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Some details in the study of viruses that affect to small animals

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Abstract

Viruses have deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) as their genome, which already makes them different from any other pathogenic agent or microorganism. Knowing all or part of its genome generally makes it possible to establish faster, more effective, simpler and lower-cost diagnostic methods.

Virology is a discipline in constant change or at least surprises, represented by the appearance of new strains or variants that can wreak some havoc on animal species, including us.

Studying animal viruses is studying viruses that emerge in humans, since *viruses are viruses* and knowing part of their genome today allows us to establish the Polymerase Chain Reaction (PCR) protocols for their detection, as two Nobel Prize winners would say.

Keywords: Viruses; PCR; Molecular diagnosis; Pathogen detection

1. Introduction

Viruses are viruses (André Lwoff, 1965), in our opinion the best definition of virus and at the Nobel Prize level. With this, Mr. Lwoff wanted to highlight the way in which viruses generate progeny, something not shared with any other known pathogen, therefore, different from microorganisms that follow the binary fission model: two originate from one bacterium; from two originate four; from four, eight originate and so on.

In contrast, viruses do not reproduce, they multiply following a process of formation by parts. The entry of a virus (virion: infecting particle, complete) is enough to generate new viruses that are released from the host cell. This constitutes the viral cycle, which has steps such as: union (virus-cell), penetration, stripping of the genome, replication of the genome with transcription and translation of viral proteins, subsequent assembly and finally the release of progeny. Notice that there is a different particle.!! This different viral particle is the annual example of new influenza viruses, for example [1].

The genome of a virus is made up of DNA (deoxyribovirus) or RNA (ribovirus). The structure of the virion (infective particle, complete) consists of genome and capsid. Some viruses have an envelope and therefore an integument (amorphous area composed of proteins). Therefore, there are two types of viruses: naked and enveloped. And regarding the genome, there are viruses with a double-stranded DNA genome and viruses with a single-stranded DNA genome. Now, if the genome of the virus is RNA, there are double-stranded RNA viruses and single-stranded RNA viruses [1, 2].

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Finally, if the virus is single-stranded RNA, there are two types: positive sense RNA virus and negative sense RNA virus. An RNA virus has a positive sense if the genome is identical to a messenger RNA, that is, it can be "read" directly by the cellular ribosome, in which case that genome is infective. Obviously, an infective genome should have a shorter viral cycle.

An exception to the rule is retroviruses, because despite being positive-sense RNA, they have an enzyme that synthesizes a complementary DNA strand and this single-stranded DNA is a template for a complementary copy, and thus the provirus (double-stranded DNA) inserts itself into the cellular chromosome of the infected cell [1, 2]. Viruses receive or are named differently, some receive the name of the place where it was isolated or where the associated disease appeared for the first time (e.g. Ebola virus, Nairobi virus, etc.), others receive it by some characteristic associated with tropism observed cell (respiratory syncytial virus, for example) or simply by some structural characteristic observed under the electron microscope, coronavirus, for example [2, 3].

Viruses can be grown in the laboratory, but for this, appropriate cells must be available, depending on the affected animal species. In other words, if we need to isolate the canine herpes virus from a sample, we must have dog cells; if we need to isolate feline calicivirus, we must have cat cells. Today with money it is possible to acquire cell lines [4].

At this point in life, the molecular detection of a virus using molecular biology techniques does not represent many difficulties [5, 6]. It would be enough to know the nucleotide sequence of a conserved gene of the virus to attempt its molecular detection, for example, through a PCR protocol, designing or copying the appropriate primers from the existing literature (Probably there is someone who knows more about PCR than I do, but: I'm the one most interested in letting you know).

2. Detection of viral genomes (PCR)

Viruses are viruses. The genome of a virus consists of DNA (Canine Herpesvirus, Feline Herpesvirus, Myxomavirus, etc.) or RNA (Feline Coronavirus, Feline Immunodeficiency Virus, Canine Distemper Virus, SARS-CoV-2, etc.) [3]. So, if the genome is known, the genes that make it up are also known, and in this way the nucleotide sequences of each one, present in the official GenBank ® database, are also known [7].

Currently, the method of choice for the detection of a pathogen of interest is the Polymerase Chain Reaction (PCR), a technique invented by Kary Mullis [8] and for which in 1993 he received the Nobel Prize, awarded by the Royal Swedish Academy of Sciences.

