Mutations in the envelope gene of naturally transmitted maedi-visna virus

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Abstract

In this project, 4 sheep were kept with infected sheep for 3 years. All 4 sheep became infected. Virus was isolated from the sheep, and DNA was isolated from these viruses to observe the mutations that had appeared during this time. We selected a sequence of the Env gene of 560 bp between 7499 and 7981, part of the principal neutralization domain (PND) of the MVV genome. The purpose of this research was to find out how the virus envelope develops in sheep that are infected by natural transmission.

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INTRODUCTION

PATHOGENESIS AND CLINICAL SIGNS:

Lentiviruses are nononcogenic, exogenous retroviruses which cause visna (wasting) and maedi (dyspnea) in sheep **(41, 96, 97),** arthritis-encephalitis in goats **(24, 25)**, infectious anemia in horses **(17, 18)**, and acquired immune deficiency syndrome in humans **(34, 37)**. These diseases are characterized by long incubation periods, insidious onset, and slowly progressive clinical courses. Infection is persistent, but only a few hosts ever develop clinical disease **(27, 28)**. Replication of the virus is restricted in the host **(42)**, and pathologic responses are immune mediated **(8, 26, 77),** both humoral and cell-mediated **(39, 55, 98, 105, 107)** rather than caused by the direct effects of the virus **(51)**.

Visna is a chronic central nervous system (CNS) infection of sheep with a naturally occurring ovine retrovirus **(41, 83, 84, 85)**. The virus persists indefinitely after infection, although at low levels, and the infected sheep develop subacute encephalitis which may progress to clinical paralysis at irregular intervals from a few months to many years after inoculation. The target cells of the virus in the persistently infected animal are monocyte macrophages **(74, 75)** and not fibroblastic cells.

Infected Icelandic sheep regularly develop neutralizing antibodies in their serum within 3 months of infection and often have high titres of neutralizing antibody in the cerebrospinal fluid (CSF) as well **(78)**. MVV establishes a lifelong infection and persists in the host despite a strong immune response. The antibodies prevent virus replication in both fibroblasts and macrophages. However, the site of neutralization differs between the two cell types. In fibroblasts, the site of virus neutralization is at the cell membrane, when the antibodies prevent virus attachment. In macrophages, virus incubated with the antibodies is phagocytized rapidly, followed by uncoating of the virions. However, virus RNA is not transcribed **(51)**.

In a MVV infection, a type-specific neutralizing antibody response appears 1–6 months after infection, whilst more broadly reacting antibodies appear up to 4 years later. These type-specific antibodies are considered the selective force which trigger the raise of antigenic variants of the virus, in other words, mutated variants of the original virus **(3)**. The unusual aspect of the life-long persistence of visna virus makes the virus undergo progressive antigenic variation in a single infected animal **(72, 73)**. Although the number of variants is substantial, there is no evidence that they replace the infecting serotype with the passage of time. Rather, parental and variant viruses appear to co-exist, suggesting that antigenic drift is not essential for virus persistence. Although antigenic variants are preferentially selected in the CNS as in the blood, they do not appear to play an essential role in the evolution of CNS lesions **(61)**.

In a visna virus-infected animal, some virus isolates obtained months after inoculation are of parental serotype. However, serologically distinct viruses can be isolated later in the course of the disease which are not efficiently neutralized by the sera obtained shortly after inoculation **(73)**.These variants are serologically distinct from one another. The infected animal subsequently develops neutralizing antibody against these variants. These antigenic variants are stable to multiply and are virulent in sheep **(73)**.

Despite the susceptibility of visna virus to neutralization in macrophages, the greater affinity of the virus for the cell than of the antibody for the virus would favor

spread of the agent in the presence of neutralizing antibodies. This provides a mechanism for the known ability of the virus to persist for years in the presence of neutralizing antibodies **(73, 104)**. It also offers an explanation for the failure of nonneutralizable mutants of visna virus to replace the neutralizable parental virus in immune sheep in which antigenic drift of the virus had occurred **(61)**. Thus, the ability of these lentiviruses to induce neutralizing antibodies in natural hosts offers no guarantee of cure of the infection. Similarly, the presence of neutralizing antibodies in the immunized host may not necessarily protect against invading virus because the kinetics of neutralization may be too slow to protect target cells **(51)**.

