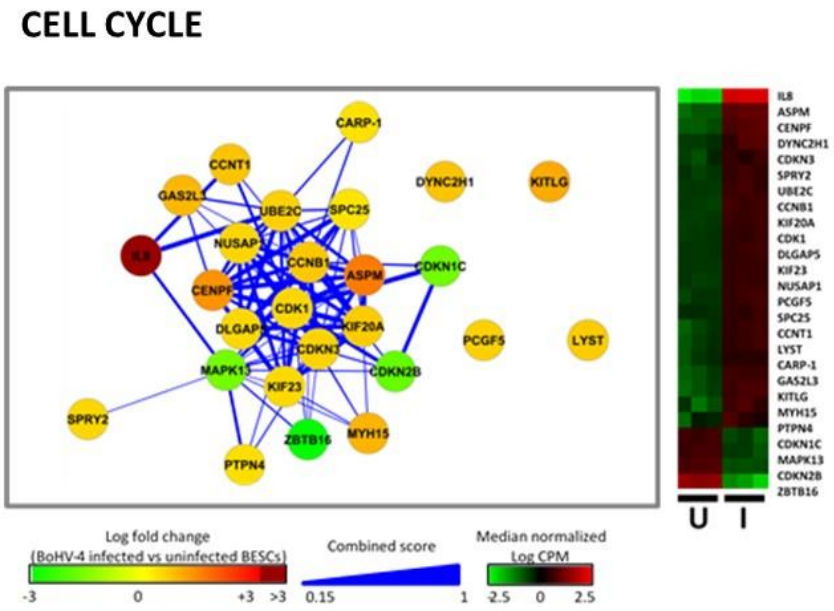


Figure 33. BESCs gene products classified as "Cell cycle" differentially modulated by BoHV-4 infection. Differentially expressed gene products functionally classified as "cell cycle" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to the score/strength of individual interactions. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

An expected result was the presence of *"Cell cycle"* and *"Regulation of apoptosis"* classes among the significantly enriched GO terms obtained by the analysis. Conversely to the IL-8 gene, other genes included in the *"Cell cycle"* class, such as ZBTB16, CDK1, MAPK13, KITLG, CDKN1C, CDKN2B and CDKN3, resulted to be down-regulated in infected samples (Figure 33, *Cell cycle*).

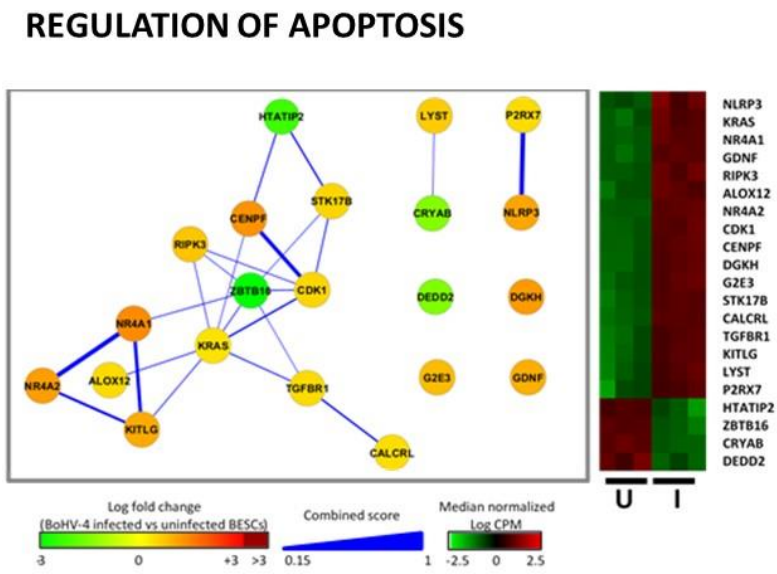


Figure 34. BESCs gene products classified as "Regulation of apoptosis" differentially modulated by BoHV-4 infection. Differentially expressed gene products functionally classified as "regulation of apoptosis" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to the score/strength of individual interactions. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

Four kinases, ZBTB16 transcription factor and both the orphan nuclear receptors NR4A1 and NR4A2, were found to be up-regulated among the *"Regulation of apoptosis"* GO term (Figure 34, *Regulation of apoptosis*).

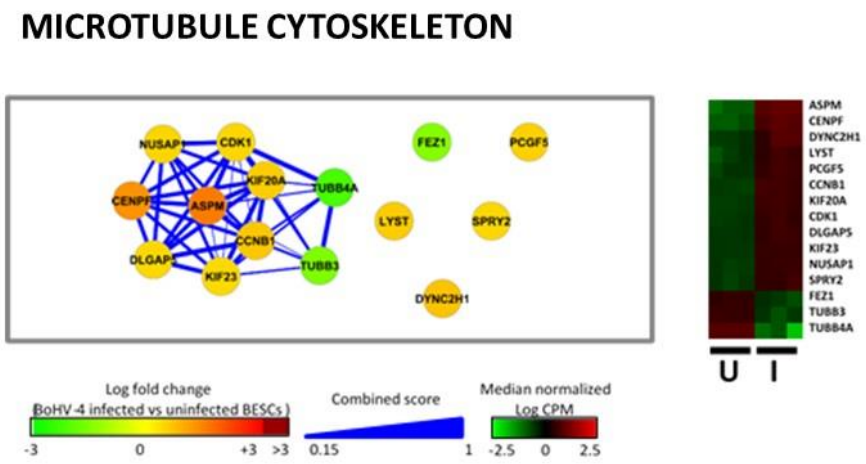


Figure 35. BESCs gene products classified as "Microtubule cytoskeleton" differentially modulated by BoHV-4 infection. Differentially expressed gene products functionally classified as "Microtubule cytoskeleton" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to the score/strength of individual interactions. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

Among the DE genes, it is possible to find also "Microtubule/cytoskeleton proteins" showing an apparent trend toward up-regulation (Figure 35, Microtubule/cytoskeleton proteins). This data, probably reflects one of the BoHV-4 hallmark which, conversely to the other Herpesviruses, is devoid of specific host shutoff housekeeping genes.