Year 1993. Kary Mullis surfs in waves of California while in Sweden it is decided who will be awarded the Nobel Prize and later he will declare "I never thought that they would award it to me so soon". That's how worried he was before such an important distinction. Kary Mullis and his development of PCR has been criticized as: "highly original and significant, dividing biology into two eras: before PCR and after PCR". Others point to it as: "Kary Mullis, perhaps the strangest human who has won the Nobel Prize in Chemistry. Raucous, eccentric, funny and iconoclastic who spins through the wonderland that is his mind" [9].

Although Kary Mullis passed away on August 7, 2019, her legacy will live on forever.

But… What is PCR? In short, it consists of the amplification up to a billion times of a DNA fragment of interest. Incredible, huh?

Kary Mullis relied on DNA replication within a cell of a living organism, but without the involvement of the formidable team of enzymes involved. Kary Mullis proposed the denaturation of DNA by applying heat (94-95°C), subsequently facilitating the binding of primers (like Okazaki fragments and at a specific temperature) to each already separated DNA strand and finally facilitating the action of a thermostable enzyme such as Taq Polymerase at 72°C. The genius of Kary Mullis is manifested when he proposed to repeat this process (one cycle) at least 30 times [8].

This repetition involves the formation of PCR products that follow the formula $P=2^n$, where n is the number of cycles. If $n=30$, $P=2^{30}$… equals 1073 million copies…!

To perform this technique, in addition to the DNA of the suspicious sample, it is required to mix in a test tube, the nucleotides (A, T, G, C), a thermostable enzyme: T*aq* Polymerase, a highly important cofactor: Mg^{+2} and a pair of pathogen-specific primers.

The tube is placed in a thermal cycler (https://www.youtube.com/watch?v=OdaPsDIhtX0) which will ultimately carry out the temperature changes proposed by Kary Mullis.

And when is RNA available as starting material? Easy, if we enlist the help of a particular enzyme, reverse transcriptase, which synthesizes DNA from RNA, discovered around 1970 in a retrovirus [10]. Thus, a previous stage that includes DNA synthesis is incorporated into the thermocycler program and from then on the normal PCR is carried out. This is called RT-PCR. Currently, a variant of conventional RT-PCR, called real-time RT-PCR (qRT-PCR), is the technique of choice for the detection of the pathogenic agent that causes COVID19: the SARS-CoV-2 coronavirus. Other known variants of conventional PCR are the nested version (involving performing two consecutive PCRs in series) and the multiplex version (involving the use of two or more primer pairs in a single PCR reaction).

This technique makes it possible to detect any pathogen with a known genome, as long as specific primers are available, complementary to a part of that genome.

2.1. Detecting a DNA virus (Canine herpes virus (CaHV-1) model)

CaHV-1 belongs to the order *Herpesvirales*, Family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, like most herpesviruses that affect domestic animals. CaHV-1 affects canids and in dogs it has been described since 1965 as the pathogen that causes fatal hemorrhagic disease in puppies under four weeks of age [2, 11].

CaHV-1 has a double-stranded DNA genome, it is enveloped (has integument), a size of around 200 nm and a genome of approximately 150 kilobases.

In Chile, it was detected in 2002 and published by our laboratory [12]. Molecular screening came later and several self genes such as *ul37*, *gB* and *gC* were detected, completing the picture. CaHV-1 is a cytolytic virus, that is, it breaks cells as part of its viral cycle and for this reason it is possible to see this effect in infected cells through a special optical microscope: an inverted microscope (https://www.medicalexpo.es/prod/zeiss-microscopy/product-104077-858579.html) that allows to see the cells (from below) adhered to the bottom of a culture flask and thus verify if the cell monolayer is destroyed or not. This characteristic is its own, which is why cells in culture should undergo this phenomenon in the presence of the CaHV-1 virion. Therefore, if this effect is not observed, it is presumably not CaHV-1. We do not see the virus, but we see the effect produced in cells. The mentioned effect must occur, but it does not constitute a guarantee of the presence of CaHV-1, since it is not the only cytolytic virus that affects small animals: a molecular check should be performed.

The genome of a herpes virus consists of several genes and in our experience we have detected at least three: *ul37*, *gB* and *gC* . The first gene codes for an integument protein and the other two for glycoproteins, and as an example the first report made in FAVET and represents the first molecular detection attempt of CaHV-1 in Chile and in FAVET in particular. In it, in addition to detection, the nucleotide identity of the generated DNA fragment was determined [13].