Even though some scientists claim an immune selection of lentivirus variants, the role of humoral immunity in infections is still unclear. The function of the antibodies under *in vivo* conditions has not been solved. It has been proposed that since neutralizing epitopes are located in regions that tolerate high variability and because of the high mutation rate of the lentiviruses, mutants with a selective disadvantage will be selected against, resulting in enrichment of variants with mutations in the neutralization epitopes. These may then acquire dominance in the virus population, either accompanying another mutation, resulting in a fitter variant, or by bottleneck transmission **(30)**. It is also possible that a change in the neutralization epitope coincides with a change in a recognition site for a receptor or coreceptor, as has been found in HIV **(68)**.

The molecular analysis of the env gene highlights a large open reading frame with the capacity to code for a polypeptide of 985 kDa. Research of the amino acid sequence identified three basic amino acid-rich regions (Arg-X-Lys-Arg) supposed to be the proteolytic cleavage sites for lentivirus envelope glycoproteins. **(93, 45).**

The envelope glycoproteins of the lentiviruses are highly variable proteins with a conserved conformation. The protein structure is maintained, with conserved cysteine residues and glycosylation **(58, 108)**. There are several potential N-linked glycosylation sites in the outer glycoprotein of MVV. Further studies found out that there are a total of around 25 N-linked glycosylation sequences (Asn-X-Ser/Thr), 21 of which resided in the surface membrane protein (SU) and 5 in the TM region **(71)**. The N-linked glycosylation addition follows some chemic rules:

- For an asparagine (Asn) to be glycosylated, it requires the context of the previous amino acid pattern Asn-X-Ser(Thr) **(63)**, where X can be any amino acid, followed by a Serine (Ser) or Threonine (Thr).
- A N-linked glycosylation site will not be glycosylated if it contains or is followed by a Proline **(35)**
- Glycosylation may be inhibited by certain combinations of Asn-X-Ser or when followed by specific amino acids **(50, 69, 94)**

It has been suggested that the role of this extensive glycosylation is to shield the virus from neutralizing antibodies **(89, 110)**, protein folding **(44, 54, 99)**, and conformation **(70)**. It seems that lentiviruses have evolved many mechanisms of immune evasion **(33, 110, 112).** Overall, the way for the lentiviruses to escape the immune response may be by continuous change of epitopes through mutation **(40)**.

MOLECULAR BIOLOGY:

The initial weight of the carbohydrate moiety of OvLv is 2.1 KDa **(57)** the molecular weight of the precursor protein is estimated at 158kDa. From the protein precursor, gp135, a cleavage at the proteolytic cleavage sites would result in protein fragments of gp100 (SU) and gp50 (TM) **(49, 10)**. From the protein fragment gp100 (SU) four hypervariable regions are characterized (V1, V2, V3, and V4), besides, V3 hypervariable region is flanked by conserved cysteines residues which is similar to the V3 loop reported for HIV **(53)** and equine infectious anemia virus **(6)**. The V4, a type-specific neutralization epitope **(100),** according to several researches, is the most variable region involving residues at the C-terminal end of the SU region and has a

