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ORIGINAL ARTICLE

EVALUATION OF LYOPHILIZED PCR MIXES STABLE AT ROOM TEMPERATURE FOR DETECTION OF VIRUSES

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Polymerase chain reaction is the most common laboratory method used in diagnosis of infectious diseases. It still requires cold-chain transportation and storage of reaction components, adequate instrumentation, as well as a skilled specialist during the preparation of reaction mixes. This study was focused on the evaluation of pre-prepared freeze-dried PCR mixes for virus detection in ready-to-use form. PCR mixes with new commercial glycerol-free DNA polymerase, cryoprotectants as trehalose, dextran or mannitol and other PCR components were lyophilized and stored at room temperature for several months. Our study revealed that 10 % trehalose, 5 % dextran and their combination stabilized PCR mixes for the detection of porcine circovirus 2 (PCV2) and hepatitis E virus (HEV) for 3 months. The freeze-dried PCR mixes detected those viruses in concentrated and 10 to 100-fold diluted clinical samples. The lyophilized PCR mixes containing commercially available glycerol-free DNA polymerase stabilized with sugar cryoprotectants were suitable for the preparation of diagnostic PCR kits.

Key words: cryoprotectant; dextran; HEV; lyophilization; PCR; PCV2; trehalose

INTRODUCTION

Polymerase chain reaction represents a gold standard in molecular biology for nucleic acid amplification and detection, and it is widely used in the diagnosis of human and animal infectious diseases [1]. Over recent decades, various modifications of PCR methods for the detection of different viruses have been developed, ranging from single PCR, reverse transcriptase PCR, nested PCR, multiplex PCR [2], quantitative PCR [3], through to digital droplet PCR [4]. However, all of these methods require cold-chain transportation and storage, adequate instrumentation, as well as skilled specialists conducting the laboratory process. Moreover, multiple pipetting steps of small volumes (1-10 µl) may lead to errors during the preparation of PCR mixes.

The pre-prepared lyophilized PCR mixes (instant PCR) present a way to simplify PCR assays for the detection of microorganisms. Such mixes may be stored at room temperature and are ready to use. Instant PCR minimizes the handling of PCR reagents, including pipetting small liquid volumes with possible errors, requires less skilled personnel, and minimizes the risk of contamination. Therefore, the preparation of dry PCR mixes for the detection of bacterial infections [5, 6, 7, 8] is not surprising. In addition, the approach has also been used for the detection of viruses such as hepatitis B virus [9] and other viruses [8, 10, 11, 12].

However, experience in different laboratories has shown that the preparation of mixes for instant PCR using lyophilization can meet problems. Glycerol, which is used for the protection of DNA polymerase during low-temperature storage (-20 °C), cannot be completely removed by sublimation because of its super hygroscopicity. To stabilize DNA polymerase activity during lyophilization, additional cryoprotectants have also been used in freeze-dried mixes, most commonly sugars, including disaccharides and polymeric saccharides [13].

Recently, commercial glycerol-free DNA polymerase has become available. This form of enzyme opens up new possibilities for the development of instant PCR assays using lyophilized reagents. The aim of this study was to evaluate the stability of freeze-dried, easy-to-use PCR mixes containing glycerol-free DNA polymerase for instant PCR to detect viruses in clinical samples. To keep the enzyme stable, three different types of cryoprotectants (trehalose, dextran, and mannitol) and a combination of trehalose with dextran were added to our PCR reaction mixes. The lyophilized mixtures were tested for the detection of the DNA porcine circovirus 2 (PCV2), which causes post-weaning multiple systemic wasting syndrome in pigs, and hepatitis E virus (HEV), a zoonotic RNA virus that infects pigs and can cause hepatitis E in humans.

MATERIALS AND METHODS

Experimental strategy and viruses

In the development stage of this project, the testing of glycerol-free lyophilized mixes was carried out with two positive clinical samples confirmed by classic PCR (without cryoprotectant) found in our previous works. One sample originated from a naturally PCV2-infected pig on a Slovakian farm [14], and the other one from liver tissue of a naturally HEV infected hunted wild boar [15]. Subsequently, optimal lyophilized PCR mixes were used for the detection of both viruses on a small collection of clinical samples which were previously positive by classic PCR.

