DOI: 10.29326/2304-196X-2023-12-3-222-227

Role of CFT and PCR in diagnosis of *Chlamydia psittaci* in experimentally infected rabbits

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SUMMARY

Specific antibodies against chlamydia were detected using complement fixation test and chlamydia genome was detected using polymerase chain reaction in pregnant rabbits experimentally infected with *Chlamydia psittaci*. The infected rabbits developed a fever and respiratory signs and the infection was confirmed by specific antibodies against chlamydia detected in their blood and by abnormalities in rabbit kindling. Complement fixation test of paired rabbit sera revealed an increase in the titers of specific antibodies against chlamydia, which on Day 7 post infection varied within 1:7.5; on Day 14, mean concentration was 1:40 and by Day 30 mean titer increased to 1:60. However, when pathological materials from the urogenital tract of the experimental animals were tested in polymerase chain reaction and in smear microscopy, it was impossible to confirm that there is an etiological link between chlamydia and kindling problems in experimental animals. At the same time, molecular and genetic tests of internal organs (liver) sampled from stillborn baby rabbits revealed the chlamydia genome, thus, proving chlamydia involvement into the pathological kindling. Therefore, such a retrospective method as complement fixation test with a chlamydia antigen is of high diagnostic value for lifetime chlamydia diagnosis.

Keywords: chlamydiosis, *Chlamydia psittaci*, polymerase chain reaction, rabbits, serological tests

For citation: Evstifeev V. V., Khusainov F. M., Yakovlev S. I., Shangaraev R. I., Eremets V. I. Role of CFT and PCR in diagnosis of *Chlamydia psittaci* in experimentally infected rabbits. *Veterinary Science Today*. 2023; 12 (3): 222–227. DOI: 10.29326/2304-196X-2023-12-3-222-227.

Conflict of interest: The authors declare no conflict of financial/non-financial interests associated with the article.

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УДК 619:616.98:579.882.11:636.92:616-076:577.2:616-078

Значение РСК и ПЦР в диагностике экспериментальной инфекции кроликов, вызванной *Chlamydia psittaci*

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РЕЗЮМЕ

Проведена индикация специфических хламидийных антител в реакции связывания комплемента и генома хламидий методом полимеразной цепной реакции у сукрольных крольчих при экспериментальной инфекции, вызванной *Chlamydia psittaci*. Развитие инфекционного процесса у зараженных кроликов сопровождалось повышением температуры тела и появлением респираторных симптомов и было подтверждено наличием в их крови специфических хламидийных антител и патологическими родами. При исследовании парных сывороток крови кроликов в реакции связывания комплемента выявили нарастание титров специфических хламидийных антител, которые на седьмые сутки после заражения варьировались в пределах 1:7,5; на четырнадцатые сутки средняя их концентрация была равна 1:40, и к тридцатым суткам средний титр увеличился до 1:60. Однако подтвердить хламидийную этиологию неблагополучных исходов окрола экспериментально зараженных кроликов при исследовании проб патологических материалов, полученных из урогенитального тракта исследуемых животных, методом полимеразной цепной реакции и микроскопией мазков-отпечатков не удалось. При этом молекулярно-генетические исследования проб внутренних органов (печень) мертворожденных крольчат позволили выявить геном хламидий, в результате чего была подтверждена хламидийная этиология патологического исхода окрола кроликов. Следовательно, при прижизненной постановке диагноза на хламидиоз такой ретроспективный метод, как реакция связывания комплемента с хламидийным антигеном, имеет диагностическую ценность.

Ключевые слова: хламидиоз, *Chlamydia psittaci*, полимеразная цепная реакция, кролики, серологические исследования

Для цитирования: Евстифеев В. В., Хусаинов Ф. М., Яковлев С. И., Шангараев Р. И., Еремец В. И. Значение РСК и ПЦР в диагностике экспериментальной инфекции кроликов, вызванной *Chlamydia psittaci*. *Ветеринария сегодня*. 2023; 12 (3): 222–227. DOI: 10.29326/2304-196X-2023-12-3-222-227.

Конфликт интересов: Авторы заявляют об отсутствии конфликта финансовых/нефинансовых интересов, связанных с написанием статьи.

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INTRODUCTION

Advances of molecular biology reported over the past decades have resulted in creation of new postgenomic technologies that facilitate development of methods indicating pathogen genome. These methods significantly improve laboratory diagnosis of infectious diseases, including those of animals. Polymerase chain reaction (PCR) has become one of such mainstream methods. Its high sensitivity and specificity, which, according to some authors, reach 99.9% [1–3] and surpasses all the other existing methods, together with the availability of test equipment, have contributed to a wide use of PCR for diagnosis of infectious diseases [4–7].

