Artykuł przeglądowy *Review*

Prospects for the use of molecular methods in the diagnosis of parvovirus type 2 (CPV-2) in representatives of the Canidae family

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Summary

Parvovirus infection is a viral disease mainly affecting young dogs, in which its course is often fatal. Despite the existence of vaccines, parvovirus remains a significant problem for dog owners. Given the ease with which the virus is disseminated, the most effective method of preventing its spread seems to be rapid and effective diagnosis. Serological methods still play the most important role in the diagnosis of parvovirus infection, but the dynamic growth of molecular biology offers hope for the increasingly widespread use of techniques enabling the detection of viral material. In recent years impressive progress has been made in this area. The PCR (polymerase chain reaction) method, which until recently was still regarded as unavailable in many diagnostic centers, is now becoming standard. Apart from ordinary amplification, numerous modifications have emerged, enabling substantial reductions in the time of the analysis, improved sensitivity and specificity, and monitoring of the reaction in real time. Due to the development of sequencing and bioinformatics, information can be obtained on an increasing number of viral genomes and on how changes in nucleotide and amino acid sequences are linked to pathogenicity. Knowledge of nucleotide sequences and phylogenetic analyses make it possible to trace the evolution of viruses and their flow between host populations.

Molecular methods seem to be the future not only of CPV-2 diagnosis, in which it is becoming increasingly common, but in virology as a whole. Owing to molecular techniques it is becoming possible to detect a pathogen at the level of just a few copies, in the early stages of infection, avoiding the limitations of the serological window. The continuing tendency towards simplification of methods, improved sensitivity and specificity, and lower costs may allow classical methods to be supplemented or even replaced by methods of molecular biology.

Keywords: CPV-2, molecular diagnostics, PCR, sequencing

Parvovirus infection is one of the most serious diseases attacking dogs. Antibodies against the pathogen are also detected in the serum of free-living representatives of the family *Canidae*, such as foxes and wolves (56, 60). The first cases of the disease were reported towards the end of the 1970s (14, 49). The etiological cause of the disease is the non-enveloped parvovirus type 2 – CPV-2 (12). The genetic material of the pathogen is single-strand DNA with a length of about 5,000 nucleotides (25, 26), encoding two non-structural proteins (NS1 and NS2), and two structural proteins (VP1 and VP2) (12, 26). The virus attacks dogs of all ages, but is particularly dangerous for puppies, in which it causes myocarditis (46). The main symptoms in adult dogs include appetite loss, apathy, vomiting and bloody diarrhea (66). In the case of adult individuals the mortality rate usually does not exceed 1% (12), although there are reports that it may reach 10%, and in puppies, up to 91% (47).

The hypothesis regarding how the pathogen emerged is interesting. It is believed to have derived from the feline panleukopenia virus (FPV), which originally attacked representatives of the *Felidae* family, including lions, tigers and lynxes, but also unrelated species, such as foxes, raccoon dogs and mink (63). A variant that attacks dogs may have emerged due to several mutations in the region encoding the protein VP2 (1, 45). Arguments in favor of this hypothesis include the high degree of similarity of the nucleotide sequence between canine parvovirus (CPV-2) and FPV, which exhibit 98% homology, while the only differences are found in the region encoding the VP2 protein (63), as well as the fact that cats can also be infected by the virus (28), or be

asymptomatic carriers (10). The high genetic variability of the virus is underscored by the emergence of genetic variants within type 2 (CPV-2a, CPV-2b and CPV-2c) (42, 47), which have supplanted the original variant. New types are more virulent and capable of infecting a wider range of hosts (2, 12). The emergence of CPV-2a-c was caused by nonsynonymous mutations in the gene encoding the protein, VP2; a single change in the amino acid enables differentiation of the pathogen into variants a and b (61). The considerable dynamics in the genome of the virus, which lead to changes in antigenic properties, may cause significant difficulties for serological diagnostic methods. For this reason it seems worthwhile to search for new methods of CPV-2 diagnosis. This paper presents molecular methods for parvovirus diagnosis and possible directions for the development of molecular diagnostics of the CPV-2 virus.

Classical diagnosis of CPV-2

Currently a frequently used method for diagnosis of parvovirus infection is the ELISA assay, enabling the detection of antibodies directed against the virus. An interesting modification of the method is commercial point-of-care ELISA kits, enabling rapid diagnosis in field conditions (36). Apart from the ELISA test, there are other methods based on serological processes. These include the hemagglutination reaction (as well as inhibition of hemagglutination) (5, 7), the indirect immunofluorescence assay (73), counter-current immune-electrophoresis (CIEP), and immunodiffusion in agarose gel (18). Moreover, methods based on electron microscopy (47) or on isolation of the virus directly from cultures (12) can be used for diagnostic purposes. All of these methods have certain limitations: isolation of the virus requires an exceptionally well-equipped laboratory, and culture and isolation are time-consuming, taking up to two weeks (62); electron microscopy has low sensitivity, is relatively expensive and does not enable quantitative diagnosis (57, 58); serological methods such as counter-current immune-electrophoresis are often based on detection of antibodies, so that they are limited by the serological window. Hemagglutination and isolation of the virus are techniques in which the virus is detectable only for a few days, which can result in false negative results (20). In addition, methods like hemagglutination and the ELISA assay are often said to have low sensitivity (17, 20, 66), which is being systematically improved by modifications of the method (22, 35).