DNA/RNA isolation

Pooled organ suspensions (tonsils, spleen, and kidney) from PCV2-infected pigs were prepared as 20 % w/v (weight/volume) tissue homogenates in PBS (Calbiochem, Darmstadt, Germany) or nuclease-free water (SERVA Electrophoresis GmbH, Heidelberg, Germany). Total DNA was extracted from 200 µl of tissue homogenate using Chelex resins (Bio-Rad Laboratories, Inc., Hercules, California, USA) according to manufacturer's recommendations.

Liver samples from wild boars and stool samples collected from human patients suspected of infection with HEV were homogenized in a 1 ml saline solution (Merck Millipore Corp., Rahway, New Jersey, USA). RNA was isolated from 400 µl of homogenate using the MagMAX™ Viral RNA Isolation Kit (Thermo Fisher Scientific, Inc., Vilnius, Lithuania) according to the manufacturer´s protocol and then stored at -80 °C.

cDNA synthesis

For HEV detection, the isolated RNA was transcribed to cDNA to be used with the lyophilized PCR mix. cDNA was synthesized using random hexamers and Premium reverse transcriptase with 1xRT buffer (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA), as described by Jacková et al. [15].

Cryoprotectants

Three different types of cryoprotectants added to the PCR mix were tested individually, namely D-(+)-Trehalose dihydrate (Merck, GmbH, Darmstadt, Germany), Dextran 100 (SERVA Electrophoresis GmbH, Heidelberg, Germany), and D-mannitol (Sigma-Aldrich, Burlington, Massachusetts, USA). They were used in different concentrations: 1, 3, and 5 % final concentration (w/v) for dextran and mannitol; 5, 10, and 15 % final concentration (w/v) for trehalose. Thereafter, stabilizers were evaluated at the optimal combination 10 % trehalose + 5 % dextran.

Preparation of lyophilized PCR mixtures

The reaction mixture was composed of 1x Standard Buffer (Ampliqon, Odense, Denmark), 0.2 mM dNTPs (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA), 300 nM of forward/reverse primers, and cryoprotectants in various concentrations (w/v). Finally, 1 U Taq DNA polymerase, glycerol-free (Ampliqon, Odense, Denmark), and molecular biology grade water (Merck, GmbH, Darmstadt, Germany) were added to give a final 23 µl volume. The published PCR primers flanking a 263 bp fragment of ORF2 for detection of PCV2 [16] and a 514 bp fragment from ORF1 for detection of HEV [17] were used in PCR assays.

The PCR mixes were frozen at -20 °C overnight, and before lyophilization they were placed at -70 °C for an hour. The lyophilization was performed according to protocol, which is generally used for the stabilization of drugs and vaccines at our university. The freeze-drying process was carried out in a CoolSafe Freeze Dryer (LaboGene, Allerød, Denmark) at -54°C and a starting pressure of 8,423 kPa for 24 h. After 24 h the pressure dropped to approximately 0,061 kPa, and then PCR mixes were lyophilized. The PCR tubes containing the dried reagent mixes were enclosed with parafilm (Sigma-Aldrich, Burlington, Massachusetts, USA) and stored at room temperature (20 °C – 23 °C).

PCR with lyophilized mixes

To the lyophilized PCR mix, 23 µl of molecular biology grade water were added, then vortexed briefly to dissolve the dried pellet. Subsequently, 2 µl of DNA (PCV2 detection) or cDNA (HEV detection) was added to the reaction. PCR was carried out under the following thermal profile: 1 cycle at 95 °C for 5 min, and 35 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C (HEV) and 62 °C (PCV2) for 40 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min using Thermocycler C1000 (Bio-Rad Laboratories, Inc., Hercules, California, USA). The PCR amplification was performed periodically on the day of lyophilization (Day 0) and on Day 30, Day 60 and Day 90 after lyophilization. Electrophoresis was carried out in 2 % agarose gels, stained with GelRed™ (Biotium, Inc., San Francisco, California, USA) and visualized by Gel Doc EZ imager (Bio-Rad Laboratories, Inc., Hercules, California, USA).

