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Investigation of DNA and RNA viruses from paraffin-embedded tissue blocks using molecular methods

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Abstract:
Polymerase chain reaction (PCR) is the most used molecular method in routine diagnosis for viral diseases. It is chosen for its high sensitivity and rapid results. One of the most important factors for reliability of results in PCR is the choice of material. Cold chain is mandatory for transporting the material. Material may start decomposing if conditions are compromised and transport time is prolonged, this is especially important if organs are used as material. Field staff may decide not to send organ samples in a case like this. Alternatively, formalin fixed paraffin-embedded tissues do not need any specific conditions in transport which poses an advantage. The aim of this study is to create awareness in value of paraffin blocks in molecular diagnostics and investigate their practical importance in PCR applications. Two different viruses were chosen in the design of the study. Bovine papillomavirus (BPV) is chosen for representing DNA viruses and canine distemper virus (CDV) is chosen for representing RNA viruses. Five paraffin-embedded tissue blocks for each virus, which are previously known to be positive, were used as material. Two different sets of primer pairs were chosen for PCR application. L1 ORF region of BPV and nucleocapsid region for CDV was targeted. Reverse transcription was performed for samples of CDV before the PCR application. Four of the BPV samples and two of the CDV samples were deemed positive following PCR and RT-PCR. According to the results, paraffin-embedded tissue blocks can be valuable, albeit more efficient for DNA viruses and less efficient in RNA viruses. Additionally, our findings indicated there might be false negative results which should be considered when planning the studies.

Keywords: Virus, Polymerase Chain Reaction, Paraffin Embedded Tissue Block

Parafine gömülü doku bloklarından DNA ve RNA viruslarının moleküler yöntemlerle tespit edilmesi

Özet:
Polimeraz zincir reaksiyonu (PCR) viral hastalıkların rutin teşhisi için kullanılan en önemli yöntemlerden biridir. Duyarlılığının hassas olması ve hızlı sonuç alınması nedeniyle tercih edilmektedir. PCR testinin sonuçlarını etkileyen en önemli faktörlerden biri de test için alınacak olan marazi maddenin doğru seçilmesidir. Marazi maddenin nakli için soğuk zincir şarttır ve uzun süren taşımalarda özellikle şartlar bozulursa organ örnekleri kokuşmaya başlayabilir. Saha personeli bu durumu göz önüne alarak organ örnekleri yollamayabilir. Alternatif olarak parafine gömülen dokular, nakil şartları için özel koşullar gerektirmemektedir. Bu durum transport için önemli bir avantaj sağlar. Bu çalışmada, parafin blokların moleküler tanı için değerlendirilmesine dikkat çekmek ve bunların PCR uygulamasında pratik olarak tanıdaki önemi belirtilmeye çalışılmıştır. Örnek olarak sık çalışılan viruslardan DNA nükleik aside sahip bovine papillomavirus (BPV), RNA nükleik aside sahip olan canine distemper virus (CDV) seçilmiştir. Her iki hastalık için pozitif olduğu bilinen vakalardan 5 ‘er adet parafine gömülü doku kullanılmıştır. Bu amaçla BPV tanısı için L1 ORF bölgesine yönelik primer seti kullanılmış, CDV için ise nükleokapsit bölgesine yönelik bir primer seti seçilmiştir. RNA nükleik aside sahip olan CDV için PCR öncesi reverse transkripsiyon basamağı eklenmiştir. Yapılan PCR ve RT-PCR sonucunda BPV pozitif dokuların 4’ünde CDV pozitif dokuların ise 2’sinde pozitiflik tespit edilmiştir. Bu durum bize parafin bloktaki dokuların özellikle DNA viruslarında daha verimli, RNA viruslarında ise daha az verimli olarak kullanılabileceğine işaret etmektedir. Bununla beraber, sonuçlar değerlendirildiğinde yanlış negatifliklerin elde edilebileceği görülmekte ve planlanan çalışmalarda bu durum öngörülerek hareket edilmesi önem taşımaktadır.

