Таблица
Исследования патологического материала из свиноводческих хозяйств РФ на эперитрозооноз

Год	Количество хозяйств, предоставивших пробы	Количество исследованных проб/из них положительно
2004	1	3/0
2005	2	2/0
2008	1	2/0
2009	3	4/0
2010	5	61/7
2011	3	33/1
2012	1	2/0
2013	3	12/0

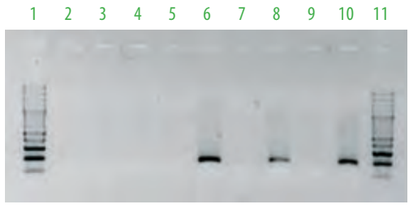

Рис. 3. Результаты исследования проб патологического материала на наличие Eperythrozoon suis методом ПЦР
1, 11 — маркер ДНК;
2 — отрицательный контроль;
3 — легкие от ремонтных свинок;
4 — аборт. плоды,
5 — плацента;
6 — кровь от ремонтных свинок;
7 — легкие от поросят-отъемышей;
8 — кровь от свиноматок;
9 — кожа;
10 — положительный контроль.

Эперитрозооноз был диагностирован только в двух хозяйствах (в одном хозяйстве из Республики Башкортостан и в одном хозяйстве из Республики Татарстан): ДНК *E. suis* была выявлена в крови у ремонтных свинок с характерными признаками анемии и абортировавших свиноматок. В образцах крови от новорожденных поросят, поросят-сосунов и поросят группы отъема с типичными признаками острой формы эперитрозооноза (бледность кожных покровов, цианоз и некроз ушных раковин) возбудитель обнаружен не был (рис. 3).

Обследуемые хозяйства из Башкирии и Татарстана обнаружили новый для себя вид патологии в 2010 г. Этот период совпал по времени с вводом в ремонтное стадо животных из-за рубежа. У свиней наблюдали массовые проявления анемии и аборты.

В заключение можно сказать, что эперитрозооноз — новое для российского свиноводства заболевание, и ситуация с его распространением пока мало изучена. Разработанный метод ПЦР является эффективным средством лабораторной диагностики эперитрозооноза.

ВЫВОДЫ

1. Разработан метод обнаружения *Eperythrozoon suis*, основанный на ПЦР с электрофоретической детекцией.

2. С использованием разработанного метода в 2004–2013 гг. проведены исследования по выявлению *Eperythrozoon suis*. Возбудитель обнаруживается в пробах цельной крови, отобранных у ремонтных свинок и свиноматок.

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DEVELOPMENT OF POLIMERAZE CHAIN REACTION BASED TEST SYSTEM FOR *EPERYTHROZOON SUIS* DETECTION

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SUMMARY
A PCR and electrophoresis based tool was developed for *Eperythrozoon suis* detection. During 2004–2013, tests for *Eperythrozoon suis* detection were performed using the developed tool. The agent was detected in whole blood samples collected from replacement gilts and sows.

Key words: *Eperythrozoon suis,* PCR, eperythrozoonosis.

INTRODUCTION

Hematrophic mycoplasmas are an individual cluster of closely related bacteria within *Mycoplasma* genus. Residing on the surface of red blood cells the bacteria damage cellular membranes thus leading to the RBC deformation and destruction, anemia and damage of blood vessel epithelium [5, 7].

As for hematrophic mycoplasmas affecting animals, study relevant is *Eperythrozoon suis* sp. which is an etiologic agent of porcine eperythrozoonosis [3]. Pigs of all ages are susceptible to the agent but the most disease predisposed are fattening pigs in which *Eperythrozoon suis* (*E. suis*) causes short-term hyperthermia, skin pallor and singular cases of jaundice [3, 5]. Clinical signs are highly variable in animals with chronic disease: weaning pigs demonstrate anemia, jaundice, apathy, anorexia; fattening pigs demonstrate stunting and retardation; reproductive function is decreased in sows [3, 4, 5, 7].

