



Research Article

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Proposal for a Conventional Polymerase Chain Reaction (PCR) Protocol for Detection of *Chlamydia felis*

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Abstract

Feline Respiratory Complex (FRC) is a common condition in young cats living in crowded conditions, such as shelters or colonies. This complex has viruses as its main etiological agents and bacteria secondarily. This respiratory complex presents a wide variety of signs, ranging from lethargy and loss of appetite to sneezing, conjunctival hyperemia, nasal and ocular secretions, hypersalivation, and even, in some severe cases, respiratory distress due to bronchopneumonia and, ultimately, death. Among the bacteria that are part of the secondary infections of CRF, there is *Chlamydia felis*, a bacteria that, together with others of the same genus, has significant zoonotic potential. *Chlamydia felis* has an affinity for the conjunctival tissue, resulting in the characteristic clinical sign of conjunctivitis, presenting a risk that mainly affects immunosuppressed individuals. Its transmission is basically by direct contact, since the bacteria is labile and only survives a few days at room temperature, being easily inactivated by most disinfectants. Prevention is mainly based on the use of the vaccine and avoiding overpopulation of cats. On the other hand, treatment involves the use of antibiotics from the tetracycline group. The prevalence of this bacteria is relatively high in many countries, including China, Italy, Japan and Slovakia, particularly among stray (>10%) and domestic cats (>3%), however, in Chile, information about its presence is scarce. Thus, in this report, a molecular protocol for the detection of *C. felis* will be proposed, and the design of *in silico* primers for the Polymerase Chain Reaction (PCR) technique will be considered, part of the objective of this protocol is to offer a diagnostic option using primers based on the most updated sequences to date of this work.

Background

Feline Chlamydiosis

Among the common pathologies that affect young cats, there are diseases of the feline upper respiratory tract. The main etiological agents of these diseases are viruses, such as *Feline Calicivirus* (CVF) and *Feline Herpesvirus-1* (HFV-1), which are often accompanied by secondary bacterial infections. The latter include *Mycoplasma felis*, *Bordetella bronchiseptica* and *Chlamydia felis*, the latter being known as *feline Chlamydiosis* [1]. *Chlamydia felis* (*C. felis*) is a Gram-negative, obligate intracellular bacterium [2]. Its main clinical manifestation is observed in the form of acute to chronic or

recurrent follicular conjunctivitis in young adult cats [3]. Although it has a zoonotic potential, there is little evidence that this is significant, since it mainly affects immunosuppressed individuals, causing conjunctivitis and/or respiratory tract diseases [4-6].

Taxonomy

C. felis belongs to the phylum *Chlamydiae*, class *Chlamydiia*, order *Chlamydiales*, family *Chlamydiaceae* and genus *Chlamydia*. The order includes eight families with varying degrees of relatedness, determined by similarities in the 16S rRNA gene. Regarding the genus, a study proposed dividing it into two: *Chlamydia* and

Chlamydophila [7]. However, this proposal has not been uniformly adopted by the scientific community. For this reason, in the edition of Bergey's Manual of Systematic Bacteriology (volume 4), it was decided to retain a single genus, *Chlamydia*, for all species of the *Chlamydiaceae* family, which are pathogens of humans or animals. Furthermore, 16S rRNA sequence identity thresholds do not consistently separate *Chlamydia* species from *Chlamydophila* species. Their genomes are remarkably similar and lack clear phenotypic characteristics that distinguish them, which reinforces the need to unify both into a single genus, this being *Chlamydia* [8-10].

Pathogeny

The development cycle of members of the *Chlamydiaceae* family is biphasic: it alternates between the Infectious Elementary Body (EB) and the Replicative Reticulated Body (RB). During infection, bacteria lodge inside a parasitic vacuole that forms from the host cell, known as an inclusion. Upon infection, an infectious elemental body differentiates into a replicative reticular body. Within the inclusion, EBs divide and then a group of RBs will differentiate again into EBs that exit the host cell by cell lysis or inclusion extrusion. In hostile environments, these microorganisms have the ability to enter a persistent stage, allowing them to survive long-term in the host cell. Conjunctivitis caused by this infection manifests after an incubation period that usually lasts between 2 and 7 days [11-13].

