Part 1. Veterinary Medicine

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DEVELOPMENT OF PCR-TEST SYSTEMS FOR SPECIES DIFFERENTIATION OF CHLAMIDIOSIS AGENTS IN FELIDAE FAMILY AND RODENTS ORDER

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Summary. According to the current classification adopted at the 2nd European Symposium on Animal Chlamydiosis and Zoonotic Implications (EMAC-2), pathogens causing chlamydioses in cats and rodents are *Chlamydia* genus bacteria of the three species: *C. felis, C. caviae* and *C. muridarum*. Three PCR test systems were developed for indication and species differentiation of the said bacteria. The basis for the developed diagnostics is the design and synthesis of three oligonucleotide primers pairs, flanking specific fragments of the chlamydial MOMP gene DNA pathogenic to cats and rodents. Analytical specificity of the developed PCR test systems is confirmed by the results of 13 biological materials samples amplification, the total of 13 ones being chlamydia-containing, namely: 4 samples contain *C. felis*, one — *C. caviae*, and one — *C. muridarum*. In addition to *Chlamydia*, DNA samples were taken from *Leptospira* and *Babesia*.

Keywords: cats, guinea pigs, mouse rodents, PCR-test system, species differentiation, *Chlamydia felis, Chlamydia caviae, Chlamydia muridarum*

Introduction. Chlamydioses form a group of infectious diseases caused by Gram-negative intracellular bacteria of the Chlamydiales order. According to the current classification adopted at the 2nd European Meeting on Animal Chlamydioses and Zoonotic Implications (EMAC-2), the above order includes 8 families (3 of them having the candidate status) represented by 13 genera (5 having the candidate status) and 25 species (among them 7 microorganisms having the candidate status). For mammals of the cat family, guinea pigs and mouse rodents, pathogenic bacteria of the *Chlamydia* genus are: *C. felis, C. caviae* and *C. muridarum* (Ksyonz and Liubetskyi, 2014; Sachse, 2013; Sachse et al., 2015).

Chlamydia felis causes conjunctivitis, rhinitis, pneumonia and urogenital injuries in domestic and wild carnivorous of the cat family (Felidae). There are known cases of human infection, on contacting with chlamydiosis affected cats, with signs of keratoconjunctivitis manifested (Hartley et al., 2001). *Chlamydia caviae* causes conjunctivitis and urogenital diseases in guinea pigs (*Cavia cobaya*) (Lutz-Wohlgroth et al., 2006). *Chlamydia muridarum* causes the disease in the Muridae family rodents (mice, hamsters, etc.) (Jayarapu et al., 2009; Frazer et al., 2013).

Currently, Ukrainian scientists and practitioners of veterinary medicine do not possess diagnostic tools capable of differentiating chlamydial infections pathogens of cats and rodents. However, such a tool is necessary to study various aspects of the above infections, in particular their etiology and pathogens.

On the basis thereof, **our work was aimed** to develop three PCR test systems for indication and species differentiation of *C. felis*, *C. caviae*, and *C. muridarum*, i.e. etiological factors of chlamydioses in cats and rodents.

Materials and methods. The study was carried out in the Laboratory of Animal Health and Genetics of the Institute of Pig Breeding and Agroindustrial Production of the National Academy of Agrarian Sciences of Ukraine.

To develop the primers design of the PCR test systems, 20 primary sequences of the gene encoding the main outer membrane protein (MOMP), belonging to three *Chlamydia* genus bacteria species, namely: *C. felis*, *C. caviae*, and *C. muridarum* involved from the nucleotide sequences databases GenBank (USA) (Korniyenko and Ksyonz, 2017).

The nucleotide sequences of the gene encoding MOMP of the three designated species of *Chlamydia* genus were aligned using the MEGA v. 4 and MEGA v. 7 software (Tamura et al., 2007). To develop the design of oligonucleotide primers, DNA fragments specific for each species of the *Chlamydia* genus bacteria have been selected.

Sequences of oligonucleotide primers with their annealing temperature parameters were obtained using the FastPCR software (Kalendar, Lee and Schulman,

2014). Among the calculated primers, a single pair (the direct primer and the reverse one) was selected for each species of the *Chlamydia* genus bacteria pathogenic to cats and rodents.

Based on the designs developed, synthesis of the oligonucleotide primers was ordered at 'Thermo Electron Corporation' (Germany). The resulting synthesized primers were diluted with sterile deionized double-distilled water to the stock concentration of 100 pM/μl and then to the use concentration of 20 pM/μl.

