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The use of rapid immunochromatographic tests and PCR for the detection of vaccine viruses (CPV, CDV CAV) in faeces and conjunctival sac swabs of vaccinated dogs

ŁUKASZ ADASZEK¹, MARIA PISAREK¹, MARTA STANIEC¹, ŁUKASZ MAZUREK¹, ANNA KUCHARCZYK¹, MAŁGORZATA RUTKOWSKA-SZULCZYK², ŁUKASZ DENEKA²

¹Department of Epizootiology and the Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Głęboka 30, 20-612 Lublin, Poland

²Vet Planet Sp. z o.o., Brukowa 36 apt. 2, 05-092 Łomianki, Poland

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Summary

The aim of the study was to determine whether the rapid diagnostic CPV Ag (VetExpert), CDV Ag (VetExpert) and CDV/CAV Ag (VetExpert) tests are able to detect the presence of vaccine viruses in faeces and conjunctival swabs (CDV) collected from dogs vaccinated against distemper, parvovirus and Rubarth's disease. The tested dogs were administered vaccines once, and then faeces and conjunctival sac swabs were collected from all animals on days: 2, 5, 7, 10, 14, 20, 25 after the vaccination for molecular testing and to perform rapid immunochromatographic tests for the detection of CPV, CDV and CAV antigens. The immunochromatographic tests were not able to detect the presence of any of the tested pathogens during the 25-day observation period in the vaccinated dogs, although CPV was detected in faeces by PCR up to 20 days after vaccination, and CDV and CAV up to 14 days after vaccination. Summing up the results of our own research, it should be stated that rapid immunochromatographic tests are not able to detect CPV, CDV and CAV vaccine viruses in the faeces of animals subjected to vaccination, and in the case of CDV also in the conjunctival swabs, but they are an excellent tool for detecting these pathogens in animals infected with field strains of these viruses and showing clinical symptoms of the diseases.

Keywords: viruses, PCR, immunochromatographic tests

Parvovirus, distemper and infectious canine hepatitis (Rubarth's disease) are infectious, potentially fatal canine diseases.

Canine parvovirus (CPV) is a disease frequently occurring throughout the world. In their study, Wójcik et al. (27) showed that the most common Polish CPV strain in the canine population is CPV-2b, detected in 80% of animals tested for it. The CPV-2a strain was detected in 17% of cases, while the CPV-2c strain is relatively the least frequently detected.

Dogs of all ages are susceptible to the infection. The most severe course of the disease is observed in puppies from about 6 weeks of age (onset of the immune gap) to 4-6 months of age (22). The mortality rate of untreated animals is very high, up to approx. 90%.

Canine parvovirus has an affinity for rapidly dividing and proliferating cells, e.g. of the bone marrow, intestinal epithelium, cardiac myocytes and cells of the developing foetus (6, 22). It enters the body via the alimentary tract, replicates primarily in the crypt cells of the small intestine and is excreted in the faeces from as early as the third day after infection. The first disease signs can be noticed one week after contact with the virus (2-4). These include vomiting, diarrhoea followed by progressive protein loss, the development of hypoalbuminaemia and severe dehydration, which can result in infected animals collapsing (20).

Parvovirus should be suspected in the case of all gastrointestinal signs, especially in young, unvaccinated dogs. Haematological examination of affected individu-

als may reveal leukopenia and very often coagulopathy develops. The most common laboratory method used to detect the virus in faeces is polymerase chain reaction, especially real-time PCR. The diagnostic process may also include a haemagglutination inhibition test and faecal CPV tests, using rapid immunochromatographic tests (20, 22).

Distemper is a highly contagious canine and canidae viral disease caused by Morbillivirus CDV (canine distemper virus). It is a highly infectious and virulent bacteria that spreads rapidly through the population of susceptible animals. In dogs, CDV leads to the development of severe systemic disease with gastrointestinal, respiratory and nervous system signs. Distemper dogs may exhibit paw pad and nasal hyperkeratosis, so another name for canine distemper is 'hard pad disease' (1).

The disease is most commonly seen in young, unvaccinated dogs less than one year of age (especially several weeks old after loss of colostral immunity), although it may also affect older dogs. Risk factors for distemper include lack of vaccination and keeping dogs in high densities (breeding facilities, shelters).