Detection of viruses in clinical samples

The lyophilized glycerol-free PCR mixes with optimal chemical composition were used for detection of viruses in different clinical samples. PCV2 was detected in four organ suspensions from naturally infected pigs. To detect HEV, four liver samples from wild boars and four human stool samples from patients suspected of having hepatitis E were chosen.

RESULTS

Evaluation of physical properties of lyophilized PCR mixtures

In preliminary experiments (data not shown), the optimal concentration of cryoprotectants was found. Finally, three various cryoprotectants at the optimal concentration were used for lyophilization in subsequent experiments.

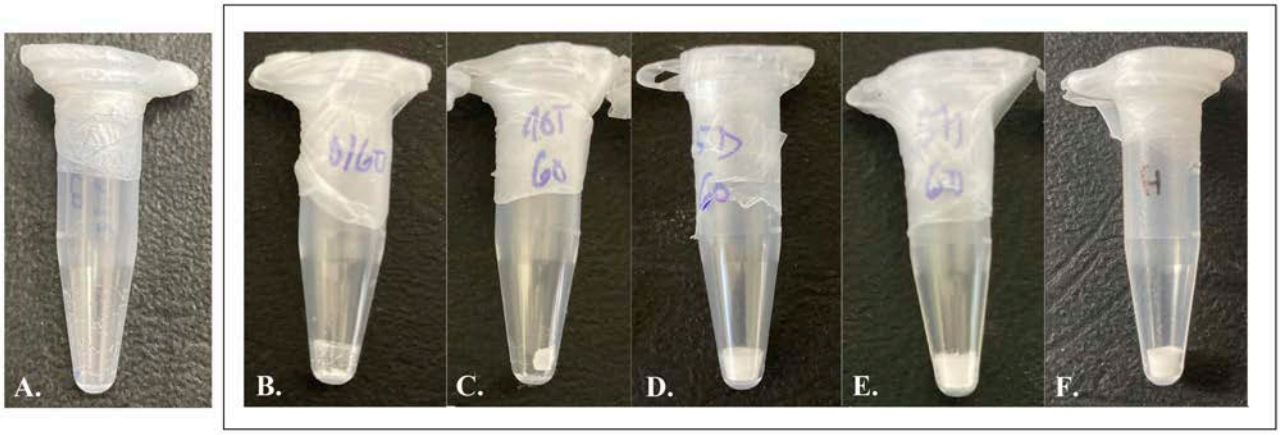

Fig. 1. Physical appearance of lyophilized PCR mixes. A. PCR mix containing glycerol. In frame (B.-F.) are glycerol-free-PCR mixes, B. PCR mix without protectants, C. PCR mix with 10 % trehalose, D. PCR mix with 5 % dextran, E. PCR mix with 5 % D-mannitol, F. PCR mix with 10 % trehalose + 5 % dextran

We observed various physical appearances of dried PCR mixes. While PCR mixes containing glycerol protected DNA polymerase seemed liquid without creating a powder (Fig. 1A), cryoprotectant-glycerol-free mixes appeared as a gentle dust (Fig. 1B). Mixes containing 10 % trehalose appeared as a light sticky, cake (Fig. 1C). Mixes with 5 % dextran, 5 % D-mannitol, and 10 % trehalose + 5 % dextran were observed after lyophilization as solid powder cakes with good appearance (Fig. 1 D – F). The dried mixes in the reaction tubes were stored at room temperature for analysis immediately after lyophilization and after 30, 60, and 90 days of storage.

The use of dried PCR mixes for detection of viruses

The PCR amplification was verified by the intensity of electrophoretic bands for DNA amplicons. To show results as clearly as possible, the electrophoretic bands for Day 30 and Day 60 have not been presented in Fig. 2 and Fig. 3, because the intensity of the bands gradually decreased to Day 90 or the bands were stable throughout 90 days.

Detection of PCV2 (Fig. 2A)

The lyophilized glycerol-free PCR mixes without cryoprotectants could detect the virus at Day 0 only but not after 30–90 days of storage. Similar intensity of electrophoretic bands was seen with concentrated as well as 10-fold and 100-fold diluted samples at Day 0 and Day 90 with PCR mixes containing 10 % trehalose or 5 % dextran. The combination of both cryoprotectants produced bands with stronger intensities than other mixes. The mixes stabilized with all concentrations of mannitol stop to work after 60 days of storage.