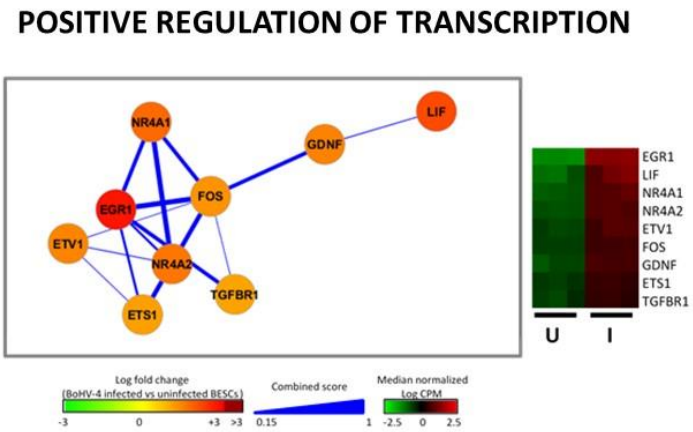


Figure 36. BESCs gene products classified as "Positive regulation of transcription" differentially modulated by BoHV-4 infection. Differentially expressed gene products functionally classified as "positive regulation of transcription" are shown on the left as a network generated with STRING v. 10, using Bos taurus ENSEMBLE gene IDs; node color indicates fold-induction in BoHV-4-infected compared to uninfected BESCs; edge thickness is proportional to the score/strength of individual interactions. The same DE genes set is shown on the right as a heat-map representation of the three biological RNA-seq replicates; up-regulated and down-regulated genes are shown in red and green, respectively. U: uninfected BESCs; I: BoHV-4-infected BESCs. Adapted from [301].

"Positive regulation of transcription" GO class (Figure 36, *Positive regulation of transcription*) encounters only infection-induced genes showing an up regulation expression, such as transcriptional activators ETV1 and EGR1, nuclear receptors NR4A1 and NR4A2, transcription factor FOS and growth factors LIF and GONF1 (or glial cell-derived neurotrophic factor) and growth factor receptor TGFB1. Overall, using TFDB annotation, 32 up-regulated sequence-specific transcription factors were detected [307].

MMP-1 up-regulation in BoHV-4-U-infected BESCs

In silico analysis allowed to discovered a broad range of DE genes, between BoHV-4 infected and non-infected BESC cells, most of which were involved both in biosynthetic and signaling pathways.

Based on what observed, there is a clear connection between BoHV-4 infection in bovine endometrial stromal cells and its pathogenic role. The expression of several genes, belonging to intracellular signaling cascade, positive regulation of transcription, glycoproteins/extracellular proteins, cell cycle, and regulation of apoptosis class, seem to be affected by BoHV-4 infection. Noteworthy, among all these DE functional classes, a key role is played by Matrix Metalloproteinase 1 (or MMP-1), whose expression level increased ≈ 8 fold in BoHV-4 infected BESC cells compared to the un infected control sample. In addition, the protein coded by MMP-1 gene is functionally interconnected in three different classes, i.e., *Glycoprotein and extracellular proteins*, Figure 27, *Pathways in angiogenesis*, Figure 30; *Cation binding*, Figure 31 and it is characterized by a trend toward up-regulation in each of these.

The MMP-1 up regulation is extremely relevant since through its proteolytic properties promotes extracellular matrix disruption as well as tissue remodeling thereby influencing endometrium-tropic virus behavior such as those of BoHV-4 in this context [310].

Based on these considerations, MMP-1 was selected as a test gene for independent validation and experimental data, obtained through semi quantitative PCR (Figure 37 B), quantitative Real Time PCR (Figure 37 A), Western Immunoblotting (Figure 37 C) and luciferase reporter system assays, confirmed MMP-1 up-regulation in host cells infected by BoHV-4-U. In order to normalize the data, coming from both semi-quantitative PCR and quantitative real-time PCR data, GAPDH expression level was taken into account since it was previously validated to be not affected by BoHV-4 infection, with the parameters (viral load and length of contact time) used for this experimental section. This assumption was further corroborated by the total absence of GAPDH up or down regulation in BoHV-4-U infected BESCs transcriptome. In addition, also MTT assay proved that BESCs metabolic activity and consequently GAPDH glycolytic activity remained completely unaltered as a result of BoHV-4 infection. To eliminate any doubt, a Luciferase assay was performed.