Anahtar kelimeler: Virus, Polimeraz Zincir Reaksiyonu, Parafin Doku Bloğu

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Intoduction

Polymerase chain reaction (PCR) for DNA viruses and reverse transcription polymerase chain reaction (RT-PCR) for RNA viruses is one of the most used routine diagnostic tool in veterinary virology (Belak and Ballagi, 1993; Yilmaz et al., 2020; Karakurt et al., 2022; Yilmaz et al., 2022). Blood, feces, swabs and organ samples are the most frequently used materials for PCR analyses (Jones, 2002; Espy et al., 2006). The choice of testing material has primary role on sensitivity of the test (Espy et al., 2006). Transport time of the chosen materials is equally important (Espy et al., 2006). Most common problem is the prolonged transport times due to long distance between area of necropsy and laboratory services. Waiting time for the material before transport should be minimal but there may be problems related to the transportation frequency, especially in rural areas. Organ samples are most affected by this problem, there may be decomposing due to disrupted cold chain caused by the prolonged transportation times (Sanchez-Romero et al., 2019). Organ samples can be processed where samples are collected with minimal equipment especially when diagnostic pathology settings are present. Tissues are fixed in formaldehyde, dehydrated with alcohol and embedded in paraffin for histopathological examinations. Tissues fixed this way can be stored in room temperatures for long periods of time and safely transported without needing cold chain. Although DNA and RNA are degraded by formaldehyde (RNA can especially be affected by these procedures), if viral load is high enough tissues embedded in paraffin can be used for molecular analyses (Howe et al., 1997; Pikor et al., 2011; Greyseels et al., 2020). Acquired immunodeficiency syndrome was first discovered in 1980s and the causative etiological agent later identified as Human immunodeficiency virus (HIV). In a current investigation, which was done by locating and testing paraffin-embedded tissues belonging to 1960s, years before disease started, revealed a near full genome of HIV (Greyseels et al., 2020). There are not many currently published molecular studies using paraffin-embedded tissues as material. This study aims to investigate the possibility of using paraffin-embedded tissues as material and to evaluate the reliability of this method and thus contribute to this topic.

Material and Methods

Material

The study material consists of ten paraffin-embedded tissue blocks, five for each, belonging to previously known cases of Bovine papillomavirus (BPV) and Canine distemper virus (CDV). Previous diagnosis was made with PCR for related virus from the same tissue before paraffin embedding. An average time of 3 months passed between first diagnosis and this study. Sections of 10 micrometer were taken from blocks and were put in polystyrene tubes. Sections of 10 micrometer were taken from blocks and were put in polystyrene tubes.

Nucleic Acid Extraction

Total DNA and RNA extraction of the tissues were achieved by method described by Pikor et al. (2011) with minor modification. For RNA isolation no RNase was added to elute total RNA as a modification. Extracts of the samples were kept at -20 0C for further analyses.

PCR and RT-PCR of the samples

MY09/MY11 primer pair was used to detect bovine papillomavirus nucleic acid with the same conditions described by Ogawa et al. Expected amplicon size was 450 base pairs (bp). Reverse transcription was performed for CDV sample extracts before performing PCR using Applied Biosystems cDNA reverse transcription kit according to manufacturer's instructions. A primer pair targeting N gene described by Frisk et al. (1999) was used to detect CDV nucleic acid. PCR conditions were programmed according to the publication. Expected amplicon size was 287 bp for CDV samples. Results of the PCR and RT-PCR test were visualized using a UV transilluminator, with running at 100V in a 1% agarose gel with ethidium bromide.

Results

Four paraffin-embedded tissues of BPV cases had correct sized amplicons and deemed as positives (Figure 1). Two of the paraffin-embedded tissues of CDV had correct sized amplicons

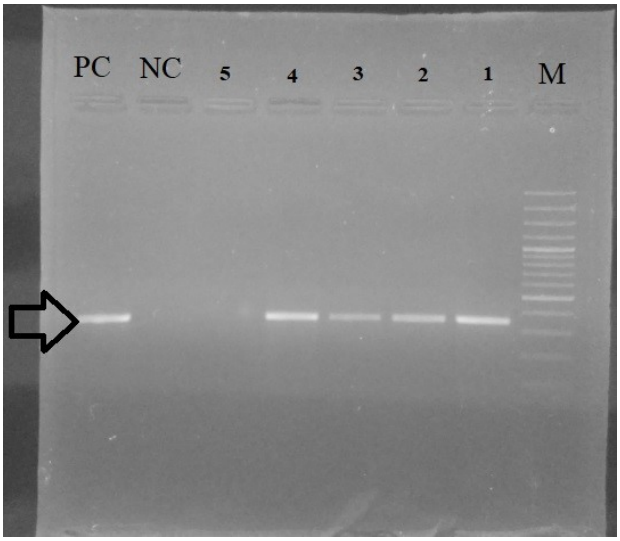

Fig. 1. Agarose gel image of BPV samples. PC: Positive Control, NC: Negative Control, Lanes 1-5 samples, M: Marker, Invitrogen 100 bp (600 bp and 1500 bp are brighter for easy reference)

