Development of a test system for detecting bovine adenovirus DNA based on polymerase chain reaction

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Abstract. The paper presents the results of the development of polymerase chain reaction (PCR) test systems for detecting the DNA of bovine adenovirus. Due the large number of classified types of bovine adenoviruses included in two separate genera of *Mastadenovirus* and *Atadenovirus*, a nested PCR variant was chosen when creating a test system using a common external pair of primers and internal primers specific for the *Mastadenovirus* and *Atadenovirus* genera. In the study of field samples from calves with a characteristic clinical picture using the developed test system, the circulation of different strains of bovine adenovirus, which are representatives of these genera, was established. Selective studies showed the presence of 1, 3, 6, and 8 subtypes of bovine adenovirus among positive samples.

1. Introduction

Adenovirus of cattle was first isolated in 1959 in the United States. It belongs to the Adenoviridae family [1, 2]. A total of 11 subtypes of bovine adenovirus (BAdV) were classified as Mastadenovirus (BAdV-1, -2, -3, -9, and -10) and Atadenovirus (BAdV-4, -5, -6, -7, -8 and -Rus) [3, 4]. Within each genus, all adenoviruses were divided into types the name of which included the type of dominant host, the name of the virus, and the Latin letter A, B, C, etc. [3]. So the type Bovine Adenovirus A (BAdV-A) included the subtype BAdV-1; BAdV-B included the subtype BAdV-3; BAdV-C included the subtype BAdV-10; BAdV-D included subtypes BAdV-4, -5, -8, -Rus [5]. At the same time, part of the subtypes of bovine adenovirus were assigned to the types in the name of which there was a different host: BAdV-2 was included in Ovine Adenovirus A (OAdV-A) type; subtype BAdV-9 was a member of the Human Adenovirus C type (HAdV-C). BAdV-6 and -7 subtypes were not assigned to any type [3].

In 2013, in order to simplify the classification of adenoviruses, the International Committee on Taxonomy of Viruses (ICTV) decided to change the name of the species of all adenoviruses (Ordinance Code: 2013.003aV), typing the name of the genus in the name. Under the new taxonomy, 4 types of bovine adenoviruses were introduced: Bovine mastadenovirus A (includes subtype BAdV-1), Bovine mastadenovirus B (BAdV-3), Bovine mastadenovirus C (BAdV-10) and Bovine atadenovirus D (BAdV-4, -5, -8, -Rus). The subtypes BAdV-2 and BAdV-9 were included in Ovine mastadenovirus A and Human mastadenovirus C types, respectively [6]. In 2015, the International Committee for the Taxonomy of Viruses was proposed to classify two more types of Bovine...
atadenovirus E adenovirus (which would include BAdV-6) and Bovine atadenovirus F (which would include BAdV-7). This proposal has not yet been approved, although the database of GenBank data already have the full genomic sequence of BAdV-6 [7, 8]. In this case, the old use of the names of viruses, strains and isolates is allowed [7].

Virions of bovine adenovirus have a typical structure for all members of the Adenoviridae family and are isometric particles with a diameter of 70-90 nm, consisting of a core containing DNA and proteins, and an icosahedral capsid; a supercapsal shell is absent [1, 2]. The capsid consists of 252 capsomers, 240 of which are hexons, and 12 are peptones. Peptones are located on the tops of the icosahedron, consist of two subunits: the base fixed in the capsid, and the appendix (10-31 nm long), protruding beyond the capsid, at the end of which there is a clavate thickening [2, 9, 10]. The best studied protein of adenoviruses is hexon, which contains the highest number of antigenic determinants, including genus, group, intergroup, subgroup and type specific antigenic determinants [5, 7, 11].

The genome of adenoviruses is represented by a single double-stranded DNA molecule (35-36 thousand base pairs) [2, 12].

2. Materials and methods

For the development of the test system, the following materials were used: the «Alpha» strain of bovine adenovirus [13], nasal and eye samples from calves obtained from farms of the central federal district of the Russian Federation. The assessment of the specificity of the test system was carried out using production strains of bovine viral diarrhea virus (BVDV), bovine herpesvirus-1 causing infectious bovine rhinotraceitis (IBR), parainfluenza-3 virus (PI-3V), bovine respiratory syncytial virus (BRSV), bovine rotavirus, bovine coronavirus and bacterial strains of P. multocida types A, B, D, M. Haemolytica, E. coli O101 and O141 serogroups.

DNA isolation from the virus-containing cell culture, nasal and eye samples was performed using a kit for the isolation of DNA based on an inorganic sorbent (Vetbiochem LLC, Russia), according to the manufacturer's method.

Selection of specific oligonucleotides (primers) was carried out using the Prime premier 6.25 computer program based on the analysis of the nucleotide sequences of different types of bovine adenovirus presented in the GenBank information database (using the DNASTAR Lasergene 11.2, BioEdit 7.0.1 programs).

PCR was performed in a volume of 25 μl. The working mixture for each reaction contained: 50 mmol KCl, 10 mmol Tris-HCl (pH = 9.0), 0.1% Triton X-100, 1.5 mmol MgCl2, 0.25 mmol of each dNTP, 10 pmol of each primer, 5 μl of isolated DNA and 1.25 units Taq polymerase.

Identification of PCR products was performed by horizontal electrophoresis in 1% agarose gel, using trisacetate buffer (pH 8.5) containing 0.4-0.5 μg/ml ethidium bromine and using a DNA marker. Analysis of the gels was carried out on an ultraviolet transilluminator (λ = 254 nm).