Detection of HEV (Fig. 2B)

The lyophilized glycerol-free reaction mixes with no cryoprotectant could detect virus in concentrated samples on Day 0 only. The presence of cryoprotectants significantly improved results of PCR amplification. Virus could be detected with similar intensity of bands at Day 0 and Day 90 in the presence of 10 % trehalose, 5 % dextran, as well as with a combination of 10 % trehalose + 5 % dextran in concentrated samples. Glycerol-free mixes with 5 % dextran were also able to detect virus in a 10-fold diluted sample. Mannitol at all concentrations did not protect PCR mixes even after 30 days of storage.

Detection of viruses in clinical samples

Following the above results, viruses in other clinical samples were detected with lyophilized solutions containing glycerol-free DNA polymerase protected with 10 % trehalose or a mixture of 10 % trehalose + 5 % dextran. Results with 5 % dextran did not confirm positive detection of viruses presented in Fig. 2 and they are not presented in Fig. 3.

The detection of PCV2 was successful up to Day 90 in almost all four clinical samples (Fig. 3A, lanes 1 – 4). PCR mixes stabilized by a mixture of 10 % trehalose + 5 % dextran provided more intensive electrophoretic bands than were observed with 10 % trehalose only.

Overall, four stool samples collected from human patients suspected of having hepatitis E (Fig. 3B, lanes 5 – 8) as well as four liver samples from wild boars (Fig. 3B, lanes 9 – 12) were used for HEV detection. On Day 0 and Day 90, virus could be detected in all four stool samples but only in one liver sample (lane 10).

DISCUSSION

This work has been initially inspired by McGoldrick et al. [18] who lyophilized in the lid of a PCR tube Taq DNA polymerase stored in glycerol together with trehalose and other reaction components for nested (second) PCR to detect pestiviruses. When the first PCR was finished, the tube was inverted, and the lyophilized reaction components were dissolved by shaking a tube to start the nested PCR with fresh chemicals. Renewed impetus for this project came when glycerol-free DNA polymerase recently appeared on the market. Here we present results that show that this form of enzyme is better for the preparation of lyophilized PCR mixes containing the cryoprotectants trehalose and dextran or their mixture. The glycerol-free lyophilized mixes stayed stable for at least 3 months at room temperature and could be reliably used for the detection of viruses in clinical samples, as demonstrated using PCV2 and HEV.

Generally, the freeze-drying method is utilized for various applications, such as stabilization of biological samples (proteins, peptides) and drug products at ambient temperature [19]. To stabilize samples during lyophilization, additives like polyethylen glycol, dextran, mannitol [20], collagen [21], bovine serum albumin [10], sorbitol,