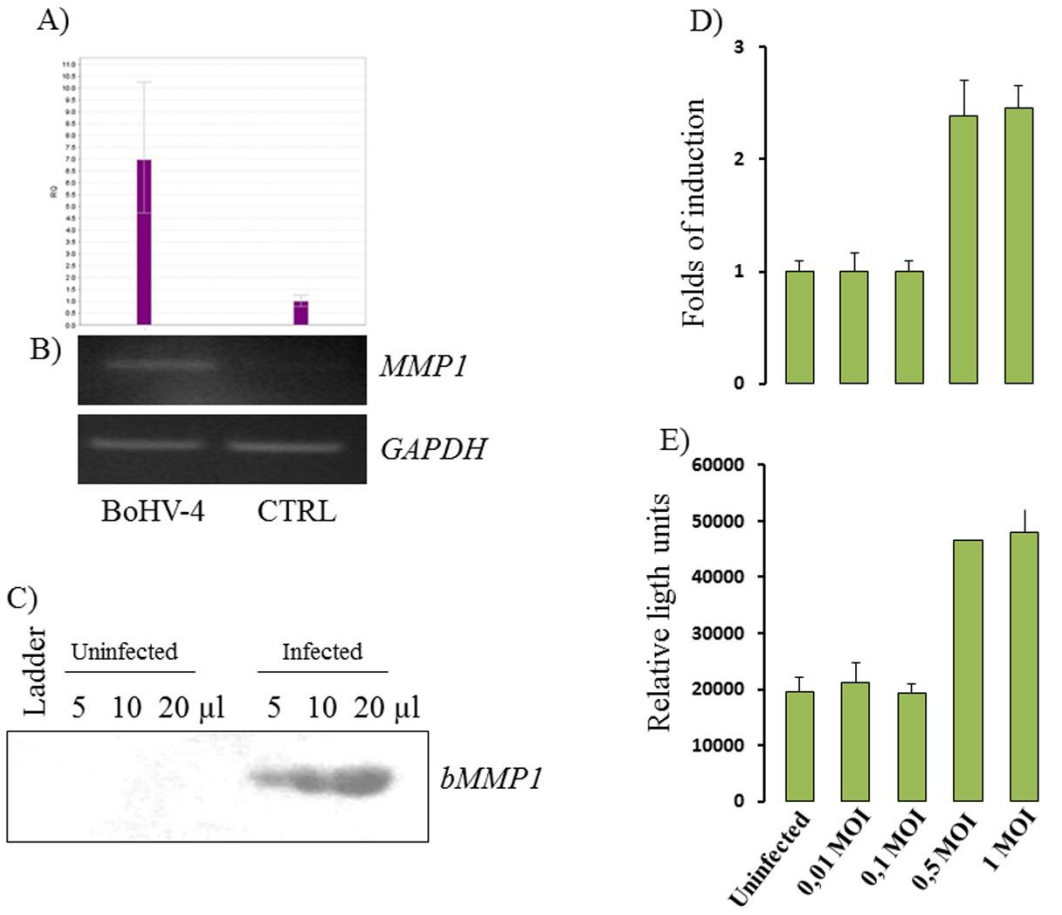


Figure 37. MMP-1 up-regulation in BoHV-4-infected BESCs A) MMP-1 transcript levels revealed by quantitative real time PCR analysis (mean ± SD of 3 independent determinations) in BoHV-4-infected (BoHV-4) and uninfected (CTRL) BESCs; a significant difference (P≤0.05) in MMP-1 expression levels between the two conditions is apparent. **B)** As in A**,** the semi-quantitative reverse transcription PCR was performed and the GAPDH gene was used as a housekeeping, internal reference gene for both analyses. **C)** Different cell supernatants (5, 10 and 20 µl, as indicated), derived from BoHV-4-U infected or uninfected BESCs, were investigated by western immunoblotting analysis. **D)** In BESCs transfected with the MMP-1 promoter-luciferase plasmid (pMMP-1-Luc) and infected with BoHV-4-U at the indicated M.O.I. values, MMP-1 promoter activity was measured. Data were indicated as relative light (luciferase activity) units and presented as the mean ± SD of three determinations. Uninfected BESCs served as a negative control and internal reference; significant differences (P≤0.001) are marked with an asterisk (*). **E)** Average (± SD) fold-induction levels, calculated by comparison with expression levels measured in uninfected controls, derived from the data shown in D. Adapted from [301].

To do that, pMMP-1-Luc construct containing a 1089 bp minimal MMP-1 promoter sequence with most of the 5'-UTR, derived from Bos_Taurus.UMD3.1.68 genome (Figure 38) and chemically synthetized upstream to the firefly luciferase gene, contained in the pGL3 basic vector, was generated.

The pMMP-1-Luc construct so obtained was used to transfect BESCs which in turn, were subsequently infected with different BoHV-4 multiplicity of infection (0.01, 0.1, 0,5 and 1 M.O.I).

MluI
Acgcgt
ATGCAGAAAGAAGAAATACTTGCTTCCTATCCCCAAGGAGCAGACATGTGCTCACACTGA
GGGGAATGAGGATGATTAAAATAAGATCTCAGACTCACACTTGATCGTCTGCTAGCCTCT
GGCTTCCCTAATGGGCCTGACAGTTTTCAGAGAAGGATTCCACTTGTCCCGTGATTCTGC
TGGGCAGTGTCTACACCCTGTAGGGAGTTACCACATACCCCACCCGTGGATGGGGGTCTT
CTCAGCCCAGATTCTCAAATTCTGGGCCTGAATGAAGATTAAGGGAAACTATGGTGCTAT
GAAGTAGTGTACAAGGCAGTTTAGCAAAGGTGGAAAGAAACCCCACTGAACCCAGAAGAA
AAAGTGAGTGTTAATTCATGTCTGATTCTTTGTGACCCCATGGACTGTAGCCTGCCAGGC
TCCTTTGTACATGAGATTCTCCAAACAAGAATACTGGAGTAGGTAGCCATTCTCTTCTCC
AAGGAATCTTCCTGACCCAGAGATGGAATCTGGGTCTCCTGCATCACAGGCAGATTCTTT
ACCATCTGGGCCACTAGGGAACCCAGGAGAGCCACCCACAAATGAGTGCCTGTCTTCGCA
GGTGCTGAGCAGCCGTGGGGCATACATGTCAACAGCTTTGCCCTTTGCTTGGAAAGCAAG
GTGTGTGTGTACCCAGAGCACTTTATGACCAGCAGAATCAAGTAGCAAAGCCTGCTATTT
TTTTTTTTTAAGTCCGTGGCGTACTCTGTGATCTGTGTATATAAGAAGTTCTCTCTCAAA
CAGGATAGAAATGAATTGGAGAAAACAATTGTTTATGTGGTGGATATGTCTCCTTTGCCC
ACGTCCTGTTCGATAGTAATGAAGATTGCAACACCAAACGACCCCAGATATTCTGCTTGG
AGTCACCATTCGTGATGATTGCTCATAGCTAATCAAAGGATGTTATGAAATGTGAGTCAG
ACAGCCGCCGGCTTCCTGGAAGTTCCAGAACCTGTATATAAAGAGGGAGCTTCCTAGTTG
AAACACTGGAGCAGCAGAGAGGTAGGAGCTATCAGATTCCTTAAGCTGAGAAGGAAGACA
GAGACCAACctcgag
XhoI

Figure 38. Bovine MMP-1 promoter sequence employed for the experimental section. The added restriction enzyme sites are represented in yellow, whereas the putative TATA box is highlighted in grey. Adapted from [301].

A direct monitoring of Luciferase activity was then executed, thereby showing (Figure 37 D and E) a statistical significant increase ($p \leq 0.001$) following virus amount.