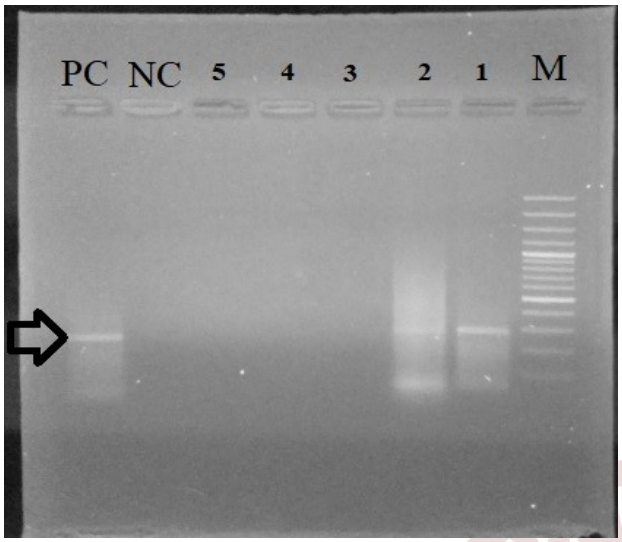

Fig. 2. Agarose gel image of CDV samples. PC: Positive Control, NC: Negative Control, Lanes 1-5 samples, M: Marker, Thermo-Scientific 100 bp plus (500 bp and 1500 bp are brighter for easy reference)

and deemed as positives (Figure 2). The positivity ratio of DNA virus was found to be 4/5 and positivity ratio for RNA virus was found to be 2/5.

Discussion

Paraffin-embedded tissue blocks are not frequently used as material for molecular studies in veterinary medicine. The primary cause of this situation is the lack of current studies which could create awareness about the diagnostic value of these material.

Detecting DNA and RNA viruses from paraffin embedded tissue blocks has both advantages and disadvantages in different aspects. Amount of incubation time is one of the disadvantages, lysis step of the tissue includes digesting the tissue with proteinase K at 57 0C. This procedure can take up to 5 days as described by Pikor et al. (2011). Especially longer waiting times at this temperature can lead to degradation of nucleic acids (especially RNA) causing low yield, this can lead to false negativity. There is also loss of genetic material in preparation steps of paraffin blocks and gradual degradation over time, this can have very significant consequences if virus load is low (Howe et al., 1997). We studied positive cases, which were tested positive before paraffin embedding procedure. There is 4 positives and one negative for BPV, and 2 positives and 3 negatives for CDV. Obviously, negatives are false negatives which can be caused by reasons mentioned above. Positive cases with higher viral loads are more likely to result in positive when paraffin embedded blocks are used. When designing studies, this fact must be taken into consideration and aim of the study should focus on finding positive cases among a selection of redundant samples. Our sample size is too small to make certain conclusions but with the previous work done (Karakurt et al., 2022) inconsistencies with results can be expected. For example one case can be identified as positive histopathologically, but can be (falsely) negative with PCR because of low viral load. Study design should be done in such a way that this situation has minimal effect on overall results. Multidisciplinary study design is one of the ways to mitigate this problem.

There may be advantageous instances when working with paraffin embedded blocks, one of which is being able to choose the histopathological region precisely and focus on areas where virus related lesions are present (e.g. inclusion bodies). Sections can be taken from places where more histopathological lesions are present for higher viral load, rather than using an organ sample where no visible lesion is present. This is especially important when working in retrospective studies, a possible histopathological lesion can lead to positive results.

In conclusion paraffin embedded tissue blocks are valuable for molecular study as material, especially when no organ material is present. However, for obtaining optimal results study design should be planned according to issues summarized in this study.

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Ethical approval

Ethical approval is not necessary for this research

Conflict of interest

Not applicable

Author contribution

Not applicable

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