Prevalence and molecular characterization of Cryptosporidium sp. in pigs in Northwestern Russia

Andrey Kryazhev1* and Artyom Novikov1

1Vologda State Dairy Farming Academy named after N.V. Vereshchagin, 2, Shmidtta, Molochnoe, Vologda, 160555, Russia

Abstract. Cryptosporidiosis is a widespread parasitic disease of many species of domestic and wild animals, as well as humans, which is a significant problem in the field of veterinary medicine and medicine. Farm animals, in particular piglets, are most often susceptible to this disease, however, the species composition of representatives of the genus Cryptosporidium in this animal species in the Russian Federation has remained unknown to this day. For the first time in the Russian Federation, in the conditions of the North-West, on the example of the Vologda region in pig farms with industrial technology of maintenance, as well as in private farms using the latest molecular genetic techniques, namely, using high-performance sequencing of amplicon libraries of fragments of the 18S rRNA gene obtained as a result of nested (nested) PCR, we have established the parasitism of C. scrofarum in all age groups of the examined animals. The infection rate of animals kept in pig farms was 34% (51/150), in farms – 32.4% (81/250). The most susceptible to infection are animals that are fattening at the age of 13-24 weeks.

1 Introduction

Cryptosporidiosis is a widespread parasitic disease of many species of domestic and wild animals, as well as humans, caused by protozoa of the Cryptogregaria subclass, the Cryptosporidiidae family, the genus Cryptosporidium. Cryptosporidia were previously considered as a monotypic family Cryptosporidiidae within the Coccidia class. According to modern data obtained as a result of phylogenetic studies performed at the molecular level, parasites form an independent high-rank grouping in the spore system, whose closest relatives are gregarins [1].

Cryptosporidiosis is currently a significant problem in the field of medicine and veterinary medicine. Not so long ago, studies have proved that cryptosporidia occupy the second place after rotavirus in the etiology of diarrhea and mortality of children [2–4]. Cryptosporidia are found in many countries of the world [5]. On the territory of the Russian Federation, they were first found in calves in 1983 [6], and then in other animal species,

* Corresponding author: kamarnett@mail.ru

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including piglets [7, 8]. Cryptosporidiosis is widespread among farm animals in the conditions of the north-west of the Russian Federation [9-12].

Not so long ago, 2 species of cryptosporidia of piglets were identified by molecular genetic methods - C. suis and C. scrofarum, which until recently were considered strictly specific for this host species [13, 14]. However, in various countries, data began to appear on the detection of the zoonotic dangerous species C. parvum in pigs, as well as on the potential zoonotic danger of the first two species [15-20].

In the Russian Federation, the detection of cryptosporidia in piglets using molecular genetic techniques has not been carried out before.

2 Materials and methods

These studies were carried out in the Russian Federation for the first time.

The studies were conducted in industrial pig farms, as well as in private pig farms located in the Vologda Region of the North-Western Federal District of the Russian Federation in the period from January to September 2022. Biological material (fecal matter) was obtained from piglets of various ages, namely suckling piglets under the age of 4 weeks, weaned piglets (5-12 weeks), feedlots (13-24 weeks) and (25 weeks and older), as well as from sows being suckled. The age groups were formed taking into account the technological parameters of keeping animals in pig farms. Fresh fecal samples in a special thermal container were transported to the laboratory, where their initial examinations were carried out. To detect cryptosporidium oocysts, identify them to the genus Cryptosporidium, as well as to determine the intensity of cryptosporidiosis infection of piglets in the laboratory on the basis of the Faculty of Veterinary Medicine and Biotechnology of the Vologda GMHA, native fecal smears, concentrated oocyst preparations were prepared using flotation and centrifuge-flotation techniques with staining of micropreparations according to Ziel-Nielsen and subsequent microscopy to identify and count cryptosporidium oocysts. The intensity of oocyst excretion in fecal samples was determined using the I. Pavlasek technique [21].

According to the number of oocysts excreted per 1 g of feces (OPG), the degree of infection of animals in crosses was determined: “+” (weak) – 1-5 oocysts in the field of view (50000-500000 in g/feces); “++” (average) – 6-10 oocysts (550000-1000000 in g/feces); “+++” (strong) – more than 10 oocysts (over 1,000,000 in g/feces) under microscopy with a magnification of 400 times.

Then the samples were sorted, deep-frozen and transported to the city of Pushkin, St. Petersburg for further research. The study was carried out using the equipment of the resource center «Genomic Technologies, Proteomics and Cell Biology» of ARRIAM.