The infection caused by *E. suis* is widely spread abroad on the farms practicing intensive pig farming. Animal health monitoring in China demonstrated that 86% of pigs are affected [6]. Prevalence of infected herds in Japan amounts to 7,5% [8]. In Germany 40,3% of tested herds were *E. suis* positive [7]. The agent in present in over 33% of pig herds in Brazil [2]. In addition, the disease is reported in England, Czech Republic, France and Canada.

Economic damage caused by eperythrozoonosis includes reduction of body weight gain and development of secondary infections due to immune deficiency [7].

Such methods as conventional PCR, real time PCR, ELISA, complement fixation test, indirect hemagglutination test are used abroad for laboratory diagnosis of the disease. Histopathological and hematological examinations [1, 3, 4, 5, 6] are also used.

Until recently there has been lack of data on eperythrozoonosis spread in the Russian Federation and this fact has been to some extent dependent on the lack of up-to-date domestic diagnostica.

The work was aimed at development of a tool for *E. suis* detection in pathological material from pigs and the tool application in the laboratory diagnosis.

MATRIALS AND METHODS

Pathological material. Fresh or frozen pieces of internal organs and whole blood from pigs demonstrating signs of anemia, fever and jaundice as well as aborted fetuses from sows with reproductive pathology were used for diagnostic tests.

Bacteria. In order to check the test specificity the following bacteria were used: *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Mycoplasma hyosynoviae, Mycoplasma*

hyopharingis, Mycoplasma flocculare, Mycoplasma bovis, Mycoplasma ovipneumoniae, Mannheimia haemolytica, Pasteurella multocida, Streptococcus suis.

Nucleotide sequences: *E. suis* nucleotide sequences deposited in GenBank were used. Nucleotide sequences were analyzed using BioEdit software application.

DNA extraction from 10% suspensions of pathological material samples was performed using 6M *guanidinium isothiocyanate* and glass fiber filters GF/F.

Polymerase chain reaction. PCR mix included 5 µl of 10× Taq polymerase buffer, 3 mM Mg^{2+}, 0.2 mM dNTPs, 2 units of Taq DNA Polymerase, primers (5 pmol each), 5 µl of DNA solution and necessary amount of water to have the final mix volume of 50 µl. PCR was performed on DNA amplifier Mastercycler (Eppendorf, Germany). The program included 35 cycles at the following temperature range: 30 sec denaturation at 94°C; 30 sec annealing of primers at 55°C and 40 sec elongation at 72°C. Reaction products were analyzed using electrophoresis in 2,0% agarose gel containing 0,001% of ethidium bromide at 50 mA.

RESULTS AND DISCUSSION

E. suis genome includes both regions common for different mycoplasma species and species-specific sites. A gene distinguished for its high conservation within species but foreign for other mycoplasma species was selected as PCR target.

Comparative analysis of *E. suis* nucleotide sequences deposited in GenBank was performed for primer calculation. Basing on the results of the comparative analysis of sequences a pair of primers was calculated which were complimentary to genome sites specific for given bacteria species. The primers flank a 180 b.p. site.

The reaction procedure is as follows. Total DNA is extracted from the test sample and it is subsequently used in amplification reaction. PCR products are analyzed in agarose gel. 180 b.p. fragment is indicative of *E. suis* presence in the sample.

During PCR optimization the reaction mix composition and temperature as well as time mode providing for maximum efficiency of *E. suis* DNA detection were determined. It was demonstrated that optimal PCR conditions are the following: primer quantity – 5 µl of each primer per a reaction; $MgCl_2$ concentration – 3 mM; primer annealing at 55°C.

Specificity of the developed method was tested using materials containing bacterial pathogens including mycoplasmas: *Eperythrozoon suis, Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Mycoplasma hyosynoviae, Mycoplasma hyopharingis, Mycoplasma flocculare, Mycoplasma bovis, Mycoplasma ovipneumoniae, Mannheimia haemolytica, Pasteurella multocida, Streptococcus suis*.

PCR with a pair of species-specific primers was performed for each sample. Synthesis of amplicons of calculated length was reported only in the sample containing *E. suis* DNA (Fig. 1). Samples containing other infectious agents demonstrated no synthesis of any DNA fragments. Thus, cross-reactivity with heterologous mycoplasma species and other bacteria was excluded.