Classical methods of CPV-2 diagnosis, such as tests based on electron microscopy or serological methods, are not always equal to the dynamic changes in viral genomes. For this reason diagnosticians are increasingly turning to molecular methods, which are becoming increasingly common in standard diagnostics.

Molecular diagnostic of CPV-2

PCR, which not so long ago was beyond the reach of many diagnostic centers, is currently accepted as a sensitive, specific, reliable and affordable technique. The method has long been used for diagnosis not only of parvovirus infections (5, 20, 41, 52). The PCR technique is based on detection of the genetic material of the virus, owing to which it sidesteps the serological window and thereby enables diagnosis in the early stages of the disease (50). Furthermore, it has greater sensitivity and specificity than commercially available tests based on serological reactions (13). The most important step which preceded the amplification is designing of the primers. Sequences which are used in diagnostics should be specific but also conservative in the primer binding region. In case of CPV-2 such a region can be found in sequences coding VP1 as well as in VP2 protein (55). One of the disadvantages of the method is that it takes a relatively long time, but modifications are being developed that considerably shorten the amplification process.

One solution is the LAMP technique (loop-mediated isothermal amplification), which enables amplification of genetic material in less than an hour (51). A significant difference in comparison with classical PCR is the use of several pairs of primers, which in the initial stages, apart from initiating amplification, have the task of preparing a suitable template for the reaction. The result can be observed as cloudiness or luminescence, depending on the kits used (29). Isothermal amplification has greater sensitivity and, owing to the design of appropriate primers, identifies specific genetic variants (33). Another unquestionable advantage of this method is the time required for the analysis, as the result is usually obtained after about an hour (8). In the case of parvovirus infection in dogs, the material for analysis is often feces, which may contain PCR inhibitors; the LAMP method is less susceptible to this type of contamination (43). Devices based on LAMP, which exploit isothermal PCR technology, can be used for point-of-need molecular diagnosis. The apparatus contains zones with different thermal profiles, and as the sample flows through successive sectors it undergoes successive stages of amplification. The sensitivity of the technique is significantly improved by the use of probes; moreover, the apparatus is portable and the analysis itself is considerably faster than in the case of classical or nested PCR (64). The technology of iiPCR (Insulated Isothermal PCR) has also been tested in diagnostics of canine parvovirus, and the detection rate has been found to be comparable to that of Real-Time PCR (71). In this method the reaction mixture is automatically passed sequentially through temperature zones (there are separate zones which refer to classical PCR steps – denaturation, annealing, and extension) in a capillary tube which is placed within the device. Both the LAMP method and iiPCR technology, owing to simplified analysis protocols, miniaturization, and considerable mobility, may become significant competition for commercially available immunochromatographic assays.

Another direction in the development of molecular methods in diagnostics is avoidance of false negative results; therefore modifications of the methods are introduced that increase their sensitivity and specificity. An example of a technique arising from classical PCR

is nested PCR, which uses two pairs of primers. During the first reaction a longer fragment containing the target sequence is amplified, and then the resulting products are used as a template for the second reaction, in which the intended sequence is amplified. The technique has also found application in detection of CPV-2 material, in which its significant advantage is sensitivity 100 times greater than that of ordinary PCR (34).

A technique continually gaining in popularity is Real-Time PCR, whose detection threshold begins with just a few copies of the virus (65). The detection of minimal amounts of the virus makes it possible to test environmental samples, which was confirmed in a study conducted on Spanish mink farms where Aleutian disease was present (54). This type of application can also be significant in the case of canine parvovirus. Large amounts of the virus are found in feces, so that the virus easily contaminates the environment, where it can persist for a long time (53); thus the introduction of an animal free of the virus into a contaminated environment can lead to infection. The real-time amplification method is increasingly used for diagnosis of numerous infectious diseases (6, 23, 70), including canine parvovirus (17, 40). A significant advantage of the technique is the fact that, in case of many diseases, amplification in real time can be used not only for diagnostics but also to monitor the course of the disease or to analyze the effectiveness of a potential treatment (24, 65, 69).

Just as the PCR method has become the basis for numerous modifications, Real-Time PCR is the source of a number of measures aimed at increasing its diagnostic power. Currently, one of the best results of such measures is a modification using minor groove binder-DNA (MGB) probes. This solution is an excellent response to the vast variability and polymorphism of the virus. While in classical PCR and nested PCR the result is a band on agarose gel, indicating the presence of the genetic material of the virus, the use of MGB allows the precise genetic variant of the pathogen to be specified. The probe is capable of detecting SNP polymorphism between genetic variants of the virus, and there are kits dedicated to parvovirus (15, 16). The method exploiting MGB probes is not specific for antibodies or antigenic proteins, but for the genome. This is especially important in the case of etiological agents with high variability, as in the case of viruses.