In addition to the primers, the test systems used reagents for the PCR manufactured by 'Fermentas UAB' (Lithuania), namely: deionized water, PCR buffer, $MgCl_2$, deoxyribonucleoside triphosphate solution (dNTP), and Taq-polymerase.

Polymerase chain reaction using the developed PCR test systems for indication and species differentiation of *C. felis*, *C. caviae*, and *C. muridarum* was carried out in 0.6 cm^3 polypropylene microcentrifuge tubes using the 'Biometra TRIO-Thermoblosk' thermocycler (Germany) in the reaction volume of 25 μl.

The ratio of the reaction mixture and the amplification program were adjusted experimentally and practically to obtain the most distinct bands on electrophoregrammes.

Fractionation of the amplification products was performed by the method of horizontal electrophoresis in the 2.0% agarose gel in the 'Cleaver Scientific Ltd.' (UK) electrophoretic chamber with a visual assessment by means of the UV transilluminator manufactured by NVO 'Progress' (Ukraine), after staining with bromine ethidium.

Restricted vector DNA *pUC19/MspI* ('Fermentas UAB', Lithuania) was used as a DNA marker.

DNA isolation from the biological samples under study was carried out using the commercially available reagents set 'PROBA-RAPID' manufactured by LLC 'NPO DNA Technology' (Russian Federation).

Material for adjusting the parameters of experimental PCR test-systems were control DNA samples of *C. felis* and *C. caviae* received from the Friedrich Löffler Institute, Federal Research Institute for Animal Health (Jena, Germany), and a control DNA sample of *C. muridarum* received from the Department of Pediatrics Infectious Disease Section, University of North Carolina (USA).

DNA samples isolated from *Chlamydia* field isolates of the cat family domestic and wild carnivores; DNA samples isolated from the *Babesia* stored in the Laboratory of Animal Health at the Institute of Pig Breeding and Agroindustrial Production of NAAS; DNA samples isolated from *Leptospira* received from the Museum of Microorganisms of the Leptospirosis Laboratory at the Institute of Veterinary Medicine of NAAS.

Results. When aligning fragments of the *C. felis*, *C. caviae*, and *C. muridarum* MOMP gene, the level of nucleotide sequences variability was 63.9% (Korniyenko and Ksyonz, 2017). That is why the primary sequences of this gene received from the international databases were used for designing the PCR test systems oligonucleotide primers for indicating and differentiating *C. felis*, *C. caviae*, and *C. muridarum.*

As noted above, the designs of oligonucleotide primers were obtained using the FastPCR software. Among a large number of primer pairs a single one for each test system was selected, taking into account size of the amplified area flanked by them (the most convenient for electrophoretic detection) and the optimum annealing temperature of the primers.

Thus, in the PCR test system for indication and species differentiation of *Chlamydia felis*, the following pair of primers is used:

ChFelMOMPL:
5'-AACTGCAAGCAACACCACTG-3',
ChFelMOMPR:
5'-CCAATCAATCCGACAAGGTT-3'.

In the PCR test system for indication and species differentiation of *Chlamydia caviae*, the following pair of primers is used:

ChCavMOMPL:
5'-TATAAAGGGACAGCGGCCAACTT-3',
ChCavMOMPR:
5'-AGGTTTCGGTGGAGATCCTT-3',
ChMuMOMPR:
5'-AGCGGGATTCTCTCTTGATG-3'.

In the PCR test system for the indication and species differentiation of *Chlamydia muridarum*, the following pair of primers is used:

MuMOMPL:
5'-AGGTTTCGGTGGAGATCCTT-3',
ChMuMOMPR:
5'-AGCGGGATTCTCTCTTGATG-3'.

PCR products are fragments of the *Chlamydia* genus bacteria MOMP gene, with sizes specific for each of the three mentioned *Chlamydia* species, namely: *Chlamydia felis* — 201 bp, *Chlamydia caviae* — 179 bp, *Chlamydia muridarum* — 205 bp.

Optimization of PCR conditions required selection of the reaction mixture composition and the temperature mode of amplification.