The disease is spread through the aerosol from the respiratory tract and other secretions and excretions of affected individuals, such as discharge from the conjunctival sac, urine or faeces. Infected dogs may shed the virus over a period of 60-90 days. CDV enters the body of a susceptible animal via the respiratory system and the gastrointestinal tract. It has been reported that in the case of domestic dogs, the infection may also spread from mother to offspring (19). The clinical signs of distemper are extremely diverse and depend on the viral strain, age of the infected animals and their immune status, as well as the comorbidities. The infection may have subclinical or full-blown presentation (23). There are two clinical forms of distemper: acute systemic disease and chronic distemper encephalitis. The incubation period usually lasts for 4-10 days.

Distemper is diagnosed on the basis of the medical history, presented clinical signs and results of additional tests. Currently, the most sensitive method used to diagnose the disease is polymerase chain reaction (PCR). Genetic material from the virus can be detected in blood, conjunctival swabs, cerebrospinal fluid (CSF) and urine sampled from affected individuals. CDV antigens in different cell types, such as conjunctival epithelia, urine sediment, leukocytes or internal organ scrapings, may also be detected using the direct immunofluorescence technique (IFAT). In their offices, veterinarians often perform rapid ELISA assays for the initial diagnosis of distemper. They are easy to perform and show a result in a short time. However, it must be remembered that their sensitivity and specificity are lower than those of PCR or IFAT.

Infectious canine hepatitis (Rubarth's disease) is a canine disease characterised by a variable course. Its aetiological factor is canine adenovirus type 1 (CAV-1), closely related to canine adenovirus type 2 (CAV- 2) (7, 10).

Puppies less than one year of age (especially those several weeks old) are most susceptible to infection, which takes a hyperacute course with sudden deaths of up to 100% (4, 16). The virus is shed in the saliva, faeces and urine (viral excretion in urine lasts from 6 to even 12 months). In the first phase of the disease (the first 34 to 35 days after the onset of clinical signs), the virus is present in all secretions and excretions. Infection occurs following direct contact between the susceptible animal and the shedder, or by indirect routes (contact with contaminated equipment, clothing, etc.).

Fever ($> 40^{\circ}\text{C}$) is the first sign of the disease. In some dogs, it resolves after 1-2 days and the animals recover. In others, the infection progresses to the acute form, accompanied initially by general symptoms, such as apathy, inappetence, increased heart rate, and hyperventilation. They may be followed by vomiting, diarrhoea, soreness and abdominal integument oedema. The latter results from the accumulation of bloody serous fluid in the abdominal cavity and liver enlargement. Occasionally, affected individuals sneeze with blood-tinged droplets and develop skin or mucosal petechiae and jaundice. One to three weeks after the primary infection, corneal cloudiness and kidney inflammation may be observed as a result of the deposition of immune complexes in these structures. The swollen cornea becomes bluish, which is commonly referred to as 'blue eye' (1). Rubarth's disease should be suspected in dogs with an unknown vaccination history showing signs of fever, gastrointestinal and ocular abnormalities.

Polymerase chain reaction (PCR) is highly sensitive in the diagnostic process. This technique allows both pre- and post-mortem detection of viral DNA. The best supravital testing material is urine, where the CAV-1 is excreted for the longest period, as well as rectal swabs and blood. Post-mortem examination is performed on kidney, lung and lymph node material. The immunofluorescence technique allows detection of virus antigens in infected cell cultures and in acetone-fixed tissue slices.

The most effective method of protecting dogs against parvovirus, distemper and Rubarth's disease is through specific immunoprophylaxis. Prophylactic vaccines against these diseases are considered core vaccines that should be given to all dogs (5, 8, 12). According to WSAVA recommendations, puppies should be vaccinated at 6-8 weeks of age, then every 2-4 weeks until 16 weeks of age. Revaccination should take place at 6 or 12 months of age, then not more frequently than every 3 years. Adults that did not receive the vaccine as puppies are vaccinated at any time, but revaccination should take place not more frequently than every 3 years.

After vaccination, vaccine viruses may be shed in the secretions and excretions of animals undergoing specific immunoprophylaxis. The study aimed at determining whether the rapid diagnostic tests CPV Ag (VetExpert), CDV ag (VetExpert) and CDV/CAV Ag (VetExpert) can detect the vaccine viruses in faeces and conjunctival sac swabs (CDV) collected from dogs vaccinated against distemper, parvovirus and Rubarth's disease.

Material and methods

The study was carried out on 40 adult dogs over 2 years of age, of different sexes, of which 30 were test animals, divided into three groups of 10 individuals. In each study group, the dogs underwent prophylactic vaccination against parvovirus, distemper and adenovirus with different vaccines. The remaining 10 dogs constituted the control group and were not vaccinated.