In the last decade of the twentieth century, genomes of almost all significant pathogens of infectious animal diseases were sequenced. Availability of nucleotide sequence databases and improvement of bioinformatic methods, along with advances in genetic engineering and artificial protein synthesis, which made it possible to obtain a polypeptide chain from amino acids, made synthesis of specific primers a routine operation.

Owing to high specificity and sensitivity of the methods based on *in vitro* nucleotide amplification, detection of the pathogen genome now plays a key role in diagnosis, pushing aside all other diagnostic methods, including retrospective diagnosis.

Wide use and availability of the method changed diagnostic algorithms, where polymerase chain reaction became the ultimate test requiring no extra confirmation and serological methods got focused only on preliminary diagnosis and monitoring with mandatory confirmation by one of direct methods, where chlamydia antigen or genome are detected in PCR[1].

However, researchers, medical and veterinary doctors, as well as laboratory workers engaged in diagnosis of infectious diseases, are familiar with such situations when diagnostic titers of specific antibodies are often detected in blood of clinically ill animals by retrospective methods, more often in complement fixation test (CFT) or enzyme-linked immunosorbent assay (ELISA) [8–11]. At the same time, titres in paired sera increased by a factor of 2 or more, which was indicative of an active infectious process, but not of a disease in the past, and PCR gave a negative result [12–15].

Thus, chlamydiosis was not confirmed, if diagnosis was based on "Methodical guidelines on laboratory diagnosis of chlamydia infections in animals", where retrospective methods are used only for preliminary diagnosis. Due to negative PCR results, many commercial breeding farms took no chlamydia eradication and prevention measures, thus leading to uncontrolled spread of chlamydia infection [16–19]. This also happened because there are simply no other diagnostic methods at disposal of veterinary laboratories, other than PCR, which detect chlamydia, antigens or DNA of chlamydia in the tested material, as prescribed in the guidelines.

Chapter 3.3.1 of the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals[2] clearly recommends to use PCR and ELISA-based methods (for antigen detection) to identify a chlamydia agent. However, there are no diagnostic ELISA kits in the Russian Federation to detect it.

Therefore, PCR has become the only method used in the veterinary laboratories to diagnose chlamydia in agricultural animals. Accordingly, the aim of this research was to determine the role of CFT and PCR in diagnosis of *Chlamydia psittaci* in experimentally infected rabbits.

MATERIALS AND METHODS

For infection we used *C. psittaci* strain "250" deposited in the FSBSI "FCTRBS-ARRVI" collection of microorganisms, isolated from an aborted bovine fetus in the Kuybyshev Oblast in 1973 and cultured in developing chicken embryonated eggs with an infectious titer of $10^{-6.5}$ LD_{50}/0.3 cm^3 [20].

Five pregnant does were used for the experiment.

Sterility (non-contamination) of the pathological material for re-isolation was determined in the nutrient media: meat-peptone broth (MPB), meat-peptone agar (MPA), meat-peptone liver broth (Kitt – Tarozzi medium), Sabouraud medium.

Concentration of chlamydia-specific antibodies in blood of experimentally infected rabbits was determined with the help of CFT, using an "Antigen and sera kit for serological diagnosis of chlamydia in farm animals" (FSBSI "FCTRBS-ARRVI", Russia).

Initially, to exclude spontaneous chlamydia infection in rabbits, biomaterial samples were tested in CFT with

[1] Methodical guidelines for laboratory diagnosis of chlamydia infections in animals: approved by the Department of Veterinary Medicine on 30.06.1999 No. 13-7-2/643. Available at: https://files.stroyinf.ru/Data2/1/4293757/4293757190.htm.

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a chlamydia antigen and in PCR with a specific primer. At the next stage of the experiment, does mated with bucks. On Day 3–7 post mating, 4 does were infected with virulent chlamydia culture. Infectious material, i.e. 10% purified suspension of elementary chlamydia bodies was administered intraperitoneally in a volume of 1.0 cm^3. One rabbit served as a control.

On Day 23–30 post infection, the effect of kindling was assessed. The kits from the does were visually examined, height and weight measured and diagnostic tests for chlamydia made to confirm the etiological link with the pathogen in case of reproductive disorders in the infected animals.