As a result of the PCR-protocol optimization, the following parameters of the reaction mixture were found to be optimal: 2.5 μl of 10-fold buffer (670 mM Tris-HCl, pH 8.8 at 25 °C, 20 mM BSA, 166 mM ammonium sulphate $(NH_4)_2SO_4$, 100 mM 2-β-mercaptoethanol) ('Fermentas UAB', Lithuania), 2.5 μl 2.5 mM dNTP ('Fermentas UAB', Lithuania), 2 μl 50 mM $MgCl_2$ ('Fermentas UAB', Lithuania), 2–3 units of Taq-polymerase (Thermus aquaticus) ('Fermentas UAB',

Lithuania), 0.5 µl (0.1 opt. unit) of each primer and a sample of the DNA under study until the final concentration in the mixture; 1 µg/cm³ deionized water to the volume of 25 µl. The amplification mixture was layered with 25 µl of mineral oil.

Optimal amplification parameters were:

94 °C	120 sec	
93 °C	30 sec	35 cycles
55 °C	30 sec	35 cycles
72 °C	45 sec	35 cycles
72 °C	300 sec	

To adjust the PCR parameters for the developed test systems and their analytical specificity testing, the following 13 biological materials were used: a sample of *C. felis* control DNA (1); a sample of *C. caviae* control DNA (2); a sample of *C. muridarum* control DNA (3); a DNA sample isolated from the chlamydia field isolate of a male cat owned by a resident of Poltava (4); a DNA sample isolated from the chlamydia field isolate of a female cat owned by a resident of Poltava (5); a DNA sample isolated from the chlamydia field isolate of a female cat owned by a resident of Poltava (6); a DNA sample isolated from the chlamydia field isolate of leopard (*Panthera pardus*) kept in the Kharkiv Zoo (7); a DNA sample isolated from the *Leptospira* strain (LSU strain, *Louisiana* serovar, *Louisiana* serogroup) (8); a DNA sample isolated from the *Leptospira* strain (493 *Poland* strain, *Polonica* serovar, *Sejroe* serogroup) (9); DNA sample isolated from the *Leptospira* strain (*Hond Utrecht IV* strain, *Canicola* serovar, *Canicola* serogroup) (10); a DNA sample isolated from the *Babesia canis* field isolate of a dog owned by a resident of Poltava (11); a DNA sample isolated from the *Babesia bovis* field isolate of a heifer belonging to a private farm in the village of Verkhy, Kamin-Kashirsky district of the Volyn region (12); a DNA sample isolated from the *Babesia divergens* field isolate of cow belonging to a private farm in the village of Sokilets, Buchach district of the Ternopil region (13).

In the first electrophoregram of the PCR products of the said 13 samples of biological material studied using the test system for indicating and differentiating *C. felis*, a 201 bp band was detected in 4 tracks — 1, 4–6 corresponding to samples of C. *felis* control DNA and to the field isolates obtained from domestic cats. In the rest 10 tracks, including negative control, there were no bands (Fig. 1).