Another brilliant solution was the use of gold nanoparticles in a buffer reaction, which gave rise to the nanoPCR method. The addition of a noble metal substantially increases the heat conductivity of the solution, resulting in greater specificity and sensitivity (from 100 to 1,000 times) (38). As the method has been used successfully in the diagnosis of enteritis parvovirus in mink (MEV) (67), parvovirus (PPV) and bocavirus infecting pigs (PBoV) (11, 68), and the pseudorabies virus (37), its application in diagnostics of canine parvovirus seems to be only a matter of time. NanoPCR is a relatively new method which is continually being improved by the use of nanoparticles produced from a variety of materials, both metallic and non-metallic, in order to further increase its sensitivity (59).

The role of sequencing in molecular virology

A single change in the nucleotide sequence can alter the antigenic properties of a pathogen; hence immunological methods can yield false negative results. A similar problem may result from changes in the hybridization sites of PCR primers. Finally, new antigen variants can be resistant to current vaccines (32). These problems point to the need not only for the use of molecular methods for direct diagnostics but also for more thorough investigation of the molecular structure of the virus. This "genome anatomy" is crucial to the development of molecular diagnostics. The PCR method becomes far less valuable when data are lacking on the nucleotide sequence of the virus, which is essential for primer design. The sensitive method of real-time amplification using MGB probes, which enables detection of SNP, also largely makes use of data on the structure of the viral genome. Molecular techniques are another source of this type of information, but in their case amplification of the material is only an indirect, preliminary stage of the actual analysis.

Examples of such a technique include sequencing by Sanger's method and sequencing using new generation methods (NGS). Sequencing by Sanger's method, though introduced in the 1970s, remains a highly informative and reliable technique. Sequencing techniques and the associated data analysis seem to remain in the shadow of molecular diagnostic methods, but it is sequencing that constitutes the core that diagnostic tests rely on. Without information on the structure of the viral genome, and whether particular sequences are conserved or variable, successful diagnostic measures cannot be taken (55).

Knowledge of the nucleotide sequence of the virus enables not only definitive confirmation of infection, but also identification of a specific variant, as well as analysis of the effect of polymorphisms on virulence, based on dynamically growing bioinformatics databases. There have been numerous reports regarding new genetic variants of canine parvovirus (9, 27, 44), the occurrence of endemic variants (48), phylogenetic relationships between isolates (39, 72), and flow between hosts belonging to different species (1, 10). A key source of these results is sequencing, which makes it possible to follow changes in the antigenic properties of the virus and potential directions of the spread of the pathogen (30, 31).

While Sanger's method does not allow for sequencing of long sequence fragments, the solution to this problem is provided by new-generation technologies that make it possible not only to sequence the genome of an individual virus in a relatively short time, but also to obtain information on the entire virome (3, 4, 21). New-generation high-throughput sequencing techniques supplemented by classical sequencing, in conjunction with bioinformatics tools, seem to be the main basis for the development of even more sensitive and specific tests for diagnosis of parvovirus infection. Moreover, knowl-

edge of the sequence of the virus and of entire viromes, which can then be linked to their specific properties, may be useful in developing effective vaccines (19), and even in attempting treatment based on the activity of interference RNA (30). The results of bioinformatic analyses of the genome of the virus can also be useful in studying the flow and variability of the parvovirus between domesticated and free-living representatives of the *Canidae* family.

Sequencing, both by Sanger's method and NGS, and bioinformatics seem to be a key stage in the development of molecular diagnostics, including diagnosis of CPV-2. Most molecular methods are based on sequencing results, which may significantly influence the specificity of the methods designed. The road to a ready-to-use test based on both amplification and hybridization leads through nucleotide sequences of pathogens. For this reason further progress in molecular diagnostics is not possible without further progress in both sequencing and bioinformatics, which seem to be essential tools for the development of diagnostics.

Recapitulation

It is obvious that further dynamic development of molecular diagnostics is essential, and we are witnesses to this. Two trends can be observed. The first aims to optimize and simplify the method and increase its sensitivity and specificity, while reducing costs and the time required for the analysis; the target seems to be a "lab-on--a-chip," which at the moment appears to be an entirely realistic goal. The second trend is high-throughput genome analyses forming the basis for diagnostic methods, but also for phylogenetic and evolutionary research. The growing interest in new-generation sequencing and its increasingly common use are making it possible to acquire information on entire viromes, which following bioinformatics analysis become a base for expanding the spectrum of diagnostic tests.

Canine parvovirus, due to its prevalence, its strong tendency to form new genetic variants and its broad host spectrum, including asymptomatic carriers, is an ideal target for molecular methods. Analysis of the parvovirus genome and comparison of the data obtained with bioinformatics databases can significantly contribute to the understanding of the evolution of parvoviruses, analysis of the effect of individual mutations on the virulence of the pathogen, and improvement of diagnostic methods.

The growing need for this type of test is increasingly driving the development not only of diagnostics, but of molecular biology as a whole, resulting in the emergence of new methods or continual improvement of existing ones. The future of diagnostics seems to belong to molecular methods, which in time may not only supplement classical methods, but supplant them.

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