Identification of Cryptosporidium species in fecal samples of farm animals was carried out using high-performance sequencing of amplicon libraries of fragments of the 18S rRNA gene obtained as a result of nested PCR. Total DNA isolated from animal fecal samples by the CTAB modified method was used as a matrix [22]. The destruction of microorganisms in the samples was carried out using a Precellys 24 ball homogenizer (Bertin Technologies, France) with a speed of 6000 shakes per minute twice for 30 seconds. Nested PCR was used to obtain libraries of fragments of the 18S rRNA gene. The first round of PCR (PCR1) was performed with a pair of F1_Zheng/R1_Zheng primers amplifying a DNA fragment of approximately 1325 bp in 15 µl of a reaction mixture containing 0.5 – 1 units of Q5® High-Fidelity DNA Polymerase activity (NEB, USA), 5 pcM of direct and reverse primers, 1 – 10 ng DNA matrices and 2 nM of each dNTP (LifeTechnologies). The mixture was denatured at 94°C 1 min., followed by 40 cycles: 94°C – 30 s, 55°C – 30 s, 72°C – 1 min. The final elongation was carried out at 72°C 3 min. Then the resulting amplification was diluted 20 times and 1 µl was used as a matrix for the second round of PCR (PCR2) with
ILL_400F/ILL_R2_Zheng primers, to which adapters developed by Illumina (Illumina, USA) were attached. The conditions of the second round of PCR were similar to those of the first, but the number of cycles was reduced to 35. The amplification size was 440 p.o. PCR products were purified according to the method recommended by Illumina using magnetic particles AM Pure XP (BeckmanCoulter, USA).

Amplicon indexing, library preparation and sequencing were carried out in accordance with the manufacturer's recommendations for operation on the “Illumina MiSeq” device (Illumina, USA) using the MiSeq® ReagentKit v3 reagent kit (600 cycle) with two-way reading (2*300 N).

The results were processed using the Illumina software (trimming and demultiplexing) and the dada2 package in the R software environment (quality filtering, data dereplication, denoising, sequence unification and ASV identification (amplicon sequence variant)). The taxonomic affiliation of the sequences was determined using blastn in the GenBank database.

Statistical processing of the obtained results was carried out using the computer program STATISTICA 10.

In total, samples from 400 animals were examined, namely 150 animals kept in industrial pig complexes (30 in each age group) and 250 (50 in each age group) animals kept in farms.

3 Results

Representatives of the genus Cryptosporidium were identified in each age group studied, both in animals with signs of digestive disorders and in piglets without clinical signs of the disease. The total infection rate of livestock in pig farms was 34% (51/150), and pigs in private farms were infected by 32.4% (81/250). Suckling piglets (up to 5 weeks of age) in pig farms were infected with cryptosporidia in 40% of cases (12/30), the intensity of Cryptosporidium infection was predominantly strong (+++), it occurred in 20% (6/30) of cases. Medium (+++) and weak (+) degrees of oocyst excretion also occurred in 3% (10/30) of cases each. In farms, the infection rate of suckling piglets was 24% (12/50), the intensity of oocyst excretion was mainly weak (+), it occurred in 32% (16/50) cases. The average (+++) degree of oocyst isolation of oocysts was 16% (8/50) of cases. In the age group of weaned piglets kept in pig complexes at the age of 5-12 weeks, cryptosporidium infection was 33.3% (10/30), a strong (+++) degree of oocyst isolation dominated, it was 26.7% (8/30) versus 10% (3/30) of average (+++) and 3.3% (1/30) of weak (+). Piglets of the same age group, kept in private farms, were infected with cryptosporidia by 42% (21/50), recorded mainly an average (+++) degree of oocyst excretion, it was 70% (35/50) versus 14% (7/50) weak (+). The most infected with cryptosporidium oocysts are fattening pigs aged 13-24 weeks, the infection rate of this group of animals kept in pig farms was 60% (18/30). The degree of oocyst excretion was mainly average (++):26.7% (8/30), and strong: 23.3% (7/30). In 13.3% (4/30) of cases, there was a weak (+) degree of oocyst excretion. The infection rate of this group of piglets kept in farms was 72% (36/50). The degree of isolation oocysts was average (+++) (42% (21/50)) and weak (+) (30% (15/50)). Animals older than 6 months in pig farms were infected with cryptosporidia in 20% (6/30) of cases. They also recorded average (+++) – 13.3% (4/30) and strong (+++) – 6.7% (2/30) degree of isolation of oocysts, weak (+) infection in this age group was not detected. Piglets of this age group kept in farms were infected with cryptosporidia in 10% (5/50) of cases. They recorded a weak (+) (4% (2/50)) and an average (+++) (6% (3/50)) degree of oocyst excretion. The sows of the industrial method of keeping were also infected with cryptosporidia. Their infection rate was 16.7% (5/30), and the degree of isolation of oocysts.
was weak (+). Infection with cryptosporidia of sows kept by farmers was 14% (7/50), and the degree of isolation of oocysts was weak (+).