However, when these induction levels were compared to those of uninfected cells (≈2.5 folds), they did not appear so striking as observed with the natural MMP-1 gene. This is probably related to the fact that, the relatively short sequence used as promoter, to induce Luc reporter expression, is missing of specific upstream regulatory elements and/or a natural chromatin context.

The putative lacking of tight transcriptional control elements was further validated by monitoring relative light units (RLU) in uninfected controls with the pMMP-1-Luc reporter construct. Obtained values, in fact, were exceedingly high (≈20.000) (Figure 37 E) considering the RT-PCR resulting data as well as the immunoblot assay, made on the natural, genome-integrated MMP-1 (Figure 37 A, B and C).

Another possible and non-mutually exclusive explanation is related to MMP-1 regulation which, although is known to occur at different post-transcriptional levels [311], in this specific experimental context, only a transcriptional analysis limited to BoHV-4-induced MMP-1 up-regulation was performed.

BoHV-4 IE2/RTA as a regulator of MMP-1 induction in BoHV-4-U-infected BESCs

As soon as matrix metalloproteinase-1 up-regulation in BoHV-4-U infected bovine endometrial stromal cells was established, further experiments were executed in order to identify which viral genes were directly or indirectly involved in MMP-1 regulation. The first candidate was the main viral trans-activator gene, IE2, also called ORF50/RTA, since it is one of the first expressed genes in infected cells initiating viral lytic replication.

In addition, the encoded protein acts also on host cellular genes thanks to the presence of RTA-responsive promoter elements binding sites, thereby promoting gene activation through protein-protein interactions with still unknown transcription factors. To verify if IE2 was really involved in MMP-1 up-regulation, a BESCs transfection with a p2xCMVeIE2 effector plasmid [309], inducing an overexpression of ORF50/RTA, was carried out.

More in detail, the plasmid is composed by two copies of the 427 bp hCMV enhancer (without the basal promoter) followed by BoHV-4 IE2 promoter (of 537 bp). The p2xCMVeIE2 transfected BESC cells revealed an MMP-1 up-regulation compared to the control cells transfected with pEGFP-C1 mock plasmid. These data were further confirmed by Real-Time RT-PCR (Figure 39 A) and semi-quantitative RT-PCR (Figure 39 B).

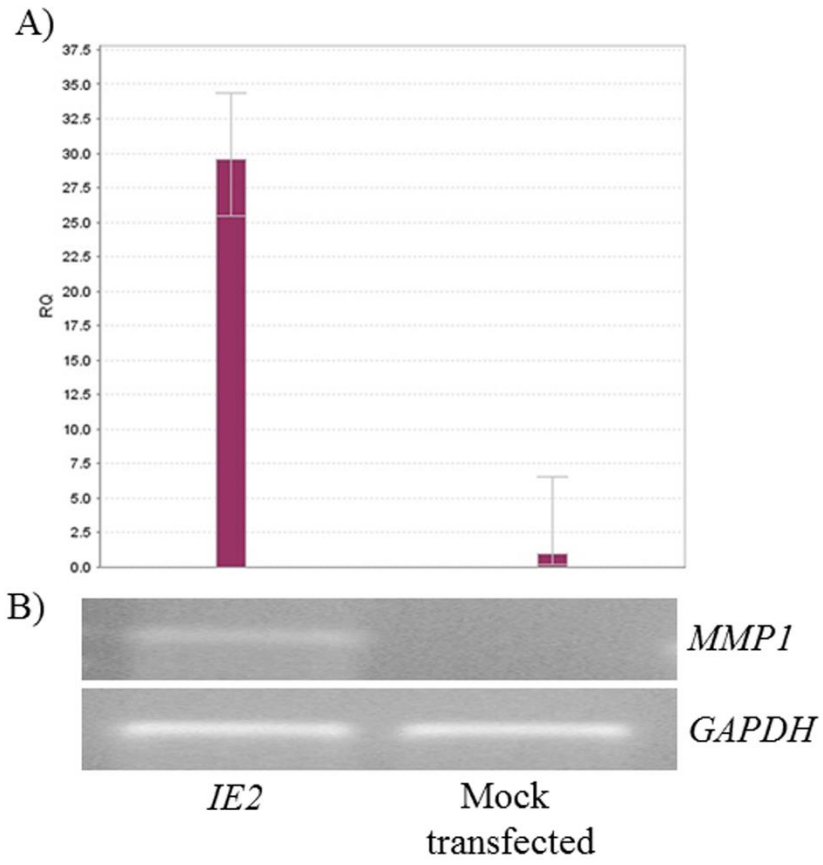


Figure 39. MMP-1 up regulation in IE2/RTA overexpressing BESCs A) In IE2/RTA-overexpressing, p2xCMVeIE2-transfected (IE2) and mock-transfected (Mock) BESCs, a quantitative Real-Time PCR analysis (mean ± SD of 3 independent determinations) was performed in order to analyze MMP-1 transcript levels; a significant difference (P≤0.05) in MMP-1 expression levels between the two conditions was observed. **B)** As in A, semi-quantitative reverse transcription-PCR was executed and GAPDH was used as a housekeeping, internal reference gene for both analyses. Adapted from [301].

However, even if the relationship between IE2 and MMP-1 up-regulation has been verified, more detailed studies are required both to elucidate the molecular mechanisms underlying the MMP-1-IE2 induced up-regulation in infected BESCs and also to better understand BoHV-4 pathogenic role.

DISCUSSION AND CONCLUSIONS

During post partum period, dairy cattle are prone to uterine infections, which are the main cause of a reduced reproductive efficiency thereby affecting dairy herds cost management.

The development of uterine diseases is influenced by the delicate balance between pathogens and the local, as well as systemic, immune status during pregnancy and parturition. When pathogens in fact, involved in the onset of metritis and endometritis, colonize cow uteri, they are generally detected by TLRs and promptly eradicated through signaling cascade activation and subsequent pathways. The stimulation of this initial endometrium defense leads to the synthesis and production of a broad range of pro-inflammatory cytokines and chemokines which, in turn, mobilize and activate immune cells [194].