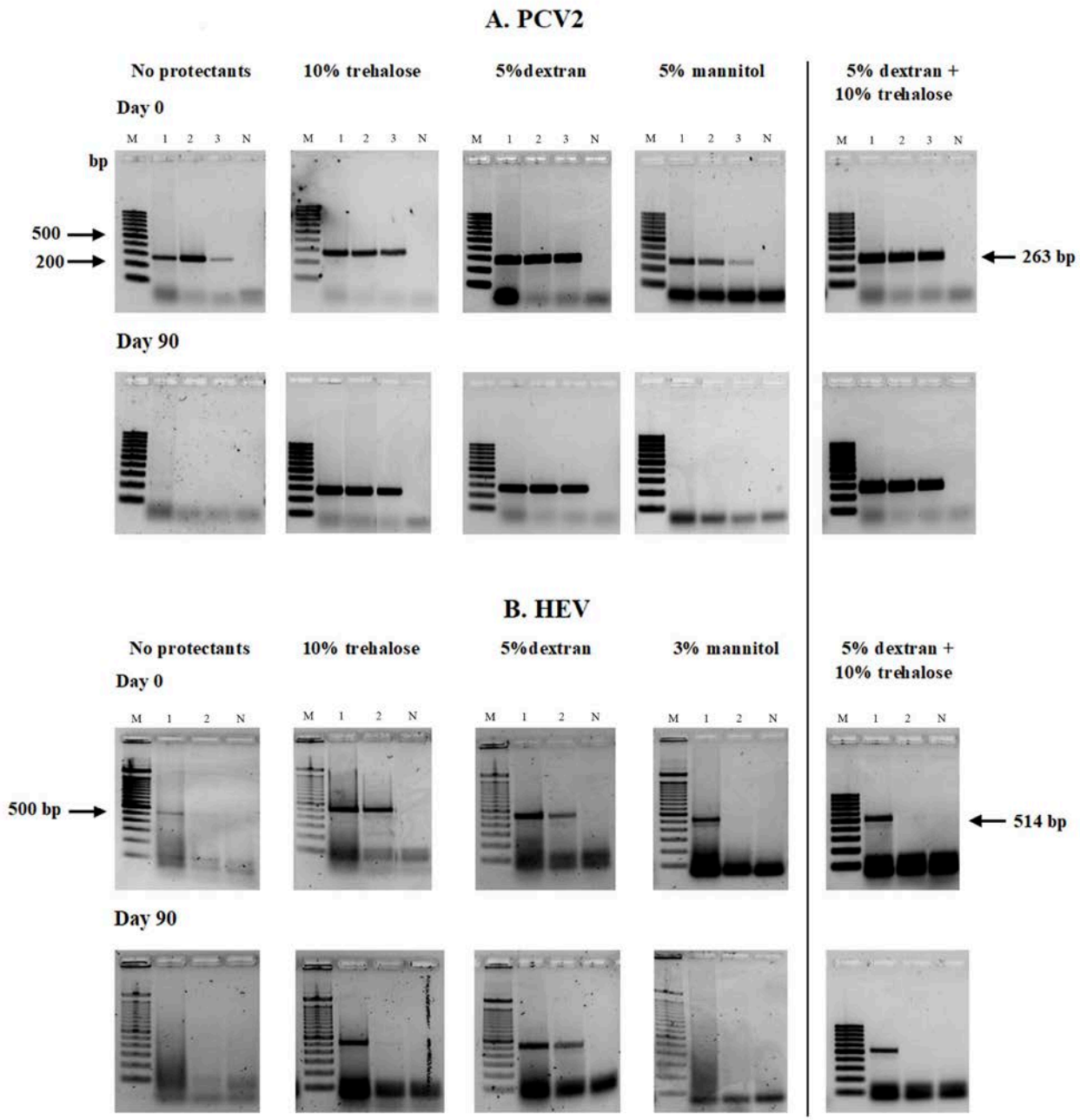

Fig. 2. Results of different cryoprotectants used on Day 0 (the day of lyophilization) and up to Day 90. A. PCV2 virus (total DNA in concentrated sample: 230 ng/µl), B. Hepatitis E virus (total RNA in concentrated sample: 138 ng/µl). Lanes: M, 100 bp ladder (Gene Ruler 100 bp, ThermoFisherScientific for PCV2 and combination of cryoprotectants for HEV; Invitrogen 100 bp, ThermoFisherScientific for HEV with individual cryoprotectant); 1, concentrated sample; 2, 10-fold diluted sample; 3, 100-fold diluted sample; N, negative control

gelatin, raffinose, Ficoll 400, and polyvinylpyrrolidone [9] have been used. At present, trehalose usually represents the most effective stabilizer during lyophilization.

In early experiments, trehalose found application for lyophilization of PCR mixes with glycerol-containing DNA polymerase for detection of various bacteria, e.g. *Mycobacteria* spp. [6], *Vibrio cholerae* [22], *Entamoeba* spp. [23], and others [5, 24, 25]. In addition, lyophilized real-time one-step RT-qPCR assays composed of glycerol DNA polymerase with sugar additives were developed to detect a few types of viruses, including foot-and-mouth disease virus [26], chikungunya virus, Rift Valley fever phlebovirus [27], as well as SARS-CoV-2 [10, 11].

