MULTIPLE PCr ASSAY FOR CHLAMYDIA-LIKE BACTERIA DETECTION

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ABSTRACT
Introduction: Waddlia chondrophila and Parachlamydia acanthamoebae are well-known and best-studied representatives of Chlamydia-related bacteria carrying a potential zoonotic threat. These bacteria are associated with miscarriage, ectopic pregnancy, diseases of the respiratory system in both humans and animals. Despite the importance of these Chlamydia-like organisms for human medicine along with veterinary medicine, studies on their prevalence in Ukraine were not conducted due to the lack of available tests.

The aim of our work was to create relatively cheap and easy method for detection Waddlia chondrophila and Parachlamydia acanthamoebae.

Materials and methods: GenBank database was used to find nucleotide sequences of the 16S rRNA gene of bacteria Chlamydiaceae. Alignment was performed using the MEGA7 software, in order to detect the presence of polymorphic hybridization sites specifically attributed to Waddlia chondrophila and Parachlamydia acanthamoebae. Primer- BLAST software was used to design oligonucleotide primers, to evaluate the critical parameters of the primer, in particular, the melting temperature, difference between melting temperatures for the primer pairs, the GC content, the self-complementarity, etc.

Results and conclusions: The amplification of control DNA of Parachlamydia acanthamoebae and Waddlia chondrophila in single PCR using the corresponding primers and subsequent gel electrophoresis of PCR products determined the size of the amplified DNA fragments 88 b.p. and 123 b.p, respectively; the fragments were in line with the expected sizes. The analytical specificity test was performed by amplifying the control DNA of 15 species of the order Chlamydiales.

KEY WORDS: Parachlamydia acanthamoebae, Waddlia chondrophila, multiplex, PCR

INTRODUCTION
Chlamydiae are gram-negative obligate intracellular bacteria sharing a unique biphasic developmental cycle and replicate exclusively within the interior of living cells in eukaryotic hosts. The members of the Chlamydiales order cause diseases of humans, mammals, birds and reptiles. Chlamydiosis in animals can cause asymptomatic infection or may result in pneumonia, abortion, rhinitis, conjunctivitis, arthritis, infertility, enteritis, and more. Humans have their own endemic chlamydial species [1].

Chlamydia trachomatis is human pathogen, the most prominent representative of Chlamydiales order, which is the most common cause of sexually transmitted diseases worldwide [2]. Chlamydia abortus, Chlamydia caviae, Chlamydia felis, Chlamydia psittaci, Chlamydia suis, Chlamydia gallinaca cause zoonotic disease. [3]. Due the development of molecular biology, the number of representatives of the order Chlamydiales has increased. In addition to expanding the Chlamydiaeae family, a number of Chlamydia-related bacteria (CRBs) are identified, some of which are associated with animal and human diseases.

The most known and currently the best described representatives of Chlamydia-related bacteria that carry a potential zoonotic threat are Waddlia chondrophila and Parachlamydia acanthamoebae. These bacteria are associated with miscarriage, ectopic pregnancy, diseases of the respiratory system in humans and animals [4, 5].

W. chondrophila was first isolated in 1986 from an aborted bovine fetus in the United States [6]. Subsequently, the association between antibodies against Waddlia chondrophila and bovine abortions was confirmed. Moreover, 1 out of 2 calves, which were experimentally infected with W. chondrophila, died within 2 weeks [7].

After that, a series of studies performed on humans the connection between the miscarriage and the presence of Waddlia antibodies in humans was observed. The pathogenic role of W. chondrophila in humans is confirmed by the strong association between seropositivity of W. chondrophila and human miscarriage. [4,8,9]. It was also proven that W. chondrophila has a negative effect on human spermatozoa [10], which suggests that W. chondrophila plays an important role in reducing the reproductive capacity of animals and humans. In addition, W. chondrophila is found in the respiratory samples of people with bronchiolitis or pneumonia [3].

For the first time, Parachlamydia acanthamoebae was isolated by Rolf Michel and Barbel Hauroder-Philippczyk in Berlin in 1994 during examining nose smears [11]. P. acanthamoebae is associated with respiratory diseases and miscarriages in ruminants. [12–14]. In humans, Paracanthamoebae is mainly associated with diseases of the respiratory system, bronchiolitis, pneumonia [15,16], also this bacterium is associated with atherosclerosis [17]
Parachlamydia acanthamoebae is widespread in nature and due to the symbiosis with Acanthamoeba, it has the ability to withstand a wide range of environmental stresses [18]. Moreover, recent studies of arthropods (mites) have shown the presence of Parachlamydiaceae DNA in mites, which, like birds, bats and mice, are considered to be the so-called “vectors” of chlamydia infection [19–22].