The immune machinery activation, leads to pathogen killing, inflammation resolution and finally to the uterine ECM remodeling that, under normal conditions, promotes the proper anatomical and functional uterine microenvironment restoration. Nevertheless, hyper-activation of more than one canonical immune response pathway may happen, thereby jeopardizing the normal uterine functionality, characterized at this point, by persistent infection, chronic tissue damage and/or healing failure. All these aspects, but especially the altered anatomical integrity, could favor viral persistence and dissemination within the uterus and also systemically, thus worsening the prognosis [194,249,312].

So, this delicate balance between normal and hyper-activated immune response is essential in order to prevent viral dissemination and the associated consequences. A key role in this context seems to be played by matrix metalloproteinase 1 (MMP-1). During the normal immune response, MMP-1 expression promotes membrane basement proteolysis thereby favoring immune cells, such as helper T lymphocytes and NK cells recruitment into the infection site via the bloodstream, as a result of their ability to modulate cytokines and chemokines gradients. Another aspect of MMP function that is extremely relevant in the particular context of bovine uterine diseases, is related to the proteolytic activation of other molecules such as IL-8, TNF-α, pro-IL1β and defensins which are pivotal players in the immune response [313].

However, when MMP over-activation and protease inhibitor down-regulation occurs simultaneously, protective mechanisms fail, giving rise to an immune-pathological state. This state can induce a myriad of complications in patients including neurological disorders in HIV and HTLV-1 (Human T-cell leukemia virus type 1) affected patients, hepatocellular carcinoma following hepatitis B viral infection, pathological conditions as a consequence of persistent bacterial infections such those induced by LPS endotoxic shock, peptic ulcer and gastric cancer caused by Helicobacter pylori infection and lung cavitation induced by Mycobacterium tuberculosis [314–319].

Another aspect to consider is that generally, MMP up-regulation, occurs normally during bovine placental tissue separation from endometrium but, also during post-partum period in cows showing LPS-induced endometritis as well as negative energy balance state, thereby assuming a pathological connotation [254,256,320]. The concept that different pathogens in different tissue districts induce MMP up-regulation, thus leading to distinct pathophysiological outcomes, is notoriously known in literature and further corroborated by our research. The transcriptome analysis performed on BoHV-4 infected BESCs not only confirmed the above, but provided supplementary data highlighting the

interconnected roles played by different MMPs in several cellular pathways during viral infection [218,232,243]. MMP-1, and to a lesser extent MMP3, were the only MMPs discovered to be up regulated by BoHV-4 infection. PI15 in contrast was the only one out of the five protease inhibitors whose expression profile increased in response to infection.

A relevant piece of evidence that emerged from our investigation is that a close relationship exists between MMP-1 up-regulation and IE2 gene activity. IE2 not only plays a key role on MMP-1 transcriptional regulation but takes part in other non-transcriptional regulatory mechanisms, thereby modulating MMP-1 activity. One of these is the pro-MMP enzyme proteolytic conversion and protease activity regulation performed by MMP tissue inhibitors (TIMP). Another aspect that cannot be overlooked and one that may jeopardize the delicate balance underlying normal and hyper-activated cellular responses, mediated by MMP-1 and other MMPs (such as MMP3), is related to TIMPs degradation executed by the same MMPs.

Other molecules taking part to this balance that are up-regulated and functionally interconnected include IL-8, whose activity increases ten fold as a result of MMPs proteolytic activity [321]. In contrast to the MMP cleaved forms that increase in or gain new functions, some cytokines experience a loss of function following similar proteolytic processing. Since this latter aspect acts by reducing immune cells recruitment, it may assume a negative connotation, especially when a timely inflammatory reaction resolution is needed [322].

Additionally, the up-regulation of many other molecule families seems to be a direct consequence of viral replication in BoHV-4 infected BESCs. Among them, integrins, other cytokines, growth factors (GDNF, KITLG, LIF, and STC1), surface receptors, signal transducers (CALCRL, GPR68, FST, RSPO3, and KRAS), and the transcription factor FOS were discovered through RNA seq. Remarkably, the fact that integrins family members, as ITGA2, ITGAV, and ITGA6, are strongly up regulated, is a clear evidence of an extensive ECM remodeling just beyond the cell surface. This is further corroborated by the dysregulation of some sterol biosynthesis regulator including both enzymes and regulatory proteins. Considering that BoHV-4 replication is strictly influenced by prostaglandin E2 presence, also PGE2 up regulation becomes relevant since is directly involved in E2 secretion [110,170]. The data presented here obtained from transcriptome analysis allowed a better understanding of the true pathogenic role of BoHV-4 through clarification of the molecular mechanisms of the physio-pathological response underlying the pathogen-host interaction. This information has lead us to a model proposal that, supports the role of BoHV-4 as a co-factor during endometritis development [108,111]. The onset of bovine uterine diseases is commonly attributed to the presence of environmental gram negative Bacteria. This occurs so frequently that it is considered a para-physiological postpartum event and it usually undergoes complete resolution within 3 weeks after parturition [196]. The reasons why this phenomenon is sometimes aggravated are explained through the putative model showed in Figure 40.