PCR sensitivity was tested on a series of 10-fold dilutions of *E. suis* DNA. All samples were tested using PCR and the results were detected in agarose gel. Fig. 2 shows the test results.

PCR allowed for *E. suis* detection in 10^3-fold diluted materials that is comparable with sensitivity of previously proposed conventional PCR [5].

As long carrier state of convalescent and clinically healthy animals is typical in case of eperythrozoonosis [7] the developed method ensures effective control of the disease by detecting *E. suis* DNA even in case of chronic infection.

The developed tool was used for diagnosis from 2004 to 2013 (table) in 199 samples of pathological materials collected in 9 RF pig farms. *E. suis* was detected in 8 samples (6,7%).

All positives were detected during testing whole blood supplemented with anticoagulant thus indicating that correct sampling is a pre-requisite for credible laboratory diagnosis of the disease. Other materials were apparently unsuitable for tests.

Fig. 1. Specificity test results of the method used for Eperythrozoon suis DNA detection
1, 13 – DNA marker;
2 – a sample containing Eperythrozoon suis;
3 – a sample containing Mycoplasma hyopneumoniae;
4 – a sample containing Mycoplasma hyorhinis;
5 – a sample containing Mycoplasma hyosynoviae;
6 – a sample containing Mycoplasma hyopharingis;
7 – a sample containing Mycoplasma flocculare;
8 – a sample containing Mycoplasma bovis;
9 – a sample containing Mycoplasma ovipneumoniae;
10 – a sample containing Mannheimia haemolytica;
11 – a sample containing Pasteurella multocida;
12 – a sample containing Streptococcus suis.

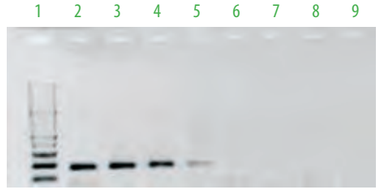

Fig. 2. Sensitivity test results of the method used for Eperythrozoon suis DNA detection
1 – DNA marker;
2–8 – 10-fold dilutions of Eperythrozoon suis DNA from initial to 1×10^{-6};
9 – negative control.

Eperythrozoonosis was diagnosed only on two farms (one farm in the Republic of Bashkortostan and one farm in the Republic of Tatarstan): *E suis* DNA was detected in blood of replacement pigs demonstrating typical signs of anemia and in aborting sows. No agent was detected in blood samples collected from newborn piglets, suckling pigs and weaning pigs demonstrating typical signs of acute eperythrozoonosis (skin pallor, cyanosis and necrosis of ear pavilions) (Fig. 3). On the tested farms of Bashkiria and Tatarstan the novel type of pathology was detected in 2010. This period was synchronous with introduction of animals imported from abroad as the replacement stock. Pigs demonstrated mass anemia and abortions.

In conclusion is may be said that eperythrozoonosis is a novel disease for the RF pig industry and its spread has been underinvestigated yet. The developed PCR tool is an effective method for eperythrozoonosis laboratory diagnosis.

Table
Testing pathological materials from RF pig farms for eperythrozoonosis

Year	Number of farms submitted the samples	Number of tested samples/ positives
2004	1	3/0
2005	2	2/0
2008	1	2/0
2009	3	4/0
2010	5	61/7
2011	3	33/1
2012	1	2/0
2013	3	12/0

CONCLUSION

1. A PCR and electrophoresis based tool has been developed for *Eperythrozoon suis* detection.

2. Tests for *Eperythrozoon suis* detection were performed in 2004–2013. The agent is detected in whole blood samples collected from replacement pigs and sows.

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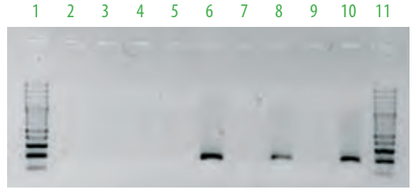

Fig. 3. PCR results of pathological material samples tested for Eperythrozoon suis
1, 11 – DNA marker;
2 – negative control;
3 – lungs from replacement pigs;
4 – aborted fetuses,
5 – placenta;
6 – blood from replacement pigs;
7 – lung of weaning pigs;
8 – blood from sows;
9 – skin;
10 – positive control.