Signology

C. felis has the ability to cause various signs, including inflammation of the conjunctival tissue, which can occur with or without ocular discharge, hyperemia in the third eyelid, chemosis and blepharospasm. Since its main affinity is in the conjunctiva, corneal involvement is rare, unless another etiological agent, such as VHF-1, is present [14,15]. The initial manifestations of the infection in cats usually occur as a unilateral eye disease, which can gradually evolve into bilateral conjunctivitis; some cats may manifest sneezing and a runny nose [16,11]. In addition to ocular signs, others that may be present include fever, lethargy, enlarged submandibular lymph nodes, lameness, loss of appetite, and weight loss [13]. The absence of clinical signs does not indicate the absence of infection [4].

Transmission

The main route of transmission of *Chlamydia* is direct contact, since the infectious form of the bacteria has limited survival in the environment, and is easily inactivated by most disinfectants [3]. Additionally, there is a lower risk of opportunistic infection through vaccination in individuals whose immune system is compromised [15].

Prevention

Because *Chlamydia* is transmitted mainly by direct contact, prevention of infection involves reducing overcrowding, reducing stress, and combining vaccination of the individual and disinfection of their environment. In relation to this last factor, it is essential to focus on the use of active products that are effective against CVF, since, unlike VHF-1, CVF is less susceptible to inactivation and both are primary agents of the feline upper respiratory complex [3].

Furthermore, quarantines are recommended, as this, together with therapies, should contribute to a decrease in the incidence of upper respiratory tract diseases [4]. In Chile, the Felocell® 4 vaccine is available as a preventive method for the disease.

Treatment

The individual health status of each cat and its environment determine the treatment strategy to follow. In some shelters, it has been observed that mild to moderate infections can resolve over time without requiring antimicrobial treatment [3]. However, when intervention is necessary, doxycycline is the preferred choice for treating *C. felis* infection, and is also effective against bacteria such as *Bordetella* and *Mycoplasma*. The recommended dose of doxycycline is 10 mg/kg per day, administered orally. In cases of vomiting, you can choose to administer 5 mg/kg orally twice a day. This treatment should be carried out for 4 weeks, and it is suggested to extend it for an additional two weeks once the clinical signs have disappeared, since there is the possibility of eventual reactivation of the infection. In addition, it is recommended to apply this treatment to all cats in the home as a preventive measure. Alternatively, amoxicillin (at a dose of 12 to 20 mg/kg orally every 12 hours) may be useful in treating secondary bacterial infections in cats with respiratory diseases, although it is not effective against *Mycoplasma* [17,3,15].

Diagnosis

For the diagnosis of chlamydiosis, it is recommended to perform the Polymerase Chain Reaction (PCR) Test, which allows the bacteria to be identified and characterized based on its different strains [6]. On the other hand, it is not advisable to use antigen tests due to their low specificity. This is because serology faces the challenge of cross-reactions between antibodies against different *Chlamydia* species. Furthermore, the intracellular nature of the bacteria may lead to a more locally restricted antibody response [18]. Cytology is another simple diagnostic method that can be used, using May-Grünwald-Giemsa staining. In this technique, the identification of *C. felis* is performed by observing basophilic inclusion bodies stained bright magenta. These bodies are round or oval and vary in size within the epithelial cells. In general, these inclusion bodies consist of particles ranging from small elementary bodies (~300nm), which stain red to purple, to larger initial bodies (1µm), which stain dark blue. However, it is important to keep in mind that diagnosis based on cytology has limited sensitivity for *chlamydiae*, so it is generally used in conjunction with PCR to support the analysis, to increase its diagnostic accuracy [13,14]. In cases of necropsy, viral inclusions can be identified in respiratory tissues [3].

Epidemiology

According to various sources, it has been observed that the highest incidence of cats positive for the disease is in the age range of 2 to 12 months [3,6,15]. The seroprevalence of *C. felis* is reported to be relatively high in countries such as China, Italy, Japan and Slovakia, with cats being the main carriers, although the possibility of dogs being potential carriers has also been identified [13]. In Spain [16] mentioned that most studies indicated a zero prevalence

in apparently healthy cats. However, in Romania [13] carried out a study with 95 stray cats, and their results showed that the frequency of detection was significantly higher in asymptomatic cats compared to symptomatic cats. In Chile, there is only one study where, through serological diagnosis, they took blood samples from 60 cats and found a positivity of 51.7% to *C. felis* [19]. However, a significant lack of other studies on the prevalence of *C. felis* in the country is identified, which highlights the need to propose diagnostic alternatives.