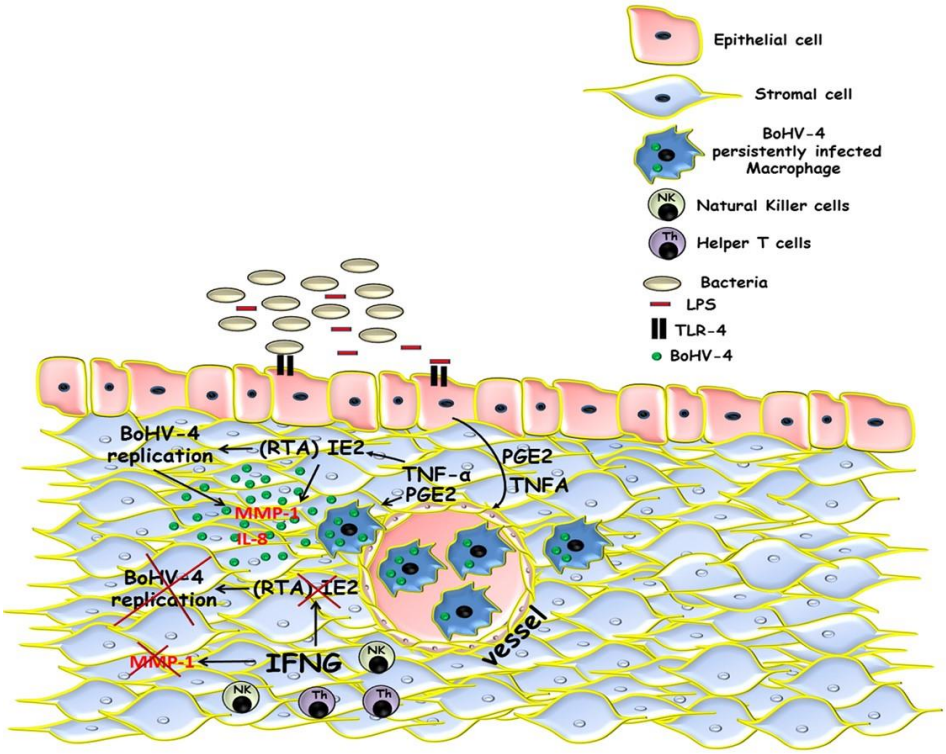


Figure 40. Putative model representation of the MMP-1-related detrimental effects associated with a persistent BoHV-4 infection. PGE2 and TNF-α are the main molecules promoting BoHV-4 replication and endometritis development through MMP-1 overexpression and IL-8 activation. Conversely, IFN-γ, produced by immune cells, has an anti BoHV-4 activity acting on IE2/RTA blockage thus leading to MMP-1 down expression. Adapted from [301].

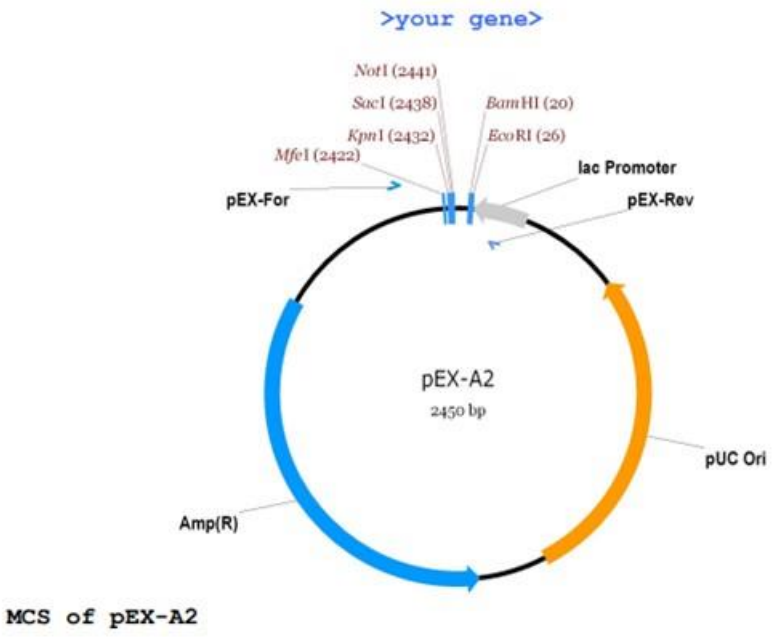
During post-partum metritis, persistently BoHV-4 infected macrophages can be chemo-attracted from the periphery to the hyperemic and inflamed site, owing to the PGE2 activity and likely exploiting the BoHV-4 pro-angiogenic effect.

The broad range of pro-inflammatory molecules, such as LPS, PGE2 and TNF-α, stimulates the IE2 promoter activity thereby promoting BoHV-4 viral replication, which in turn spreads and infects the neighboring permissive endometrial stromal cells [108–110,170]. The IE2 up-regulation, especially in the newly infected BESCs, leads to the up-regulation of several cellular pathways such as those involving IL-8 and MMP-1 (Figure 40).

These factors are known to cause endometrial tissue destruction and hamper the healing process leading to inflammatory cells recruitment and sustenance of the inflammation state [302]. The fact that MMP-1 expression is favored by inflammatory and stromal cells exposed to exogenous (as LPS) and/or endogenous (as TNF-α, IL1β, and PGE2) stimuli, further corroborates this model [323,324]. Noteworthy is that if all these molecules positively induce MMP-1 regulation, mediated by BoHV-4 IE2 activity, other molecules act counteracting this effect. Among them, IFN-*γ* stands out for owning anti BoHV-4 activity and down-regulating IE2 stimulation thereby dampening BoHV-4 replication [111].

Based on this model, even though many great strides were reached, many other efforts must be made in order to elucidate additional molecular mechanisms involving BoHV-4 IE2 activity. Considering the above, the IFN-*γ* role as well as those of other cytokines, has to be considered and further investigated with the aim to find new potential therapeutic agents to treat post-partum cows showing a complex clinical condition. Another interesting aspect, that deserves to be

more carefully studied, is related to which precise steps, during the immune response against BoHV-4 infection, are involved in triggering cellular pathways responsible for a positive (thereby promoting the healing process), and/or negative (thereby favoring a delayed repair and endometritis state) effects associated to MMP-1 stimulation.



MCS of pEX-A2

GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGC
TGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACcaattgGG
TACCgagctcGCGGCCGCAAGC>your_gene>ACCTGCTTTTGCTCGCTTgg
atccGAATTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACG
AGCCGGAAGCATAAAGTGTAAAGCCTG

Figure 41. Schematic representation of pEX-A2 vector map, multiple cloning site and full sequence. Adapted from Eurofins genomics.