To the foregoing it should be added that Waddlia chondrophila and Parachlamydia acanthamoebae have been detected in drinking water [23,24], which suggests that water can be a source of infection for animals and humans.

Despite the importance of these chlamydia-like organisms for human and veterinary medicine in Ukraine, studies on their prevalence among animals and humans have not been conducted due to the lack of available tests.

THE AIM
The aim of our work was to create relatively cheap and easy method to detect Waddlia chondrophila and Parachlamydia acanthamoebae, which will be used for widescreen monitoring in current challenging economic situation in Ukraine.

MATERIALS AND METHODS
GenBank database was used to find nucleotide sequences of the 16S rRNA of the order Chlamydiales. Alignment of 111 nucleotide sequences of 36 bacteria was performed using the MEGA7 software, in order to detect the presence of polymorphic hybridization sites specifically attributed to Waddlia chondrophila and Parachlamydia acanthamoebae. Primer-BLAST software was used to design oligonucleotide primers, to evaluate the critical parameters of the primer pairs, the GC content, the self-complementarity, etc. The verification of the specificity of polymorphic fragments determined for each type of chlamydia with the nucleotide sequences of microorganisms, both opportunistic and infectious agents, was implemented using the online service Primer-BLAST. [25, 26, 27, 28].

The studies were carried out in the laboratory of animal health and the laboratory of genetics of Institute of Pig Breeding and Agro-Industrial Production, NAAS, which is certified for genetic analysis at the DNA level (Compliance certificate “state of the measurement system” number 021-19 from 01/31/2019).

DNA amplification was performed using Thermo Fisher Scientific reagents according to the manufacturer’s protocol. Oligonucleotide primers for the identification of Waddlia chondrophila and Parachlamydia acanthamoebae were synthesized by Metabion international AG, Germany.

The resulting primers were diluted with sterile deionized water. Following reagents were used in PCR tests: 100 ng of DNA template, primer mix, deionized water, 10x(NH4)2SO PCR buffer, 25mmMgCl2, deoxyribonucleoside triphosphate solution (2mmNTP) and Taq polymerase (Thermo Fisher Scientific) according to the manufacturer’s specifications. PCR amplification was performed in a final volume of 25 μL.

DNA amplification using PCR was performed on a “Tercyc-2” multichannel thermocycler (DNA technology, Russia). The cycling conditions to amplify consisted of an initial denaturation at 94°C for 5 min, 30 cycles of melting at 94°C for 30 s, annealing at 63°C for 30 s, and elongation at 72°C for 1 min and a final extension at 72°C for 5 min.

PCR products were separated using 2% agarose gel electrophoresis in 1 × TBE buffer for 2 hours at a current of 50 mA in an electrophoresis chamber (Cleaver Scientific Ltd). Plasmids pUC19 hydrolyzed with Msp I endonuclease (Thermo Fisher Scientific) were used as a molecular weight marker. After the end of the electrophoresis process, the gel was stained with a solution of ethidium bromide (10 mg/cm3) and results of electrophoresis were captured using gel documentation system (Cleaver Scientific Ltd. UK).

Control DNA samples: Parachlamydia acanthamoebae strains “Berg17” and “Bn9” were kindly provided by Dr. Michel Rolf (Central Military Hospital Koblenz, Germany), DNA of Parachlamydia acanthamoebae, strain Hall obtained from Prof. Gilbert Greub (l’Institut de Microbiologie Médecin chef des laboratoires de microbiologie diagnostique Institut de microbiologie de l’Université de Lausanne, Switzerland), DNA samples of Waddlia chondrophila, Chlamydia avium, Chlamydia pecorum, Chlamydia abortus, Chlamydia psittaci, Chlamydia suis, Chlamydia caviae, Chlamydia trachomatis, Chlamydia abortus, Chlamydia felis, Chlamydia muridarum, Chlamydia pneumoniae, Chlamydia gallinarum, were obtained from Dr. Christiane Schnee (Institut für molekulare Pathogenese, Jena, Germany). Clavochlamydia salmonicola and Piscichlamydia salmonis samples were obtained from Dr. Heike Schmidt-Posthaus, (Center for Fish and Wildlife Health, Bern). Control DNA samples were used to test and verify the analytical specificity of the developed PCR tests.