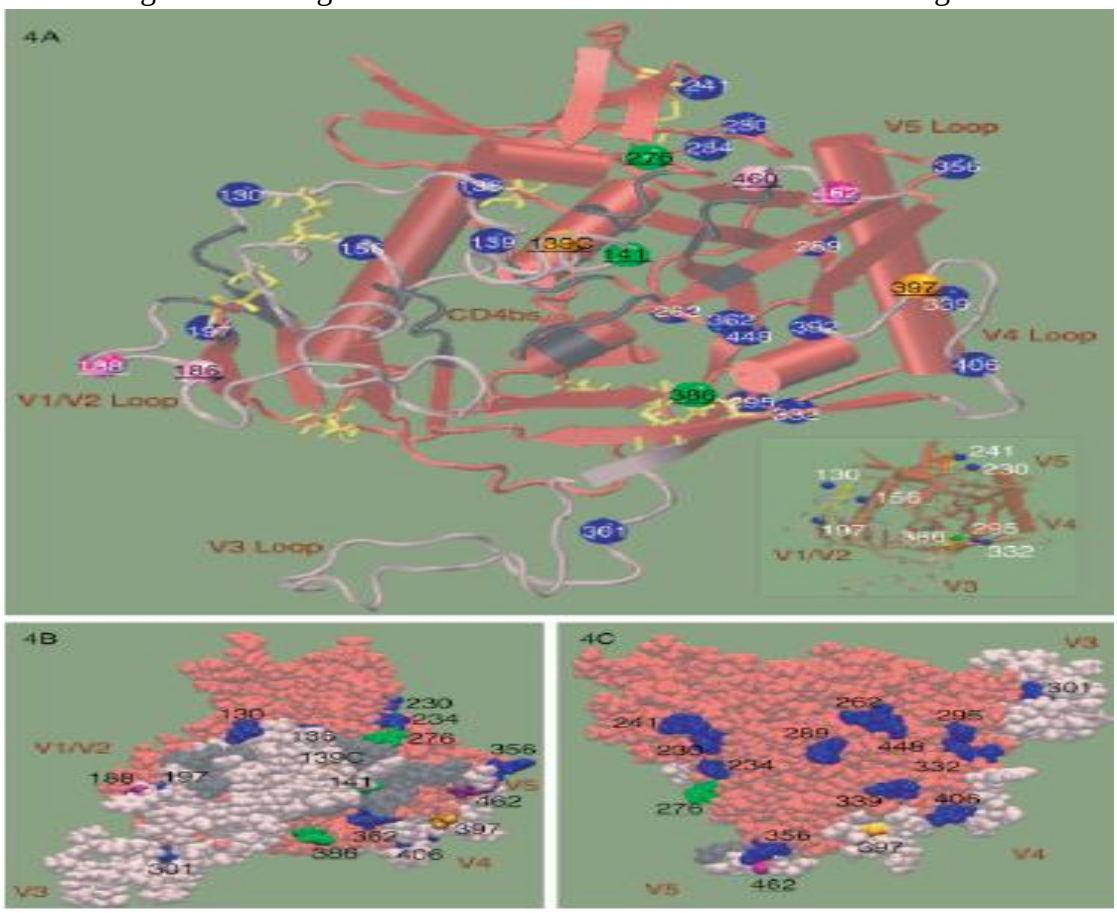


Fig. 1

neutralization conformational epitope; this means that the glycans of this region induce conformational changes in the env protein **(100)**.

The initial type-specific neutralizing antibodies detected in the sera of HIV-1-infected humans are mostly directed to the V3 regions on gp120, hence the term 'principal neutralization domain' (PND) **(48)**. Resembling to gp135 in MVV, the major exterior glycoprotein, gp120, and a glycosylated transmembrane protein, gp41 **(1, 88, 109)**. Gp120 has been shown to be essential to the interaction of the virus with the CD4 receptor **(60, 62, 66)**. Indeed, even the coreceptor binding site is only available after conformational changes induced by CD4 **(21)** and even the gp41 ectodomain is only thought to be available after conformational changes induced by coreceptor binding

(21), it could be discussed that the CD4bs is the most vulnerable region of the viral Env and thus that mutations that shield it from antibodies would provide a fitness advantage to the virus **(29, 115).** Gp120 may also contribute to the cytopathogenicity of HIV through its involvement in syncytium formation **(59, 101),** and, in analogy to other retroviruses, HIV gp120 represents the main antigen of the virus as well **(1, 7, 52).**

Gp120, as gp135 is for MVV, is heavily glycosylated, comprising more than 20 potential N-glycosylation sites. Thus, carbohydrates represent approximately one-half of the molecular weight **(1, 88).** Aside the V3 loop, the majority of the potential N-linked glycosylation sites fall within limited windows, or "hot spots" (which corresponds to residues within the variable loops that are proximal to the CD4bs), of the HIV envelope. These hot spots include the N-terminal side of V1, a small region on the C-terminal side of V2, most of V4 and a small region of V5 **(113)**.

Therefore, carbohydrates play a role in the determination and/or the accessibility of the structural domains involved in receptor binding. It has been reported that virus-induced cell fusion is inhibited by native gp120, but not by unglycosylated recombinant gp120 fragments, although the latter are still led to induce antibodies neutralizing HIV infection in vitro **(87)**.

The V3 loop region plays a central role in determining coreceptor usage and viral tropism. Indeed, it influences the specific chemokine receptors used by different primate immunodeficiency viruses **(23)**. Early antibodies are also directed to the V1/V2 variable loops, whereas later, more broadly reacting antibodies are probably directed mostly to receptor binding site surfaces **(112).**

In overall, the majority of neutralizing antibodies present in sera from individuals infected with HIV type 1 (HIV-1) or immunized with recombinant HIV-1 proteins or in experimentally infected animals are directed either to the V3 loop of envelope gp120, which play an important role in physical interactions with the CD4 coreceptor and its strong net positive charge can facilitate binding to the negatively charge CC5 coreceptor **(114)**; or to epitopes overlapping the CD4- binding site of gp120, predominately, V1 and V2 **(12, 16).** For SIV, the V1 and V4 domains appear to contain the principal neutralizing determinants **(11, 16, 90, 91).** It is generally accepted that anti-V3 loop antibodies are type or sequence specific, whereas anti-CD4-binding-site antibodies are broadly cross-neutralizing **(13, 79, 102, 106).**