Difficulty with sublimation of glycerol can be minimized by using glycerol-free PCR enzymes. There have been few reports where glycerol-free DNA polymerase has been used for the preparation of instant PCR. Khazani et al. [5] used such a form of polymerase for lyophilization in a

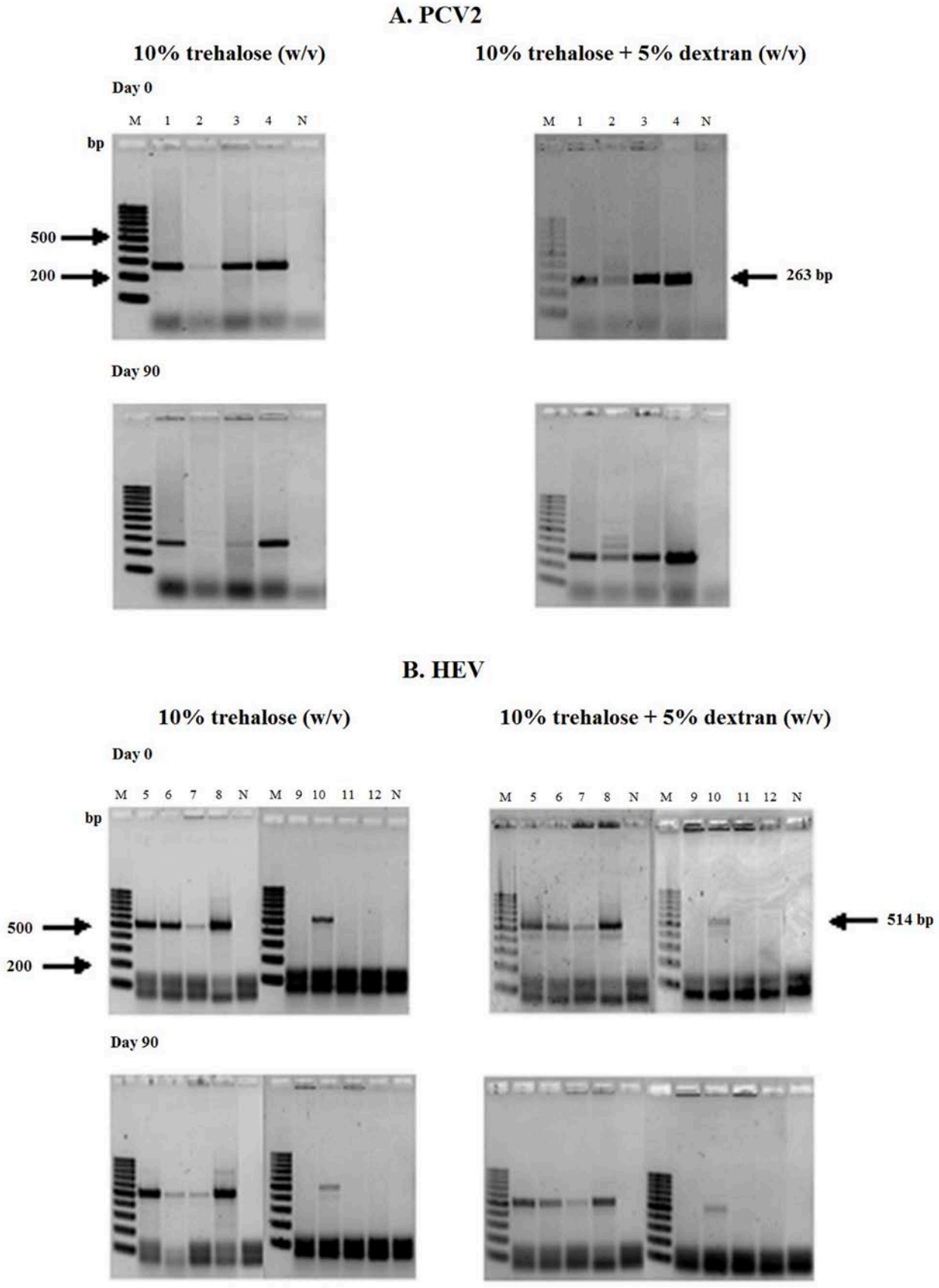

Fig. 3. Results of the presence of two types of cryoprotectants on clinical samples at Day 0 and up to Day 90. A. PCV2 virus, B. Hepatitis E virus. Lanes: M, 100 bp ladder (Gene Ruler 100 bp, ThermoFisherScientific); 1-4, organ suspensions from pigs; 5-8, human stool samples; 9-12, liver samples from wild boars; N, negative control

one-step PCR assay for detection of *Klebsiella pneumoniae* and *Haemophilus influenzae.* For virus detection, Yang and Wen [9] used self-prepared glycerol-free components for lyophilization of real-time PCR mixes for detection of the hepatitis B virus. Now commercially available glycerol-free DNA polymerase simplifies the whole process of instant PCR preparation.