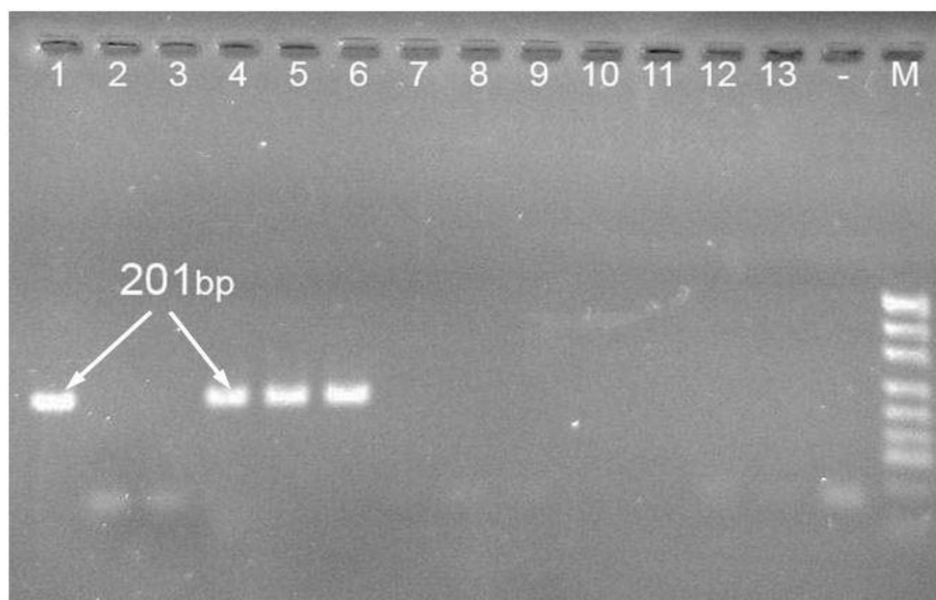

Figure 1. Electrophoregram of PCR products of the 13 biological material samples studied by means of the PCR test system for *Chlamydia felis* bacteria indication and species differentiation, namely: 3 control samples of *C. felis, C. caviae,* and *C. muridarum*; 4 DNA samples isolated from field isolates of cat family domestic and wild carnivores; 3 DNA samples isolated from the *Leptospira* strains; 3 DNA samples isolated from the *Babesia* genus Protozoa field isolates of dogs and cattle. Tracks: 1, 4–6 — band length 201 bp (*C. felis*); 2, 3, 7–13 — negative result; «-» — negative control; M — a DNA size marker *pUC19/Msp*I ('Fermentas UAB', Lithuania)

The absence of 201 bp length band in track 7 corresponding to a DNA sample isolated from a field isolate of a leopard belonging to the Kharkiv Zoo, is due to the fact that the mentioned pathogen was differentiated as *Chlamydia pecorum* according to previous studies. In the second electrophoregram of the same 13 biological material samples' amplification products studied using the test system for indication and species differentiation of *C. caviae*, 179 bp length band was detected in the only track 2 corresponding to the sample of *C. caviae* control DNA. In the rest 13 tracks, including negative control, there were no bands (Fig. 2).

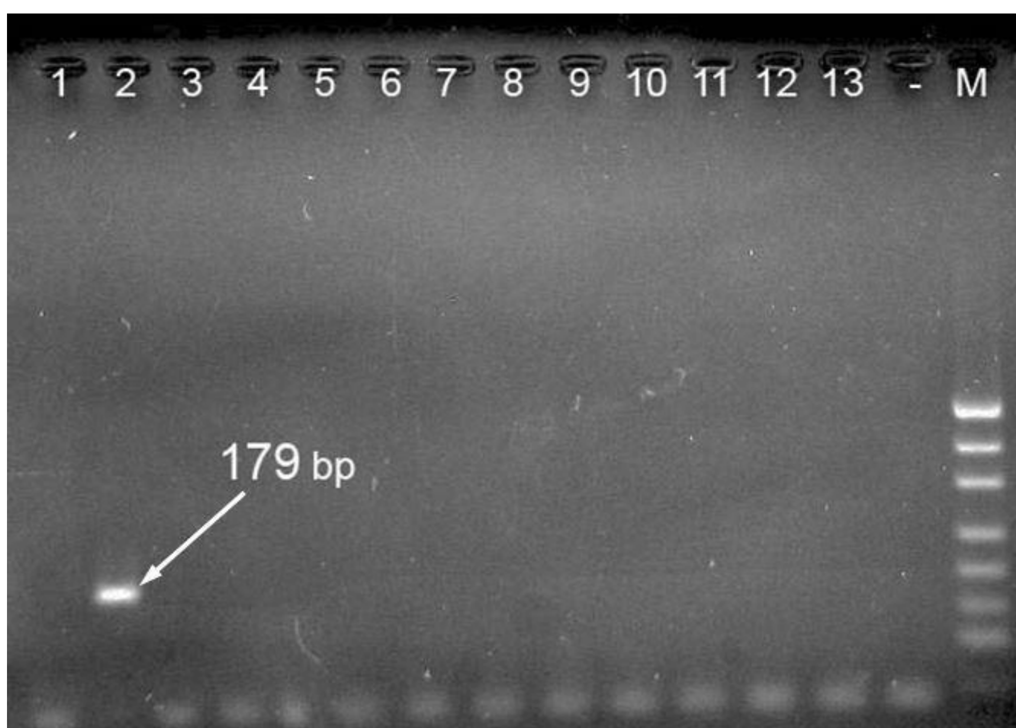

Figure 2. Electrophoregram of PCR products of the 13 biological material samples studied by means of the PCR test system for *Chlamydia caviae* bacteria indication and species differentiation, namely: 3 control samples of *C. felis, C. caviae*, and *C. muridarum*; 4 DNA samples isolated from field isolates of cat family domestic and wild carnivores; 3 DNA samples isolated from the *Leptospira* strains; 3 DNA samples isolated from the *Babesia* genus Protozoa field isolates of dogs and cattle. Tracks: 2 — band with 179 bp length (*C. caviae*); 1, 3–13 — negative result; «-» — negative control; M — a DNA size marker *pUC19/Msp*I ('Fermentas UAB', Lithuania)

In the third electrophoregram of PCR products of the above 13 biological material samples studied using the test system for indication and species differentiation of *C. muridarum*, a band sizing 205 bp was detected on the only track 3 corresponding to the sample of *C. muridarum* control DNA. In the rest 13 tracks, including negative control, there were no bands (Fig. 3).