Neutralization resistance can be acquired either directly by a point mutation within the antibody-binding site that reduces or abrogates the binding of the antibody or indirectly by a point mutation elsewhere in the envelope gene that alters the conformation of the antibody-binding site **(4, 64, 67, 81, 103, 111).**

In HIV, the amino acid substitutions in the V3 domain that confer neutralization resistance create a potential glycosylation site **(22)**. It is possible that the presence of N-linked carbohydrates at this position of the loop shields the virus from immune recognition **(22)**. In fact, this N-linked glycosylation site within the V3 loop appears to be dispensable for virus replication and yet is highly conserved **(56)**.

The amino acid substitution in the V1 domain, however, results in the removal of a potential glycosylation site **(22)**. Elimination of glycosylation sites in the V1 domain of HIV-1 and SIV has also been reported to affect the ability of monoclonal antibodies (MAbs) to bind and subsequently to neutralize viral infectivity **(15, 32, 38, 91).** It may happen due to the loss of one N-linked glycosylation site could result in tighter packing of glycoprotein regions involved in neutralization epitopes, reduce accessibility, and so also facilitate immune escape. For example, a change in sequins in HIV-1 transmembrane envelope (Env) protein gp41 induces conformational changes in the associated Env gp120 surface protein that dramatically diminish the binding of

many gp120-specific antibodies **(95)**, or sequence changes in the V1 domain have been reported to alter V3 and CD4-binding-site recognition **(9, 14, 38).** The latter mutation of V1 and V2 as well affect the charge becoming more negative, and it could influence the packing of variable loops around the CD4bs **(31, 86)**

PROTEIC STRUCTURE AND NEUTRALIZATION SITES:

Structurally, the gp120 core of HIV is divided into an inner domain and an outer domain with a short bridging sheet. In the trimeric form, the inner domain binds antibodies that are non-neutralizing and is referred to as the non-neutralizing face; part of the outer domain is heavily glycosylated and is probably recognized by the host immune system as 'self' and is not immunogenic, it is considered the "silent face" even though few antibodies are known to recognize it. However, the surface that interacts with neutralizing antibodies involves parts of both domains and the bridging sheet and includes the V1/V2 and V3 variable loops **(46, 47, 112),** which have provided evidence for similarities in the structures of MVV gp135 and HIV-1 gp120. The heavily glycosylated region on gp135 probably forms the outer region, as suggested by Hotzel et al. **(47).** Several studies have shown that removal of carbohydrates from this region renders the virus more susceptible to neutralization **(65)** The inhibition of syncytium formation by concanavalin A in gp120 focuses attention in this respect to oligomannosidic glycans as one of the key glycans in the attach of the conserved receptor with the virus. Hence, both gp120 and gp135 conserved receptor binding sites reside in depressions where oligosaccharide side chains are located which are inaccessible to further action of glycosidases and glycosyltransferases **(36).**

According to maedi-visna virus, type-specific neutralization domain is located within a 39 aa sequence in the fourth variable domain of gp135 **(100).** Neutralizing antibodies detected early in infection are directed to this epitope, suggesting an immunodominant nature of this domain. The region contains two conserved cysteines suggested to form a cysteines loop, due to disulfide bridges, and a conserved potential glycosylation site. This cysteines loop belongs to the V4 region, resembling to the V3 HIV-loop **(100).** A mutation in the conserved cysteines results in antigenic escape **(43)**. Researches in this region find out that mutations in this region between amino acids 559 and 597 in the outer envelope glycoprotein gene of MVV result in escape from neutralization and creation of a new type of neutralization specificity. This is a variable region in the MVV SU glycoprotein **(2, 92)** in an analogous position to V4-V5 in SIV and in FIV **(80).** Mutations in the region between amino acids 559 and 597 in the outer envelope glycoprotein gene of MVV result in escape from neutralization and creation of a new type of neutralization specificity. This data suggest that V4 epitope of MVV is a binding site of receptor or coreceptor, having an important role in MVV infectivity **(100).**

Therefore, the glycosylation site at aa 568 in VMV gp135 is highly conserved in all MVV and CAEV strains that have been sequenced **(43)**. A frequently occurring mutation, removing a potential glycosylation site, had no effect on its own on the neutralization phenotype of the virus. However, adding an extra potential glycosylation site in the region resulted in antigenic escape **(43)**.