RESULTS
Amplification of control DNAs of Parachlamydia acanthamoebae and Waddlia chondrophila with corresponding

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Product size</th>
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<tbody>
<tr>
<td>Waddlia chondrophila</td>
<td>WADCHOFGAAGCGATGTGCCTTTGAGT</td>
<td>123 b.p.</td>
</tr>
<tr>
<td></td>
<td>WADCHORCCTCTACGACCATATCCGG</td>
<td></td>
</tr>
<tr>
<td>Parachlamydia acanthamoebae</td>
<td>PCHARFCAAGTAGCCTATCGGAGAAGAT</td>
<td>88 b.p.</td>
</tr>
<tr>
<td></td>
<td>PCHARGCTTGGCCACACCTCGGAAGAT</td>
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Table 1. Primers used for detection Waddlia chondrophila and Parachlamydia acanthamoebae
MULTIPLEX PCR ASSAY FOR CHLAMYDIA-LIKE BACTERIA DETECTION

primers using PCR and the following gel electrophoresis of PCR products determined the size of the amplified DNA fragments as 88 base pairs and 123 base pairs (b.p.) respectively, the fragments corresponded to the expected sizes of the DNA fragments of 16S rRNA gene \textit{Parachlamydia acanthamoebae} and \textit{Waddlia chondrophila} (Fig. 1.)

At the next stage, the primers for identification of \textit{Waddlia chondrophila} and \textit{Parachlamydia acanthamoebae} were mixed and used in multiplex PCR with the DNA of \textit{Waddlia chondrophila} and \textit{Parachlamydia acanthamoebae}, after which the electropherogram showed products with fragment sizes of 123 b.p. and 88 b.p. PCR was performed as a duplex to show the different sizes of the amplicon genes of the two organisms and the possibility of using them in a multiplex combination. (Fig. 1) The analytical specificity test was performed by amplifying the control DNA of 15 species of the order Chlamydiales with a primer-mix for the identification of \textit{Waddlia chondrophila} and \textit{Parachlamydia acanthamoebae}. The test for analytical specificity showed the absence of PCR products. (Fig. 1)

**DISCUSSION**

During the development of PCR test for \textit{Waddlia chondrophila} and \textit{Parachlamydia acanthamoebae} detection, conservative 16S rRNA genes were selected as target genes for which the corresponding primers were selected. The key step in primers development was to find the most variable regions within each of the 16S rRNA gene sequences and use these regions as species-specific. \textit{Waddlia chondrophila} and \textit{Parachlamydia acanthamoebae} are \textit{Chlamydia}-like bacteria that are associated with diseases of animals and humans. These bacteria are isolated from animals and humans, so they can be considered as a potential zoonotic threat [4,8–10,12–15].

Due to the difficult economic situation in Ukraine deterioration of living conditions and food occurs. Healthcare reform, which, on the one hand, destroyed the Soviet health care system, on the other hand, did not come to an effective completion has led to the unsupervised use of antimicrobials, the lack of proper epidemiological surveillance along with shortness of veterinary and sanitary control over the quality of livestock products. Armed conflict ("hybrid war"), which is being fought in eastern Ukraine and mass impoverishment, generates a high level of migration and population concentration [29,30,31]. All of the above are driving factors for the occurrence of infectious diseases in general. Therefore, there is a need for simple, affordable tests that do not need expensive equipment. Higher sensitivity of PCR tests is their main advantage compared to culture technique and other methods which are too time-consuming, difficult to perform and sometimes inaccurate. PCR diagnostics of chlamydial infections available for clinical use is the best and recommended method for detecting chlamydial infections. [32]

The 16S RNA gene was chosen as the molecular target for the differentiation of \textit{Waddlia chondrophila} and \textit{Parachlamydia acanthamoebae}, since this conservative gene is the most universal among bacteria on the one hand, and on the other has hypervariable regions, which allows to choose a species-specific region for each species[33]. \textit{Waddlia chondrophila} and \textit{Parachlamydia acanthamoebae} primers were designed with the same physical characteristics to ensure simultaneous amplification under the same conditions in single or multiplex PCR. To be able to
visually assess the amplification product, the primer pairs differ in the length of the amplicons, the gap of the target fragment is 35 bp, which meet the resolution requirements for electrophoresis in a 2% agarose gel. To prevent the appearance of non-specific products, the melting point of the primers was 60 °C, and their length was 20–21 base pairs. In the process of optimizing PCR tests using temperatures from 55 °C to 63 °C, the optimum temperature was 60 °C with standard concentrations of PCR components. Despite the fact that there are foreign-made commercial PCR tests to identify these bacteria, in the current economic situation in Ukraine, the widespread use of these diagnostic kits appeared to be almost impossible, due to the high price, or the need for expensive equipment (for real-time PCR tests) [34,35].

CONCLUSIONS
An affordable and relatively simple instrumentality to identify Waddlia chondrophila and Parachlamydia acanthamoebae was created, which, after testing on clinical material, can be used for extensive monitoring.

REFERENCES


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Authors’ contributions:
According to the order of the Authorship.

Conflict of interest:
The Authors declare no conflict of interest.

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