Systematic analysis focused on the stability of lyophilized PCR mixes at -86 °C, -20 °C, 4 °C, 37 °C, and 56 °C showed that higher storage temperatures lead to reduced PCR efficiency [6, 8, 11, 25-27]. Since lower temperatures require refrigeration, we stored our reaction mixes at room temperature. In our hands, glycerol-free TaqDNA polymerase protected with sugar stabilizers in lyophilized mixes was stable for at least 3 months. In contrast, Agel and Sagcan [7] published only 2 weeks of stabilization of lyophilized mixes containing glycerol DNA polymerase and sugar additive for the diagnosis of tuberculosis using instant PCR assay. The lyophilized real-time one-step RT-qPCR mixes utilizing glycerol DNA polymerase and sugar stabilizer for detection of various viruses maintained activity at room temperature up to 30 days [10, 11, 12].

We have to point out that detection of HEV, which is RNA virus, was not carried out with lyophilized PCR mix in one-step due to problems with sublimation of glycerol protected reverse transcriptase. Therefore, we prepared cDNA by classical reverse transcription of HEV RNA to cDNA, which was used in the next instant PCR step. Meanwhile, glycerol free reverse transcriptase becomes available on the market. This form of enzyme will simplify its application in the freeze-drying process.

The combination of different cryoprotectants has synergic action [28, 29]. In our study, we also observed a synergic protective effect using trehalose with dextran in comparison to mixes with a single stabilizer.

We also tested pre-prepared glycerol-free PCR mixes on a limited collection of clinical samples. In all cases, viruses were detected in lymphoid organs (PCV2), liver homogenate, and human patient stool samples (HEV). These encouraging results show that preparation of diagnostic kits using lyophilized PCR mixes in the future is possible.

We believe that freeze-dried glycerol-free PCR components protected by sugar cryoprotectants can be used soon in the field where conditions for preservation of classical reaction mixes are minimal. Application of pre-prepared mixes is possible with portable PCR machines for the rapid diagnosis of various diseases directly under field conditions. In addition, such mixes can find application in countries where refrigeration capacities are limited.

CONCLUSIONS

In summary, our work demonstrated that the preparation and long-time storage at room temperature of lyophilized PCR mixes with glycerol-free DNA polymerase is a promising direction for the development of instant PCR assays. The cryoprotectants such as trehalose, dextran, and their combination can effectively protect enzymes and pre-prepared lyophilized glycerol-free PCR mixes and make them suitable for the detection of viruses in clinical samples.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Statement

The protocol for collection of animal clinical samples followed the guidelines stated in the Guide for the Care and Use of Animals (protocol number 3323/16–221/3) which was approved by the State Veterinary and Food Administration of the Slovak Republic and by Ethics Commission of the University of Veterinary Medicine and Pharmacy in Kosice, Slovakia. The collection of human stool samples was carried out in accordance with the International Code of Medical Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethics Committee of the Louis Pasteur University Hospital in Kosice, Slovakia (No. 104/2011).

Data Availability

The preprint of this manuscript is openly available in the online collaborative writing tool, Authorea, at http://doi.org/10.22541/au.172959374.48638715/v1.

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Generative AI Statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Authors' Contributions

Božena Kočíková (Investigation – Equal, Methodology – Equal, Validation – Equal, Visualisation – Lead, Writing – original draft – Equal, Writing – review and editing – Equal), Alica Pavlová (Writing – review and editing – Equal), Anna Jacková (Funding acquisition – Equal, Supervision – Equal, Methodology – Supporting, Validation – equal, Writing – review and editing – Equal), Štefan Vilček (Conceptualization – Lead, Funding acquisition – Lead, Investigation – Lead, Methodology – Lead, Project administration – Lead, Supervision – Lead, Validation – Lead, Visualisation – Equal, Writing – review and editing – Lead).

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