Therefore, both MVV and HIV neutralization resistance can be conferred by glycosylation masking the epitopes **(5, 22, 89, 110)** or conformational changes **(70)**.

The Icelandic MVV strains K1514 and K1772 induce a much stronger neutralizing antibody response than other strains of MVV or CAEV **(19, 20, 76).** An

outline of the Env gene of MVV strain KV1772 strain (one of the more virulent one) is highlighted here, showing the conserved and variable regions (Fig 1)

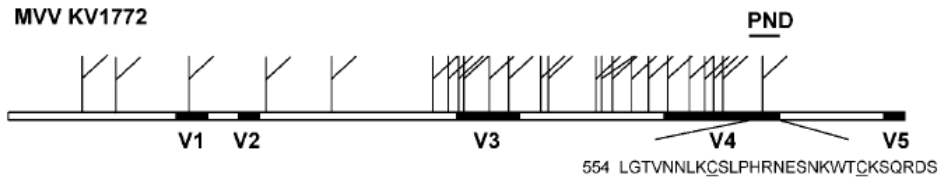


Fig. 2 Schematic representation of the surface unit of the envelope gene, showing variable regions as determined by Valas *et al.* (2000) (black boxes labelled V1–V5) and potential sites for *N*-linked oligosaccharides (denoted by forks). The amino acid sequence of the principal neutralization domain (PND) is shown. The two conserved cysteines that are proposed to form a cysteine loop are underlined.

MATHERIAL AND METHODS:

VIRUS AND CELLS: The MVV strain K796 is a virus isolate from transmission experiment where virus was passed from sheep to sheep **(97, 100)** Virus was propagated in monolayers of sheep choroid plexus cells (SCP) **(98)**. Choroid plexus cells were grown at 37 °C in a humidified atmosphere of 5% CO# in Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 unit/ml penicillin and 100 unit/ml streptomycin, 2mM glutamine and either 10% or 1% lamb serum (for expansion of uninfected cells or for virus propagation, respectively).

VIRUS ISOLATION: Virus was isolated from lungs, spleen or blood of the sheep by explants and coculture with SCP **(98)**.

INFECTION OF CELLS AND LYSIS FOR PCR: SCP cells were infected with MVV at an m.o.i of 0.5 $TCID_{50}$. When cythopathic lesions started to appear, usually 2-3 days after infection, the cells were harvested and resuspended in 100µl lysis buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.5% Triton X-100, 0.001%SDS, 300µg/ml proteinase K) and incubated overnight at 37ºC. After 15 min of inactivation at 96ºC, 3 µl of the lysate were used in a 20 µl PCR reaction **(Transmission of maedi-visna virus by natural routes: Selection for neutralization resistance).**

PCR AND ENV SEQUENCE: The sequence was amplified from the DNA of the sheep with a PCR. It was performed with Phusion Hot Start Polymerase. Two different approaches were done depending on the primers

The first one was:

- **3** ML OF THE VIRUS **DNA.**
- **10** ML OF **PHUSION HF BUFFER 5X**
- **5** ML OF **DNTPS**
- **5** ML OF **20MM PRIMER 7499 BAM HI FORWARD**
- **5** ML OF **20MM PRIMER 7981 REVERSE**
- **0.5** ML **HOT START PHUSION POLYMERASE.**
- **21.5** ML **DH_2O**

- **50** ML **TOTAL**

The other one was:

- **3** ML OF VIRUS **DNA**
- **10** ML OF **PHUSION HF BUFFER 5X**
- **5** ML OF **DNTPS**
- **5** ML OF **20MM PRIMER 7499 BAM HI FORWARD**
- **5** ML OF **20MM PRIMER 7945 BAM HI REVERSE**
- **0.5** ML OF **HOT START PHUSION POLYMERASE**
- **21.5** ML OF **DH_2O**

Both of the reactions followed the same temperature-cycle protocol, with a variation in the annealing temperature of 65-50ºC due to two possible reasons; the low quality of the virus DNA or the high specify of the sequence. The descent of the temperature until 50ºC worked, achieving the desired sequence