Vaccines were administered once to the dogs in the study groups, after which faeces and conjunctival sac swabs were collected from all animals (on days 2, 5, 7, 10, 14, 20, 25 after the vaccinations) for molecular testing and for rapid immunochromatographic tests for CPV, CDV and CAV antigens.

Faecal PCR testing for parvovirus was performed according to the method described by Adaszek et al. (2). The PCR reaction was performed in a Biometra thermocycler (Biometra, Göttingen, Germany) using the primer pair: P1: 5'-CTACTCAGCCACCACTAAAG-3', P2: 5'-ATTTTCTAGGTGTAGTTGAGA-3', complementary to the respective sequences of the VP2 gene, allowing amplification of its 1,278 base pairs. The PCR reaction involved 30 consecutive cycles, each consisting of three steps: denaturation of the DNA strand (94°C for 60 s), primer attachment (58°C for 60 s) and strand extension (72°C for 60 s). The test was carried out using positive and negative controls. The positive control was the CPV genetic material isolated from the vaccine while the negative control was total DNA isolated from the faeces of a healthy dog.

Faecal PCR test and conjunctival sac swabs for distemper were performed according to the method described by Adaszek et al. (3). In the studies, a pair of primers specific to the nucleoprotein gene of CDV – P1 (5'-ACAGGATTGCTGAGGACCTATT-3') and P2 (5'-TTTTCAGATAACCATGTACGGTGC-3') – were used, which enabled the amplification of the gene fragment consisting of 293 base pairs (14).

In Reverse Transcription Reaction a mixture of 5 µl of isolated total RNA, 9.5 µl of water and 1 µl of p(dN)6 (Invitrogen) was denaturated at a temperature of 65°C for 5 minutes and then transferred to an ice bed for 5 minutes. Next, 5 µl of reverse transcriptase (Fermentas), 2.5 µl of dNTP (2 mM), 1.0 µl of ribonuclease inhibitor (10 u/µl, Fermentas) and 1.0 µl of reverse transcriptase (200 u/µl, Fermentas) were added. The synthesis of cDNA was performed at a temperature of 50°C for 30 minutes in a Biometra thermocycler. The reaction mixture was incubated at a temperature of 94°C for 5 minutes.

In PCR reaction the amplification of the resulting cDNA was carried out in 50 µl of the reaction mixture containing: 31.5 µl of water, 5 µl of Tag polymerase buffer, 1 µl of dNTP (10 mM), 1 µl of each of the starters P1 and P2 (at a final concentration of 0.2 µM), 5 µl of MgCl₂ (25 mM), 0.5 µl of Taq DNA Polymerase at a concentration of 5 u/µl (Fermentas), 5 µl of the matrix (earlier synthesised DNA strand). PCR reaction involved 40 cycles, each consisting of the consecutive phases of: denaturation of the strand at a temperature of 94°C for 30 seconds, binding of the starters at a temperature of 55°C for 30 seconds and strand prolongation at a temperature of 68°C for 1 minute.

Faecal molecular test for adenovirus was performed according to the method described by Kalinowski et al. (17). The genetic material of CAV-2 was isolated using a Genomic mini DNA isolation kit (A&A Biotechnology, Gdańsk, Poland) according to the procedure provided by the manufacturer. Purified DNA was suspended in 100 µl of Tris buffer to be used in further analysis. Amplification of the E1B-19K CAV-2 gene was performed using the pair of primers CAV-F 5'-GAGCAGGTAGTATGGAC-3' and CAV-R 5'-TCAGTAAAAGRAGCAAC-3'. The primers made it possible to amplify a gene fragment of 235 base pairs (15). The 50 µl PCR reaction mixture consisted of: 33 µl of water, 5 µl of buffer for Taq polymerase, 1 µl of dNTP (10 mM), 3 µl of MgCl₂ (25 mM), 1 µl of each primer: CAV-F and CAV-R with a concentration of 50 pM, 1 µl of Taq DNA Polymerase 5 u/µl (Fermentas) and 5 µl of matrix (DNA isolated earlier). The PCR was performed using a Biometra apparatus (Göttingen, Germany). It covered 40 cycles, each consisting of a stage of DNA denaturation at 94°C for one minute, annealing of primers at 50°C for 1 minute and strand extension at 72°C for 1 minute.