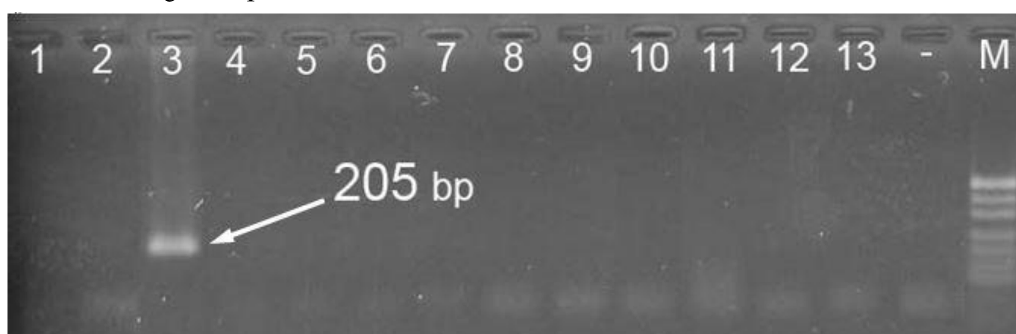

Figure 3. Electrophoregram of PCR products of the 13 biological material samples studied by means of the PCR test system for *Chlamydia muridarum* bacteria indication and species differentiation, namely: 3 control samples of *C. felis, C. caviae,* and *C. muridarum*; 4 DNA samples isolated from field isolates of cat family domestic and wild carnivores; 3 DNA samples isolated from the *Leptospira* strains; 3 DNA samples isolated from the *Babesia* genus Protozoa field isolates of dogs and cattle. Tracks: 3 — a band sizing 205 bp (*C. muridarum*); 1, 2, 4–13 — negative result; «-» — negative control; M — a DNA size marker *pUC19/Msp*I ('Fermentas UAB', Lithuania)

The results of studying the above said 18 DNA samples by means of the three developed PCR test systems prove that the chlamydia DNA indication occurs exclusively in the samples corresponding to each diagnosticum specificity: in the electrophoregrams, the amplification products obtained using the test system developed for *C. felis* indication and species differentiation, four bands sizing 201 bp were detected in

the tracks corresponding to the *C. felis* control DNA sample and to field isolates DNA samples isolated from domestic cats; in electrophoregrams of amplification products obtained by means of the test system developed for indication and species differentiation of *C. caviae*, only one 179 bp band was found in the track corresponding to the *C. caviae* control DNA sample; in the electrophoregrams of amplification products obtained by means of the test system developed for indication and species differentiation of *C. muridarum*, one 205 bp band was found in the track corresponding to the *C. muridarum* control DNA sample. None of the electrophoregrams contained any other bands, in particular, they were absent in the tracks corresponding to the appropriate DNA samples of *Babesia* and *Leptospira*.

Study of the 13 above mentioned DNA samples using each of the three PCR test systems was performed in 3 replicates, with similar results (Tables 1–3). However, it should be noted, that the intensity of the bands was somewhat reduced with the concentration decrease of the DNA under study in the reaction mixture.

Conclusions. The developed PCR test systems, which include oligonucleotide primers flanking different sized DNA fragments encoding MOMP of the three *Chlamydia* genus bacteria species, allow to detect DNA of *Chlamydia felis*, *Chlamydia caviae*, and *Chlamydia muridarum*, which are, respectively, pathogens of cat family mammals, guinea pigs and mouse rodents. Indication and differentiation of chlamydia by species is provided by visual assessment of amplified fragments by various length of the bands in an electrophoregram in the 2.0% agarose gel.

Testing of the developed PCR test systems for indication and species differentiation of the chlamydial infections pathogens in cats, guinea pigs and mouse rodents proves their sufficient sensitivity and analytical specificity.

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