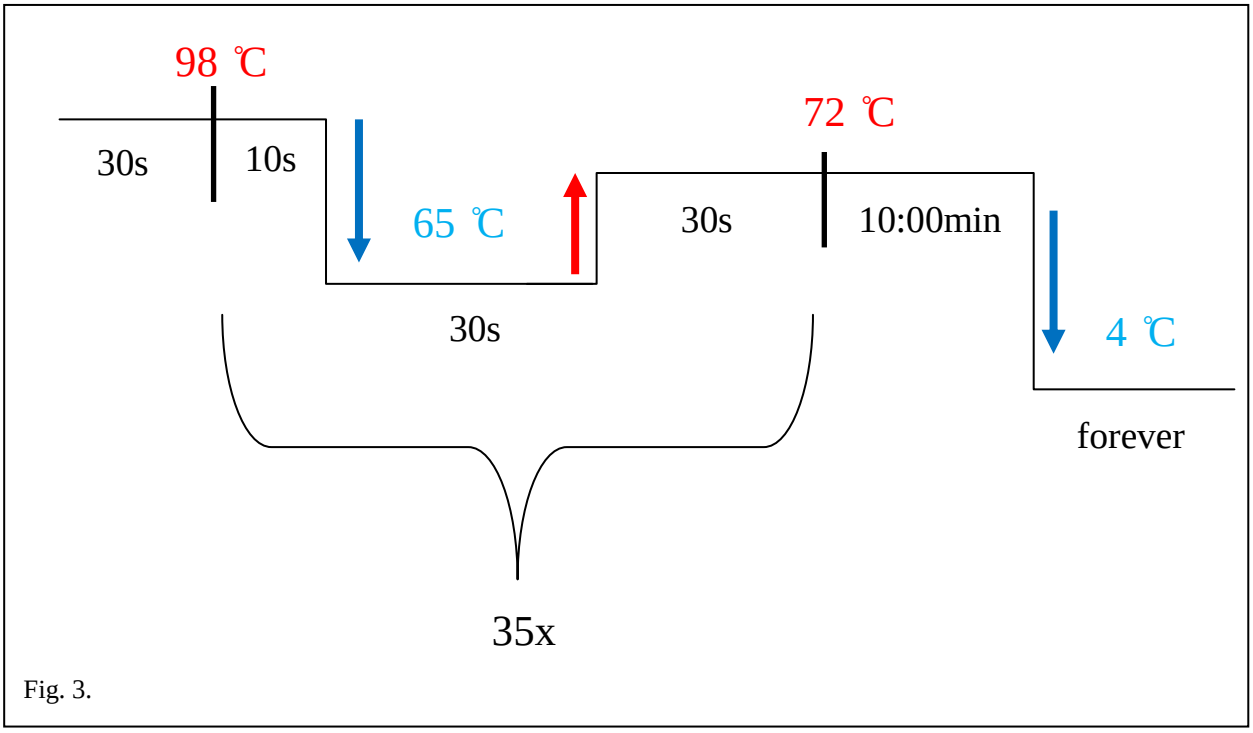


Fig. 3.

RESTRICTION ENZYMES AND CLONING: The desired sequence, after be isolated with "QIA gen® quick kit to extract DNA in agarose gel" were split and introduced in a pUC 19 vector.

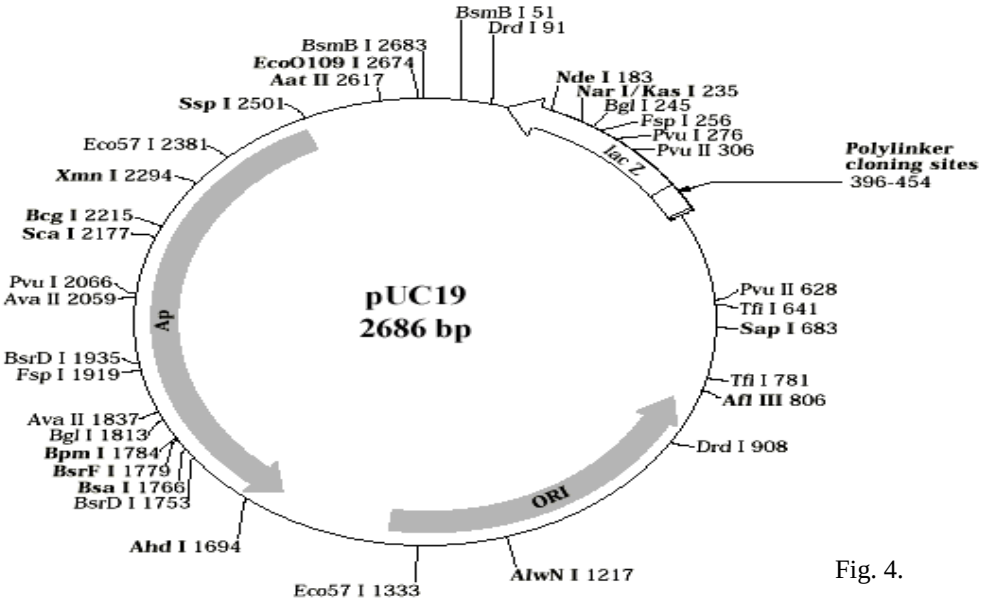
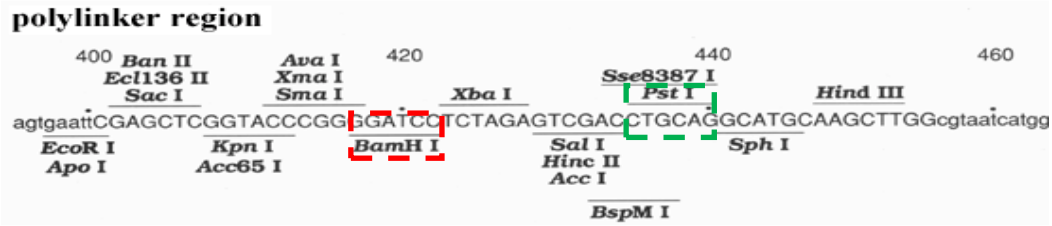