Thus, in this proposal, the application of the primer design is proposed for the detection of the *C. felis* ompA gene present in the Baker strain, using the conventional PCR technique. This gene is highly conserved [2] and has been used in reference studies for the detection of Chlamydia and discrimination between its different species [20]. Its sequence is available in GenBank. The purpose of the proposal is to provide a diagnostic protocol that can be replicated for future research with updated information.

Materials and Methods

This proposal can be carried out in any Animal Virology Laboratory in the third world that has the basic infrastructure for the molecular detection of pathogens of veterinary interest.

Design Of Primers for The Molecular Detection of *C. Felis* Using the OmpA Gene (Baker Strain) As A Target

The design of primers contemplates the use of the Genbank® database to access the sequences of the ompA gene of *C. felis*, which currently has a total of 9 sequences [21]. With these sequences, a consensus sequence will be obtained to use for the design of primers with the Oligoperfect Design® program from Invitrogen, which provides a ranking of optimal starters considering a GC percentage greater than 50% and a temperature delta less than 3°C. In the design of the primers, the generation of a DNA fragment of at least 500 bases will be established as a condition, with the purpose, when implementing this protocol, of knowing the percentage of nucleotide identity of the fragment amplified with the ompA gene of *C. felis*. A company will also be proposed to which to send to synthesize the proposed starters, for example: Integrated DNA Technologies (IDT®).

Primers selection criteria:

- Length of the primer: between 18-25 nucleotides
- GC content (% GC): 50%
- Alignment temperature (Tm): Between 50-60°
- Absence of secondary structures: the starters should not form dimers (self-dimers or hetero-dimers) or hairpins.
- Specificity: OmpA gene region.

Proposal A Protocol for The Detection by Conventional PCR Of *C. Felis*

The proposal of specific conditions for molecular detection will consider the use of the primers designed in the previous point, in

conjunction with the generic conditions in every PCR reaction, such as:

PCR Reaction Mixture: The PCR reaction mix will consider using DNA (suspect sample, vaccine, positive control, negative control and reagent control), the designed primers and a Master Mix solution (Taq Polymerase, nucleotides ([A, T, C, G]) and Mg²⁺) according to the manufacturer's instructions.

PCR Reaction: The PCR reaction will consider a protocol compatible with the design of the primers obtained and generally includes three steps: denaturation at 94°C for 30 seconds; alignment at a temperature defined by the design of the starters, initially (Tm-5)°C for 30 seconds and the polymerization process at 72°C for 1 minute. Repeating the above 35 times, finally a final extension stage at 72°C for 10 minutes.

Visualization of Amplified Product: Once the PCR reaction is completed, the product must be visualized through electrophoresis in a 2% agarose gel in TAE (Tris-acetate-EDTA) buffer. This electrophoresis can be performed at 90 Volts for 40 min, using a molecular weight marker. Subsequently, the agarose gel will be incubated in a GelRed solution for 30 minutes at room temperature. Finally, the gel will be observed in an ultraviolet light transilluminator and a photographic record will be obtained. The positivity criterion contemplates the visualization of an expected DNA fragment (for example 400 bp) in comparison with the migration of the marker used. Although the diagnostic protocol does not consider the determination of the percentage of nucleotide identity of the amplified fragment, it is suggested in the first instance and consists of sending the positive fragments for the described PCR to a Sequencing Center. The resulting nucleotide sequences can be aligned using the Clustal Omega program, obtaining a consensus sequence for each fragment. Subsequently, these consensus sequences can be entered into the BLAST program to know the percentage of nucleotide identity, with respect to the Genbank® database.

Biosafety Measures: The procedures carried out in the laboratory will be carried out in accordance with the biosafety levels established for Microbiology and Virology laboratories, which contemplate the use of clean material, the correct disposal of waste, the use of a closed apron and gloves in practical work. Visualization of the amplified product will involve the use of red gel and a UV light transilluminator, therefore, when viewing the gel, an acrylic plate and glasses with a UV filter will be used.

Discussion

In this case, the detection of *C. felis* included the use of the OmpA gene. However, for a similar detection any conserved gene of the indicated bacteria could be used, for example, the gatA gene or another. The important thing will be to follow the aforementioned methodology to design the matches that allow the unequivocal detection of the bacteria using some programs and algorithms available on the Internet today.

Conclusion

Thus, the molecular detection of pathogens of veterinary inter-

est is not a major obstacle in third world veterinary laboratories. We are able to apply Kary Mullis' fabulous invention [22], both in veterinary medicine and in human medicine, for example, in the detection of SARS-CoV-2, the etiologic agent of COVID19.

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Conflict of Interest

None.

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