Then, the detection of the glycoproteins *gB* and *gC* of CaHV-1 was carried out and in the second case (gC) the *design of in silico* primers was used, that is, the detection was carried out by means of a PCR reaction with "own" primers [14].

2.2. Detecting RNA virus (Canine Distemper Virus (CDV) model)

VDC is a pathogen that causes a highly contagious disease in several animal species, terrestrial and marine mammals. To date there are many works published from various countries of the world. One of the strange things about this virus is its ability to have crossed the species barrier, because although it was initially described in canines, today it is already documented that it affects big cats and seals [15, 16].

The VDC has a single-stranded RNA genome, negative polarity and a size close to 15,700 nucleotides. The virion is enveloped, has a size between 150 and 300 nm and its genome consists of 6 genes: N, P, M, F, H, L. Each gene gives rise to a protein: the N gene codes for the Nucleoprotein, the P gene for the Phosphoprotein, the M gene for the Matrix protein, the F gene for the Fusion protein, the H gene for Hemagglutinin and the L for Large Polymerase.

The RNA genome is surrounded by the Nucleoprotein generating the nucleocapsid. Glycoproteins H and F participate in the union and fusion with the cell to be infected and are the proteins against which the organism generates antibodies. Of all, the H gene is the most variable, which will bring some inconveniences at the time of the generation of canine distemper vaccines. The N gene is the most conserved and therefore would be the most appropriate at the time of establishing or defining a molecular detection method by RT-PCR and in the same way the H gene would not be the most appropriate for this purpose, since it is defined as the most variable gene [1, 2, 17].

In FAVET we have started the molecular detection of CDV using RT-PCR reactions in different situations some time ago. Our first reports made were targeting the H gene [18] and subsequently it has been scanned the genome and genes of VDC to determine the best detection method [19, 20, 21]. As we have already mentioned, the main idea of this course aims to elucidate the question: why do dogs vaccinated against canine distemper get sick and die? and others such as: will the vaccine used have something to do with it? Haven't they followed some basic rules for the conservation of the vaccine? Has the genome of the virus changed? By the way, in the CDV genome the gene sequence follows the order N, P, M, F, H, L and of all, the L gene is the longest in nucleotides (around 6500 nt).

(https://www.ncbi.nlm.nih.gov/nuccore/AY443350.1?report=graph). There is a Japanese author in the literature: Mochizuki who began the study of the various CDV genotypes around the sequences of the H gene, the most variable. We invite you to read the article: https://jcm.asm.org/content/37/9/2936.long indisputably cited in any subsequent article that deals with the existence of the almost fourteen different genotypes of VDC And at the national level, what happens?... How many genotypes exist in Chile? [22].

2.3. Detecting special RNA virus (A Feline Leukemia Virus (FELV) Model)

A retrovirus is a virus that, having single-stranded RNA genome and positive polarity, chooses to follow a path of multiplication or generation of progeny different from that expected. The reason? The existence of a structural protein called reverse transcriptase or reverse transcriptase that allows it to synthesize cDNA and subsequently integrate into the genome of the infected cell, where it remains as a provirus.

The existence of this enzyme made it possible to complete the Dogma of Molecular Biology. This discovery is due to Renato Dulbecco (Caltech) and two of his former disciples: David Baltimore (MIT) and Howard Temin (University of Wisconsin-Madison), for which they were awarded the Nobel Prize in Medicine in 1975.

In addition, the existence of this enzyme has allowed the amplification of RNA virus genes through the brilliant idea of Kary Mullis, by incorporating a previous phase of reverse transcription. Thus, when cDNA is synthesized, the next step (PCR) does not have any inconvenience. Among the most important retroviruses for the human species, the *Human Immunodeficiency Virus* (HIV) stands out; however, there are several other retroviruses that affect other animal species such as the ape or the domestic cat and within them, on this occasion, we will focus attention (as an example) on the *Feline Leukemia virus* is a retrovirus that infects cats. FeLV is transmitted between infected cats through saliva or nasal secretions. If not counteracted by the animal's immune system, infection with the virus can be fatal. This virus causes a type of cancer of the blood cells, the lymphocytes, that is, leukemia.