Fig. 4.



BamHI cleavage site

PstI cleavage site

The sequences were cut with following two different approaches, according with the previous set of primers that were used in the PCR step; in the first one, with 7499 Bam HI forward and 7981 reverse the restriction enzymes were:

- BamHI
- PstI

With the primers 7499 BamHI forward and 7945 BamHI reverse was:

- BamHI

(All the restriction enzymes are from New England Biolabs Inc).

Digestion with the latter set of primers needed special treatment due to just one endonuclease was used, hence, the protocol for Dephosphorylation of DNA 5`-termini was chosen.

In this method, an alkaline phospatase (hydrolase enzyme) removes phospatase groups from nucleotides. As DNA possesses phosphate group on the 5`end, removing these prevents the DNA from ligating, and, thereby, keeping DNA molecules linear until the next step of the process.

The protocol to follow is:

1. Prepare the following reaction mixture containing:
    a. 1-3 μg DNA
    b. 1x restriction enzyme buffer
    c. 2 u of restriction enzyme for 1 μg of DNA.
2. Incubate at 37ºC for 1 hour.
3. Add 1 unit of SAP (Shrimp Alkaline Phosphatase) per 1 picomole of DNA 5`-termini. Incubate at 37ºC for 30 min.
4. Stop reactions by heating at 65ºC for 15 min or at 80ºC for 20 min (whether restriction enzyme cannot be inactivated at 65ºC)

Again, cut sequences were isolated with the QIAgen© product due to the extraction avoids the "mismatch", in other words, the union of wrong pieces of the overhang cleavage digestion sequence with the vector. Therefore, it increases the likelihood of obtaining the desired ligation.

The mixture of the ligation between the desired sequence and the pUC 19 vector was:

- 7 μl of virus-sequence-DNA
- 1 μl of pUC 19 vector (cut)
- 1 μl of T4 DNA ligase 10x Buffer
- 1 μl of T4 DNA ligase

The sample was incubated overnight at 16ºC.

Transformation was performed with thermal-shock method: Competent *Escherichia coli* strain DH5α cells were grown with ampicillin. This is the antibiotic which pUC 19 is resistant for because it contains a resistant-gene inside of it, so petri plates were made with the latter antibiotic in. Since a few non-cut plasmids could grow in the petri plates, a technique to avoid picking the wrong colony out was selected.

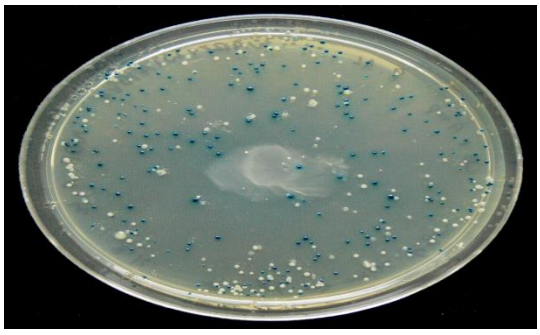


Fig. 5.

The blue-white screen is a molecular technique that allows for the detection of successful ligations in vector-based gene cloning. DNA of interest is ligated into a vector. The vector is then transformed into competent cell (bacteria). The competent cells are grown in the presence of X-gal. If the ligation was successful, the bacterial colony will be white; if not, the colony will be blue.

This technique allows for the quick and easy detection of successful ligation, without the need to individually test each colony. An example of such a vector is the artificially reconstructed plasmid pUC19.