VetExpert immunochromatographic tests were used to detect faecal parvovirus (CPV Ag), distemper virus (CDV Ag) and adenovirus (CDV/CAV Ag) antigens. In addition to faeces, the distemper test material included conjunctival sac swabs.

The testing material was collected with a hygienic stick or swab. Samples of material were then placed in tubes containing dilution buffer. Then the samples were mixed with the buffer and the solids of the mixture fell to the bottom of the tube. On a flat surface, 3-4 drops of the test liquid were placed on a t-test window with a dropper. The result was read out after 10 minutes.

Results and discussion

With rapid immunochromatographic tests, no vaccine virus antigens were detected in any of the test samples taken at an interval of 2-25 days after the vaccinations. In the control group this test gave a negative result.

In the group of dogs tested for CPV, the virus genetic material was detected by PCR in all 10 dogs (in two dogs, the virus was detected in the faeces between 2 and 10 days after the vaccination, in seven between 2 and 14 days after the vaccination and in one between 2 and 20 days after the vaccination). On the follow-up on the 25th day, CPV was not detected in the faeces of any of the vaccinated individuals. The virus was also not detected in the faeces of the control animals via the PCR technique.

The CDV genetic material was found in all animals vaccinated against distemper in both conjunctival sac swabs and faeces. In conjunctival sac swabs, it was detected in 3 animals between the 2nd and 10th day after the vaccination, while in the remaining seven between 2nd and 7th day after the vaccination. The faecal test detected viral RNA in nine dogs between the 2nd and 14th day after the vaccination, and in one between the 2nd and 10th day after the vaccination. In the control group, CDV RNA was not detected in any individual tested

with the RT-PCR technique, either in the conjunctival sac swabs or faeces.

CAV DNA was detected in the faeces of all vaccinated animals, but in no individual from the control group. Adenovirus genetic material was present in the faeces of vaccinated animals between the 2nd and 7th day after the vaccination (3 animals), the 2nd and 10th day after the vaccination (5 animals) and between the 2nd and 14th day after the vaccination (2 animals).

Our observations indicate that rapid immunochromatographic tests have low sensitivity in detecting CPV, CDV and CAV strains in faeces and, in the case of CDV, also in conjunctival swabs, irrespective of the vaccine used. The tests were unable to detect either of these pathogens in the test material over a 25-day follow-up period in any of the vaccinated dogs, although CPV was detected by PCR up to the 20th day after the vaccination, and CDV and CAV up to the 14th day after the vaccination.

No studies are available on the use of rapid immunochromatographic tests to detect vaccine CDV and CAV strains in faeces and secretions of vaccinated dogs. The available literature describes only of a few experiments using this diagnostic tool for CPV detection in the faeces of dogs undergoing vaccination against parvovirus.

The results of studies similar to those described above confirm our own observations that 'point of care' tests are unable to detect parvovirus vaccine strains in faeces. Schultz et al. (25) vaccinated 64 beagle dogs with six different parvovirus vaccines. None of the dogs' faeces showed CPV after the vaccination procedure. Similar observations apply to feline parvoviruses. Of 64 cats vaccinated against panleukopenia, FVP was detected in the faeces by rapid test in only one individual (21).

In contrast, vaccine parvovirus genetic material can be detected by PCR in canine faeces and tissues up to 28 days after the vaccination (13, 24).

In conclusion, while rapid PCR tests are not able to detect vaccine CPV, CDV or CAV in the faeces of vaccinated animals and, in the case of CDV, also in conjunctival swabs, they are an excellent tool for detecting these pathogens in animals infected with field strains and developing clinical signs of the disease (11). This sensitivity difference is probably caused by insufficient amounts of vaccine virus in the faeces and conjunctival sac swabs, being below the detection limit of the tests. In disease cases, virus titres are much higher, so they can be identified by the tests (9).

Low sensitivity in detecting vaccine viruses is not a disadvantage of the discussed diagnostic kits. When a vaccinated animal becomes infected with a field strain of, e.g., CDV, knowing that rapid tests cannot detect the vaccine virus, confirmation of CDV in the faeces of such patients will indicate that we are dealing with a virulent pathogen. In other words, these tests allow us to differentiate vaccinated patients with non-CDV, CPV and CAV diarrhoea from vaccinated patients infected with these pathogens and developing parvovirus, distemper or Rubarth's disease (9).

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Corresponding author: Marta Staniec, Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Głęboka 30, 20-612 Lublin, Poland; e-mail: marta.staniec@up.lublin.pl