Microscopic tests included sampling, preparation of smears, carbol-fuchsin staining (Stamp's method) and micro-slide examination under the immersion system of a light microscope at a magnification of 100×.

The does were sampled in accordance with the PCR sampling rules. Urogenital probes were used to sample vaginal mucosa of the infected animals, after that the samples were put into sterile tubes containing transport medium. Samples for microscopy were also taken with urogenital probes and applied onto slides to prepare smears.

All tests in animals were carried out in strict compliance with intergovernmental standards on laboratory animal keeping and handling adopted by the Intergovernmental Council for Standardization, Metrology and Certification as well as in accordance with Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

PCR test was used for molecular and genetic purposes. Chlamydia DNA was detected in samples from the infected animals using PCR-based "CHLA-KOM" test kit for chlamydia detection in animals and birds (FBIS "Central Research Institute of Epidemiology" of Rospotrebnadzor, Russia), developed to detect DNA of *Chlamydiaceae* family microorganisms in biological material.

DNA was extracted from the tested material using a set of reagents "DNA-sorb-B" (FBIS "Central Research Institute of Epidemiology" of Rospotrebnadzor, Russia). Amplification products were detected in agarose gel electrophoresis using the "EF" kit for preparation of agarose gel (FBIS "Central Research Institute of Epidemiology" of Rospotrebnadzor, Russia). The results were read judging by the presence or absence of a specific band of amplified DNA in the electrophoregram.

Chlamydia was isolated from pathological and clinical material taken from developing 6-day-old chicken embryoned eggs. For this purpose, 20% suspensions were prepared from the liver of stillborn rabbits (2 fetuses from each doe), which were subsequently administered into the yolk sac of developing chicken embryonated eggs. Embryos that died earlier than Day 4 post infection were culled (as nonspecific). Yolk membranes were sampled from the embryos that died between Day 4 and Day 14 post infection, smears were prepared from them, stained using modified Stamp's method and examined under an immersion light microscope in order to identify elementary chlamydia bodies in the form of pink dots on a green mounting media.

RESULTS AND DISCUSSION

The experiment was conducted in the Department of Virology of the FSBSI "FCTRBS-ARRVI".

At the first stage, general health status of the experimental animals was assessed.

Observation of animals before insemination and on Days 3–7 after insemination, as well as before infection revealed that the rabbit health parameters were within the physiological norm, as confirmed by the body temperature (38.5–39.5 °C) and animal behavior.

No specific antibodies were detected in sera samples tested in CFT with a chlamydia antigen. No chlamydia genome was detected in PCR-tested clinical material from these animals.

Prior to the experiment, 4 bucks were clinically examined; then used for mating with experimental does and subjected to similar laboratory tests for spontaneous chlamydia infection. The results were also negative.

As thermometry showed, infection with a virulent chlamydia strain caused a short increase in body temperature in all 4 experimental rabbits, which began on Day 2 after infection and lasted until Day 6, when the average body temperature in the group was equal to 39.62 °C. In the following days, the temperature began to stabilize. In addition, the infection affected the overall health status of the rabbits. Starting from Day 3, the animals lose appetite, look lethargic and unkempt. On Day 5, three infected rabbits (Nos. 1, 2 and 3) developed a cough.

Assessment of kindling post infection with virulent chlamydia is given in Table 1.

Table 1
Effect of experimental infection with *C. psittaci* "250" on pregnancy in rabbits

Animal number	Group	Effect on pregnancy	Kits born in total	Kits died		Kits survived	
				number	%	number	%
1	Experimental	s/b	6	6	100	0	0
2		s/b	5	5	100	0	0
3		kindling, s/b	6	2	33	4	67
4		s/b	5	5	100	0	0
Total for the group			22	18	82	4	18
5	Control	kindling	6	0	0	6	100

"s/b" – stillbirth.

Stillbirth cases were reported in three infected animals. Only four out of six kits from Doe No. 3 survived. The survival rate in the group was 18%. The control doe gave birth to 6 healthy kits.

The next stage was to study humoral immunity dynamics in rabbits. The results of serological tests of sera from experimental and control animals are given in Table 2.

It was found that, prior to infection no antibodies to chlamydia antigen were detected in blood of all rabbits. Consequently, they lacked specific anti-chlamydial immunity, which was important for purity of the experiment. On Day 7 post infection, complement-fixing antibodies were detected in sera from experimental rabbits; mean titer was 1:7.5. In the future, there was an increase in their concentration. Therefore, on Day 14, mean titer was equal to 1:40 and by Day 30, it increased to 1:60. An increase in the antibody level in the paired serum samples from the infected rabbits suggests development of an infectious process of chlamydial etiology. No complement-fixing antibodies were detected in blood of the control animals during the whole experiment.