The molecular explanation of this procedure is that *E. coli* bacteria, which cannot produce the enzyme β-galactosidase (coded by *lacZ* gene of the *lac* operon), are transformed by absorbing the plasmid vectors, which contain an insert in the *lacZ* open reading frame. A successfully transformed bacterium has a truncated β-galactosidase gene, causing white colonies on the plate. Bacteria transformed by empty vectors, which does not contain an insert in the lacZ open reading frame, are now able to produce the enzyme β-galactosidase which can then cleave the X-gal present within the nutrient agar, resulting in a blue colony. Bacteria colonies that grew from bacteria that were not transformed do not contain the antibiotic-resistance, and thus, die. The plasmid vectors can also be coded to disrupt a different bacteria's ability to produce β-galactosidase causing the desired bacteria colonies to grow to be white and non-transformed colonies to grow to be blue.

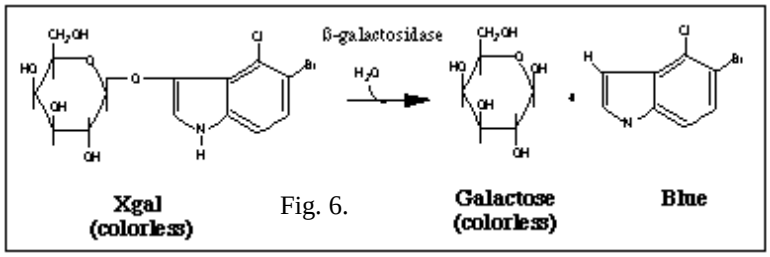


Fig. 6.

SEQUENCING: After extraction of DNA from the *E.coli* colonies with the kit "Plasmid DNA purification using the QIAprep Spin miniprep kit and a microcentrifuge" from QIAgen©, the sequencing procedure was set by ABI PRISM® 310 from Applied Biosystems™ . First of all, the purified DNA is measured with a spectrophotometer which is neccesary to know the concentration of DNA in the sample. With the known amount of DNA, sequencing preparation may be done. Sequencing is running with the desired sample which has been prepared before. The sequencing outcome is displayed with the program "Sequencher 4.9".

RESULTS:

DNA SEQUENCING:

4 sheep were chosen: Sheep-36, sheep-296, sheep-898 and sheep-900. Several PCRs for the desired sequences were made, but, they did not amplify during all these tests. Several difficulties might be involved in this fact: The quality of the virus-DNA from the cell transfection might be not good enough for the amplification with the PCR method. Moreover, if there are mutations in the strand corresponding to the primers,, annealing of the primers with the strand might be not specific enough and therefore sequences could not replicate. For this reason, lower annealing temperature was used, because this also decreases the anneal specificity between primers and strands.

However, lower specificity is a risk method to obtain a band. "Pollution" of the reagents might emerge within the sample, confusing our desired band with traces of unknown DNA from other experiments or reactions.

The main trouble of this wrong-picked out sequence is that it might not be detected until sequencing of the latter. In my research, this trouble came up in two of the four samples, Sheep-36 and Sheep-900. It is thought to be DNA from the strain KV1772 of MVV, due to several researches are on currently with the aforementioned strain of the virus.

The samples sequenced have exactly the same pair of bases that sequenced genome of KV1772 published, without mutations and hence, showing that is not the right one.

On the other hand, high anneal temperature causes the lack of bands in the PCR, reaching a double-way problem hassle. In one way, pick out of pollution DNA in the reagents, in the other way, lacking of results.

DISCUSSION

In this study, MVV strains from four sheep were studied. These sheep had been housed together with sheep inoculated with the virus strain K796 and had become infected by aerosol transmission **(82)**. A number of virus strains from other sheep that also had been housed with the same infected sheep had been studied previously and it was discovered that all viruses of the sheep had mutations in some "hot spots" mostly in glycosylation sites and charged amino acids of the PND. The aim of this study was to sequence part of the envelope gene and find out if viruses from these naturally infected sheep had acquired the same or similar mutations in the same "hot spots" as the virus strains that had been examined previously. The region was amplified with PCR. In two of the strains, no bands were obtained, despite multiple attempts, and the bands that were obtained from the two other strains turned out to be the strain 1772 when sequenced.

There can be many reasons for the PCR not working. One reason might be that the DNA corresponding to the primer region had accumulated many mutations so that annealing was not effective. The annealing temperature was lowered to 50ºC, but still no bands appeared in two of the samples. There was a band in the negative control, which explains the appearance of the 1772 strain in the other two samples, since this is the strain that is routinely used in the laboratory. However, the lack of bands in two of the samples can best be explained by some sort of inhibition of the PCR in the samples.

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