The FeLV belongs to the *Retroviridae* family, *Orthoretroviridae* subfamily, and the *Gammaretrovirus* genus [2]. It is an RNA virus with two copies of the genome, has positive polarity, icosahedral capsid and envelope. Its size ranges between 80 and 120 nm.

FeLV was first described at the University of Glasgow School of Veterinary Medicine [23]. It consists of three genes: *gag* (structural), *pol* (enzymes), and *env* (envelope and transmembrane), and the total genome is approximately 9,600 bases.

In the detection of this and other retroviruses, better results have been obtained using the PCR technique, mainly due to its unique infective cycle, which involves the DNA *provirus* state. Thus, its molecular detection may be using primers described in the literature or by *In Silico* design, by selecting one of the retrovirus genes.

A general outline of the Feline Leukemia Virus is as follows: you can choose one of the three main genes: *gag, pol* or *env*, as well as some other conserved area of the genome.

Undoubtedly, a virus that does not follow the typical sequence of an RNA virus, single stranded, positive polarity is worth highlighting and attempting its molecular detection...!!

Finally, if you like pussycats, you can enjoy watching how Marlon Brando masterfully improvised the scene with a cat that suddenly appeared on the set of the film directed by Francis Ford Coppola: The Godfather (The Godfather, 1972)... until you feel the purr of the cat... https://www.youtube.com/watch?v=UjAPCvkRo4M

2.4. Choincing the primers

The successful development of a PCR protocol has no excuses at present, since the brilliant idea of Kary Mullis, who occupies exclusive primers for the pathogen to be detected, has been within our reach for some years.

Although the primers correspond to short-sequence oligonucleotides (generally 20) complementary to a genome sequence of the pathogen to be detected, their sequence can be defined "by hand" considering this last characteristic. For example, if the "target" nucleotide sequence is: ATGCTCTCCTACCAAGACAAGGTGGGTGCCTTCTATAAGGACAAT GCAAGAGCTAATTCACCCAACTGTCCCTAGTGACAGAAGAGCAAGGGGGAAGGAGACCACCCTATTTGCTGTTTGTCCTTCTCA TCCTACTGATTGGAATCCTGGCCTTGCTTGCCATCACTGGAGTTCGATTTCACCAAGTATCAACTAGCATATGGAATTTATCGT TAAGGCTTAAGCGGC and we want to generate an amplicon of about 100 bp, there are several possibilities to find a pair of primers that meet that condition.

For example, the primer P1: TACGAGAGGATGGTTCTGTT complies with being complementary and has been "designed" without much problem (it joins perfectly to the beginning of the original sequence) and then a second primer can be P2: CTCCTTCCCCCTTGCTCTTC, since when used they generate a fragment of 100 nt. OKAY... But are they the optimal primers to use? won't we lose money by sending them to synthesize? (it is most likely) because a pair of primers must not only be complementary, but also some thermodynamic characteristics must be met, such as the type of bond and its associated energy or the % of GC in each primer. In this example: P1(45%); P2 (60%).

To ensure the optimum choice of primers, there are some online programs such as *Oligoperfect Design* ® from the company Thermofisher Scientific, in which the target sequence is entered and the program provides a ranking of the best pairs of primers that generate a fragment of around 100 bp. and it is possible to know the sequence of the primer, its length in nucleotides, its % GC, its Tm (*melting temperature*) and where it is located (start and end). The sequences are copied into a Word format and sent to a company dedicated to the chemical synthesis of oligonucleotides and usually after a week, they reach us. In Chile, the approximate cost is USD16 (the pair of *primers*). This type of design is an alternative, that is, it can be used when the primers are not known in the literature or to compare results with those described in the literature.

Thus, one of our last qualified students proposed an RT-PCR protocol for the detection of *Feline Calicivirus*, but with a primer design (24).

2.5. Sequencing and identification

Currently there are genomic databases that allow the identification of a particular pathogen. The Clustal and BLAST softwares are very useful [25, 26] and allow us to corroborate the percentage of identity between our sequences and those already officially published.

3. Conclusion

Today there is no excuse for not implementing or developing a PCR (or RT-PCR) protocol in a diagnostic laboratory, much less to identify the genome of a virus that affects humans or other animals.

Compliance with ethical standards

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D*isclosure of conflict of interest*

We have no conflict of interest.

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