Mucus scrapes from vagina of infected animals were taken together with the samples of internal organs from the stillborn and normal kits (liver) for microscopic, molecular and genetic analyses aimed at detection of chlamydia elementary bodies and DNA on Day 30 post infection, after kindling in all does.

Table 3 summarizes data on fetuses and clinical materials tested after kindling, as well as on chlamydia re-isolation from pathological materials.

Microscopic tests showed no chlamydia in vaginal smears of the infected does. Microscopic tests showed elementary bodies of chlamydia in smears prepared from the internal organs of stillborn kits. PCR revealed no DNA chlamydia in samples of vaginal mucosa. PCR revealed *C. psittaci* genetic material in liver samples from stillborn kits. Similar tests of the pathological material taken from the control rabbit gave negative results.

To identify the pathogen and confirm chlamydial etiology of the abnormal kindling observed in does, the pathogen was re-isolated from samples of internal organs (liver) of the stillborn fetuses with positive PCR results in developing 6-day-old chicken embryonated eggs, followed by a smear microscopy to detect elementary bodies of chlamydia. Chlamydia were already isolated in the first passage. Specific embryonic death was reported on Days 4–8 after infection with a 20% suspension of pathological materials. In addition to confirming the chlamydial etiology of stillbirths in rabbits, it also suggested a high virulence of chlamydia isolates re-isolated from rabbits. Administration of pathological materials from the liver of control rabbit fetuses into the yolk sac of developing chicken embryonated eggs showed a negative result.

Analyzing the data obtained during the experiment, it can be concluded that the use of PCR alone gives an incomplete picture for the chlamydia lifetime diagnosis. It is advisable to use retrospective methods, such as CFT with a chlamydia antigen, which makes it possible to identify chlamydia specific antibodies. An increase in the antibody titre in the animal blood may suggest an active infectious process and may be used as a basis for a diagnosis.

Table 2
Level of complement-fixing antibodies in blood of infected rabbits

Animal number	Group	Chlamydia antibody titer			
		before infection	Day 7	Day 14	Day 30
1	Experimental	–	1:10	1:20	1:40
2		–	1:5	1:20	1:40
3		–	1:10	1:80	1:80
4		–	1:5	1:40	1:80
Mean titre in group		–	1:7.5	1:40	1:60
5	Control	–	–	–	–

Table 3
Re-isolation of *C. psittaci* "250" from clinical and pathological material from infected rabbits after kindling

Animal number	Group	Test material	Microscopy results	Re-isolation of chlamydia in chicken embryonated eggs	PCR results
1	Experimental	mucus scrape from vagina	–	not tested	–
		fetal liver	+	+	+
2		mucus scrape from vagina	–	not tested	–
		fetal liver	+	+	+
3		mucus scrape from vagina	–	not tested	–
		fetal liver	+	+	+
4		mucus scrape from vagina	–	not tested	–
		fetal liver	+	+	+
5	Control	mucus scrape from vagina	–	not tested	–
		fetal liver	–	–	–

"–" – negative test results;
"+" – positive test results.

CONCLUSION

The conducted experiments showed that infection of pregnant does with virulent *Chlamydia psittaci* strain "250" resulted in an active infectious process, characterized by a fever, respiratory signs and deterioration in the general condition of the tested animals. In addition, all experimental does had abnormal kindling. Only 4 out of 22 kits survived. The offspring survival rate was 18%. Only serological tests (CFT) made it possible to confirm chlamydial etiology of the pathological process in the infected animals. Imprint smear microscopy and PCR of urogenital tract scrapes from the infected animals gave negative results. However, the chlamydial etiology of abnormal kindling was confirmed by PCR, used to test internal organs from fetuses of the infected animals and by chlamydia re-isolation in 6-day-old chicken embryonated eggs.

Thus, the use of PCR alone does not give a complete picture for chlamydia lifetime diagnosis in animals. Consequently, it is advisable to use not only molecular, but also retrospective methods, such as CFT with a chlamydia antigen, which helps to identify specific chlamydia antibodies, and the growing antibody titre can serve as a basis for the diagnosis.

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Received 14.03.2023
Revised 12.05.2023
Accepted 15.06.2023

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