A recombinant plasmid for assessing the sensitivity of the test system was constructed using the vector cloning system of the pGEM T-Easy PCR products and the primers developed. We used the culture adenovirus BAdV-1 (genus Mastadenovirus) and the field virus (positive sample) as a matrix, which was previously characterized by the sequencing method, as bovine adenovirus (genus Atadenovirus). Transformation of the plasmid into competent E. coli cells, selection of positive clones, isolation and purification of the plasmid were performed according to the instructions of the manufacturer of the vector system.

Amplified DNA fragments were isolated from the gel using the Silica Bead DNA Gel Extraction Kit (TermoScientific, USA) for purification of PCR products, according to the manufacturer’s method.

The mass concentration of the recombinant plasmid solution in the sample was determined on a spectrophotometer. The molecular concentration of n (molecules/μl) was calculated from the mass by the formula:

\[ n = \frac{(C \times NA)}{(X_b \times p \times M_1 b \times p)} \]
where \( n \) - molecular concentration of plasmid (molecules/μl); \( C \) - mass concentration of plasmid in solution (g/μl); \( NA \) = Avogadro number; \( X \) b.p. = number of nucleotide base pairs (b.p.) in the plasmid; \( M1 \) b. p. = 660 (g/mol) - the average molar mass of 1 b. p.

Some positive field samples were identified by Sanger sequencing using primers selected for PCR. Sequencing of the amplified fragment of the virus genome was performed using fluorescently-labelled terminating nucleotides on an ABI Prism 3130 automatic sequencer (Applied Biosystems, USA) according to the manufacturer-device instructions.

Statistical processing of the results was performed using generally accepted methods by means of Microsoft Office Excel 2010, Stat Plus 2009.

3. Results

All nucleotide sequences taken from the GenBank resource database were pre-aligned and cut to the size of the region encoding adenovirus hexon, which is the most conservative for all types of bovine adenovirus [8, 12, 14, 15]. Since a large number of subtypes of cattle adenovirus belonging to the Mastadenovirus and Atadenovirus genera were classified, we decided to use the nested PCR variant, while using a common primer pair (BALF and BARF) for the first amplification [14], and for the second one - individual pairs for each genus (Mast-F, Mast-R, and At-F, At-R, respectively), table 1.

![Table 1. A set of primers for DNA detection of cattle adenovirus.](image)

In the process of optimizing the conditions for PCR, optimal temperature-time regimes were selected for all reaction stages, table 2.

![Table 2. Temperature-time modes of PCR.](image)

To assess the sensitivity of the developed test system, the molecular concentration of each plasmid was determined, and then sequential tenfold dilutions of the samples were prepared, the last plasmid dilution at which a positive PCR result was observed was considered the limit of sensitivity.
The sensitivity of the developed test system is $5.4 \times 10^3$ mol/ml for Mastadenovirus genus and $4.3 \times 10^3$ mol/ml for Atadenovirus genus.

The specificity of the developed test system was tested using a panel of samples containing DNA, the most common viral and bacterial pathogens causing diseases with a similar clinical picture, and nasal samples obtained from a clinically healthy cow (the animal was kept in the private sector). Samples containing adenovirus BAdV-1 and BAdV-8 DNA were used as a positive control, table 3.

**Table 3.** The sensitivity of PCR test systems for detecting the DNA of bovine adenovirus.

| Probe   | K+ | nasal samples from cattle | BVDV | IBR | PI-3V | BRSV | Rotavirus | Coronavirus | P. multocida type A | P. multocida type B | P. multocida type D | M. haemolytica | E. coli O101 | E. coli O141 |
|---------|----|---------------------------|------|-----|-------|------|-----------|-------------|---------------------|---------------------|-------------------|----------------|-------------|-------------|
| BAdV-1  | +  | -                         | -    | -   | -     | -    | -         | -           | -                   | -                   | -                 | -             | -           | -           |
| BAdV-8  | +  | -                         | -    | -   | -     | -    | -         | -           | -                   | -                   | -                 | -             | -           | -           |

The results obtained demonstrated a 100% specificity developed by the test system. Subsequently, using the developed test system, 124 field samples of the material (nasal and eye samples obtained from calves with a characteristic clinical picture, obtained from livestock farms of the central federal district of the Russian Federation) were used to detect adenovirus DNA (figure 1).

![Figure 1. DNA detection of cattle adenovirus in field samples.](image)

Thus, the DNA of bovine adenovirus from the genus Mastadenovirus was found in 11.3% of the studied samples, the DNA of bovine adenovirus from the genus Atadenovirus in 7.2% of the samples studied.

Some of the samples that gave a positive result were subjected to genomic sequencing of the isolated DNA fragments. A comparative analysis of the sequencing results of the obtained PCR amplicons with nucleotide sequences of different types of bovine adenovirus presented in the GenBank resource database showed the presence of 1, 3, 6, and 8 subtypes (BAdV-1, -3, -6, -8) among the positive samples.

**4. Conclusion**
The results of the research indicate that the developed PCR test system for detecting bovine adenovirus DNA is sensitive, specific, and allows routine diagnostics.
In the study of field samples of DNA bovine adenovirus was detected in 18.6% of samples. Among circulating subtypes of bovine adenovirus in cattle-breeding farms of the Russian Federation, 1, 3 and 6 and 8 subtypes were identified.

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