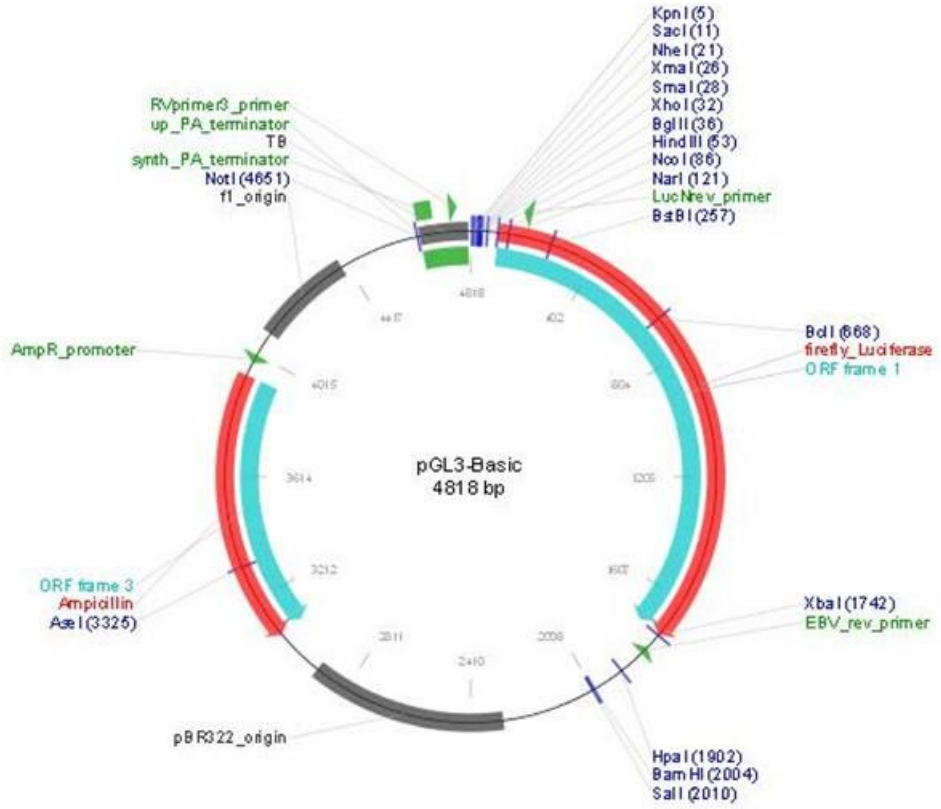


Figure 42. Schematic representation of pGL3-Basic vector (Promega).

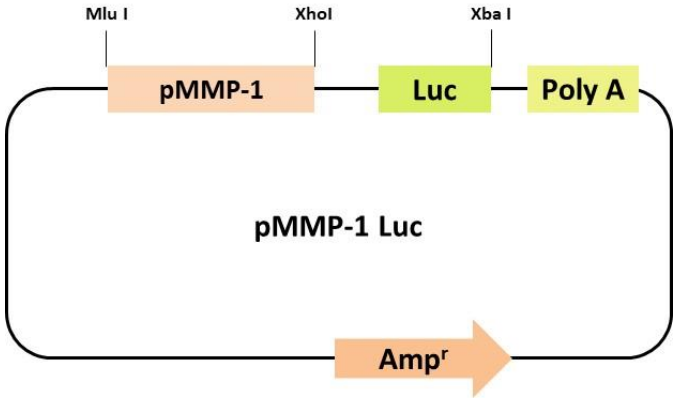


Figure 43. Schematic representation of pMMP-1-Luc plasmid vector that was obtained by sub-cloning the 1089 bp MluI/XhoI MMP-1 promoter sequence, cut with the same restriction enzymes from pEX-A2 (Eurofins) and inserted in Mlu/XhoI-digested pGL3 basic vector (Promega).

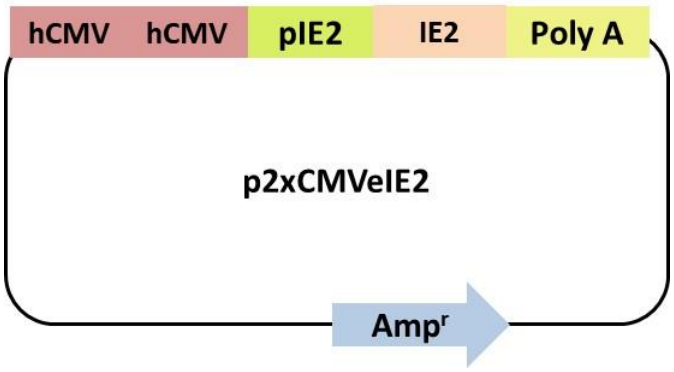


Figure 44. Schematic representation not in scale of p2xCMVe*IE2* vector plasmid which is composed by two copies of the 427 bp hCMV enhancer (without the basal promoter) followed by BoHV-4 IE2 promoter (of 537 bp).