Based on the literature data [23, 24], a system of primers (Table 1) for nested PCR was created, amplifying a potentially species-specific section of the 18S rRNA gene with a size of 393 nm and satisfying the capabilities of high-performance sequencing using Illumina technology. The sequence of the ILL_R2_ Zheng primer has been modified with the introduction of degenerate positions in order to make the primer more versatile.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of the primer</th>
<th>Primer sequence (5′-3′)</th>
<th>Amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F1_Zheng</td>
<td>TTCTAGAGCTAATACATGCAG</td>
<td>1325</td>
<td>Zheng S. et al, 2019</td>
</tr>
<tr>
<td>2</td>
<td>R1_Zheng</td>
<td>CCCATTTCCTCGAAACAGGA</td>
<td></td>
<td>Zheng S. et al, 2019</td>
</tr>
<tr>
<td>3</td>
<td>ILL_400F</td>
<td>tcgtgccccagctagtaggtgagagacag* GTTGTGCAGTTAAAAAGCTCGTAG</td>
<td>507 (393 without adapters)</td>
<td>A.Kaupke et al, 2017</td>
</tr>
<tr>
<td>4</td>
<td>ILL_R2_ Zheng</td>
<td>ttcgtgccccagctagtaggtgagagacag* AARGAGTAAGSGAACAACCTCCA</td>
<td></td>
<td>Zheng S. et al, 2019; This work</td>
</tr>
</tbody>
</table>

*The Illumina adapters are indicated in lowercase letters

Table 1. System of primers used for sequencing.

To clarify the possibility of differentiating cryptosporidium species based on the nucleotide sequences of this site, homologous sequences belonging to different species of the genus Cryptosporidium were extracted from the GenBank database, and these sequences were aligned.

The analysis of nucleotide polymorphism and its distribution in sequences demonstrated that all types of cryptosporidia demonstrate a quite high specificity and can be identified with high probability. However, it should be noted that nucleotide polymorphism within species also occurs. It is interesting that, apparently, the polymorphism of indels is somewhat more specific than the polymorphism of nucleotide substitutions. Moreover, it is possible that the creation of a diagnosticum using the features of the indel distribution may turn out to be a rather convenient tool for cryptosporidia.

As a result of sequencing of libraries of fragments of the 18S rRNA gene obtained using selected primers and subsequent taxonomic analysis of the obtained nucleotide sequences, it was shown that representatives of only one species of C. scrofarum are present in all the samples studied. An insignificant nucleotide polymorphism present in all the presented sequences indicates either the presence of allelic variations, or the existence of unknown very closely related species.

From the presented results it can be seen that the selected primer system is very specific to the sequences of the 18S rRNA gene of the genus Cryptosporidium, however, in some cases the percentage of nucleotide sequences not related to cryptosporidia exceeds 50%, which indicates the correctness of the chosen method for the identification of microorganisms. The Sanger sequencing method in these cases would not allow to obtain any positive result.

4 Discussion

As a result of our research, it was found that in the conditions of the north-west of the Russian Federation, on the example of the Vologda region, both with industrial technology of keeping and in conditions of private farms, pigs of all age groups are infected with C.
scrofarum. The infestation of animals with both methods of keeping is approximately within the same limits, the difference is not statistically significant (p ≤ 0.05). At the same time, there was a significant difference in the degree of isolation of cryptosporidium oocysts with different content technologies. If, in the conditions of industrial pig breeding, the intensity of oocyst excretion was predominantly strong (+++) and average (++), then in the conditions of farms, on the contrary, it was predominantly weak (+) and only sometimes average (++) with a relatively equal degree of infection (p ≤ 0.05). It was also noted that cryptosporidium is isolated by animals both with signs of diarrhea and without any symptoms, and the manifestation of diarrhea was found mainly among piglets kept in pig complexes, and in farm conditions, diarrhea was practically not present in animals. This fact is consistent with the research of other scientists [19, 25-27].

It was found that animals that are fattening at the age of 13-24 weeks are the most susceptible to infection while the majority of foreign researchers in Europe [19, 25, 26], America [27] and Australia [28] report the greatest infection with Cryptosporidium infection in piglets aged 1-3 months.

In addition, our studies have shown that all animals are invaded by only one species of cryptosporidium (C. scrofarum). A similar fact is also reported by a group of Chinese scientists [29] while a number of researchers write about the presence in piglets of two, and sometimes even three species of cryptosporidia, such as C. suis, C. scrofarum, and C. parvum [13-20].

It is interesting that our studies have established the fact of infection of suckling piglets with C. scrofarum, while it is mainly reported that animals of older age groups are infected with this type of cryptosporidium [15, 19, 30]. However, there are reports that coincide with the results of our studies when this type of pathogen was registered in piglets under the age of 5 weeks [5, 31, 32]. In addition, cryptosporidia found in sows have been identified as C. scrofarum, which allows us to draw conclusions about the influence of sows on the infection of suckling pigs, in this case, sows should be considered as almost the only source of cryptosporidiosis infection.

5 Conclusions

For the first time in the Russian Federation, in the conditions of the North-West, on the example of the Vologda region, in industrial pig-breeding enterprises, as well as in private farms, parasitisisation of C. scrofarum in all age groups of piglets was established using the latest molecular genetic techniques. Animals aged 13-24 weeks are most susceptible to infection.

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