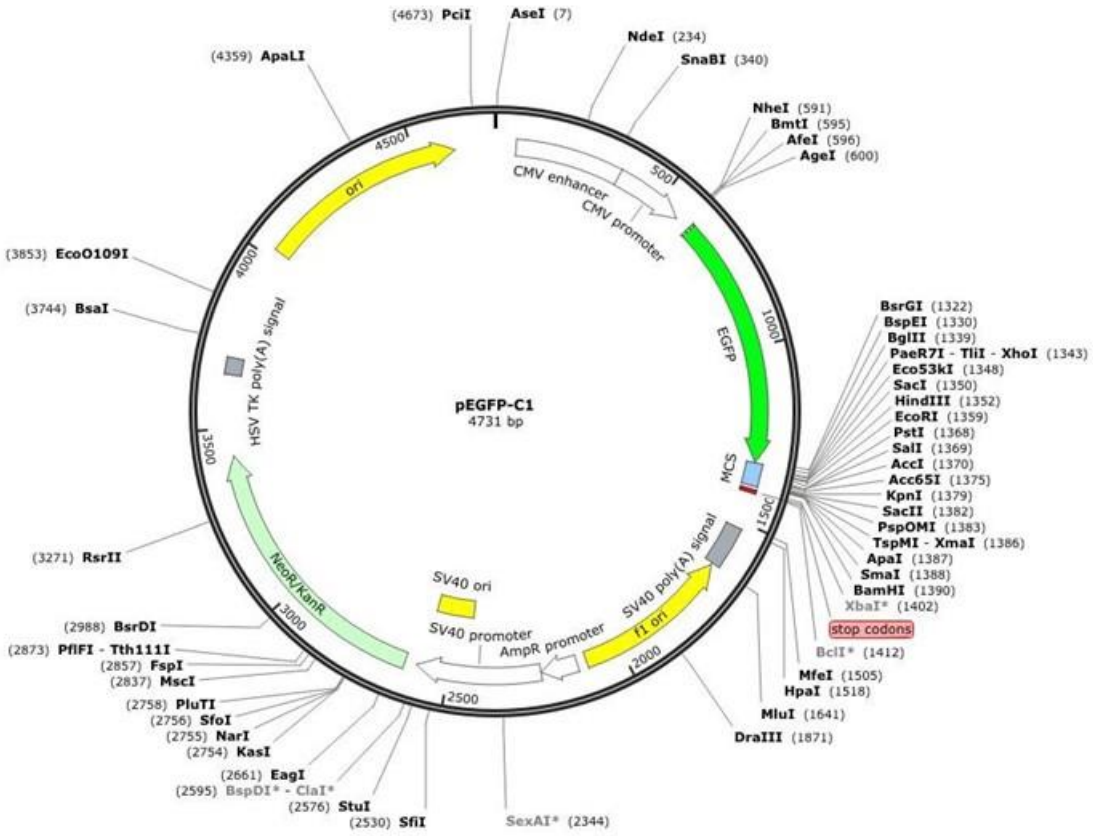


Figure 45. Schematic representation of pEGFP-C1 (Clontech) vector used as a mock plasmid to transfect BESCs cells.

List of papers published during the PhD in Medical-Veterinary Science

1. **Heterologous Matrix Metalloproteinase Gene Promoter Activity Allows In Vivo Real-time Imaging of Bleomycin-Induced Lung Fibrosis in Transiently Transgenized Mice.**
   Stellari FF, Ruscitti F, Pompilio D, Ravanetti F,**Tebaldi G**, Macchi F, Verna AE, Villetti G, Donofrio G.
   Front Immunol. 2017 Mar 1;8:199. doi: 10.3389/fimmu.2017.00199. eCollection 2017 Mar 1.
   PMID:28298912

2. **BoHV-4-based vector delivering Ebola virus surface glycoprotein.**
   Rosamilia A, Jacca S,**Tebaldi G**, Tiberti S, Franceschi V, Macchi F, Cavirani S, Kobinger G, Knowles DP, Donofrio G.
   J Transl Med. 2016 Nov 24;14(1):325.
   PMID:27881138

3. **Virus-Mediated Metalloproteinase 1 Induction Revealed by Transcriptome Profiling of Bovine Herpesvirus 4-Infected Bovine Endometrial Stromal Cells.**
   Tebaldi G, Jacca S, Montanini B, Capra E, Rosamilia A, Sala A, Stella A, Castiglioni B, Ottonello S, Donofrio G.
   Biol Reprod. 2016 Jul;95(1):12. doi: 10.1095/biolreprod.116.139097. Epub 2016 Jun 8.
   PMID:27281703

4. **Assessment and optimization of Theileria parva sporozoite full-length p67 antigen expression in mammalian cells.**
   Tebaldi G, Williams LB, Verna AE, Macchi F, Franceschi V, Fry LM, Knowles DP, Donofrio G.
   PLoS Negl Trop Dis. 2017 Aug 11;11(8):e0005803. doi: 10.1371/journal.pntd.0005803. eCollection 2017 Aug.
   PMID: 28800590

5. **Induction of Antihuman C-C Chemokine Receptor Type 5 Antibodies by a Bovine Herpesvirus Type-4 Based Vector.**
   Verna AE, Franceschi V, **Tebaldi G**, Macchi F, Menozzi V, Pastori C, Lopalco L, Ottonello S, Cavirani S, Donofrio G.
   Front Immunol. 2017 Oct 25;8:1402. doi: 10.3389/fimmu.2017.01402. eCollection 2017.
   PMID:29118763

6. **Bovine Herpesvirus-4-Based Vector Delivering Peste des Petits Ruminants Virus Hemagglutinin ORF Induces both Neutralizing Antibodies and Cytotoxic T Cell Responses**.
   Macchi F, Rojas JM, Verna AE, Sevilla N, Franceschi V, **Tebaldi G**, Cavirani S, Martín V, Donofrio G.
   Front Immunol. 2018 Mar 5;9:421. doi: 10.3389/fimmu.2018.00421. eCollection 2018.PMID:29556236

7. **Bovine herpesvirus 4-based vector delivering the full length xCT DNA efficiently protects mice from mammary cancer metastases by targeting cancer stem cells.**
   Gaetano Donofrio, **Giulia Tebaldi**, Stefania Lanzardo, Roberto Ruiu, Elisabetta Bolli, Andrea Ballatore, Valeria Rolih, Francesca Macchi, Laura Conti & Federica Cavallo
   OncoImmunology·September 2018 DOI: 10.1080/2162402X.2018.1494108

8. **Molecular and antigenic properties of Theileria parva antigen Tp9 expressed in mammalian cells**

   Reginaldo G. Bastos , Valentina Franceschi, **Giulia Tebaldi** , Timothy Connelley, W. Ivan Morrison, Donald P. Knowles, Gaetano Donofrio , Lindsay M. Fry
   Submitted to Frontiers Immunology

CONFERENCES :

1. **VI NATIONAL VETERINARY VIROLOGY WORKSHOP, CAVALLERIZZA REALE, Turin, 13-14 October 2016 : oral presentation**
2. **LXXI SISVET CONGRESS IN VETERINARY MEDICINE, Naples, from 28 june -1 JULY 2017: oral presentation**
3. **LXXII SISVET CONGRESS IN VETERINARY MEDICINE, Torin, from 20 June-22June 2018: oral presentation**

   I received the "Young Researchers" award conferred by C.S. SISVet

EUROPEAN RESEARCHERS' NIGHT : The event aims at creating opportunities to bring together researchers and citizens in order to disseminate scientific culture and information about research careers in an informal and stimulating context

1. 30 September 2016
2. 28 September 2018

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