Ribonucleic Acid Degradation and Diagnostic Testing

Subjects: Veterinary Sciences

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Successful downstream molecular analyses of viral ribonucleic acid (RNA) in diagnostic laboratories, e.g., reverse transcription-quantitative polymerase chain reaction (RT-qPCR) or next-generation sequencing, are dependent on the quality of the RNA in the specimen. In swine specimens, preserving the integrity of RNA requires proper sample handling at the time the sample is collected on the farm, during transport, and in the laboratory until RNA extraction is performed. Options for proper handling are limited to maintaining the cold chain or using commercial specimen storage matrices.

Keywords: swine viruses ; viral RNA ; RNA stability ; diagnostic specimens ; sample storage ; molecular diagnostics

1. Introduction

Common swine ribonucleic acid (RNA) viruses, e.g., porcine reproductive and respiratory syndrome virus (PRRSV), porcine coronaviruses, swine influenza A virus, and others, are a threat to pig health and welfare. Measures taken to assess their presence on the farm require collecting specimens, e.g., serum, oral fluid, processing fluid, feces, environmental samples, semen, swabs, and tissues [1][2] for molecular testing, e.g., reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In turn, these test results form the basis for decisions concerning their prevention and control. Regardless of sample status at the time of collection on the farm, RT-qPCR results reflect the quality and quantity of the target nucleic acid in the sample at the moment it is processed for testing in the laboratory [3][4]. However, between the time the sample is collected on the farm, packaged, shipped, and finally tested in the laboratory, it may have been exposed to handling conditions that adversely affect the RNA in the specimen and, therefore, the subsequent RT-qPCR test results. Notably, RNA is of more concern than deoxyribonucleic acid (DNA) in this regard because RNA molecules are susceptible to degradation via the hydrolysis of the 2′ and 3′ hydroxyl groups on their ribose residues.

2. Ribonucleic Acid and Ribonucleases (RNases)

In vivo, RNA is continuously produced, which means that an active process of catabolism is necessary to eliminate defective or obsolescent molecules and maintain population equilibrium. For the most part, this process involves RNA-degrading enzymes, i.e., ribonucleases (RNases) [5][6][7]. RNases are hydrolytic enzymes that catalyze the cleavage of phosphodiester bonds to degrade RNA molecules into smaller fragments [8]. They are classified into two main groups with several types in each group: endoribonucleases, which cleave RNA molecules internally, and exoribonucleases, which digest RNA molecules from either the 3′ or 5′ end [5][9][10]. RNases are present in all cells and found in most secretions/excretions from living organisms. For that reason, RNases are ubiquitous in the laboratory environment, i.e., on human skin, laboratory glassware, metalware, and in laboratory working solutions [11][12][13][14]. RNases are heat-tolerant, stable over a wide range of pH, and resistant to many denaturing agents [15][16]. This justifies the requirement for working with samples in laminar flow hoods, wearing personal protective equipment, using RNase/DNase-free solutions, and treating labware and working solutions with potent RNase inhibitors such as diethyl pyrocarbonate (DEPC) or ribonucleoside-vanadyl complexes [13][17].

RNase A is the enzyme of main concern because it is ubiquitous [3][18]. A heat-resistant endoribonuclease, RNAse A, was first identified in 1920 [19], although it was not recognized as a ribonuclease until the 1930s [20][21][22][23].

3. Ribonucleic Acid Degradation and Testing

RNA includes both coding RNA or messenger RNA (mRNA) and non-coding RNAs, i.e., transfer RNA, ribosomal RNA, and small and long RNAs. Both coding and non-coding RNAs are recovered through the nucleic acid extraction procedure and targeted through polymerase chain reaction (PCR) primers and probes in the amplification step [24][25][26]. Hence, the responsibility of the veterinarian and the diagnostician is to protect the integrity of all RNA present in a diagnostic specimen. The “minimum information for publication of quantitative real-time PCR experiments (MIQE)” guidelines

recognize sample storage as a key component in generating reliable and reproducible quantitative PCR (qPCR) data [27]. After diagnostic specimens are collected, and at any point during transport and storage, RNA degradation can occur through the action of ubiquitous, extracellular RNases that cleave RNA into fragments that are no longer recognizable by PCR primers and probes [5][28]. During cell lysis, RNases may be released from any specimen [12] but particularly from specimens with high RNase activity, e.g., pancreas, spleen, and lung [29][30][31]. Thus, extracellular RNases represent the primary threat to RNA integrity in molecular diagnostics [32][33].

Data on the effect of storage temperature on pathogen-specific RNA are sparse in the refereed literature, but the general effect is well established: RNA stability increases as temperature decreases; hence, the rule to keep samples at low temperatures, e.g., 4 °C, −20 °C, or −80 °C. A further complication is the fact that the temperature-dependent RNA decay rate varies among specimen types. For example, PRRSV RNA was relatively stable in serum at 4, 10, and 20 °C for 7 days, but a constant decline in PRRSV RNA concentration was observed over time in oral fluids and feces held at the same temperatures [34].

The need to preserve targets of interest in diagnostic specimens has been a topic of research since the 1920s [35][36]:

- Freeze-drying (lyophilization). With the goal of finding a method to "*send active virus in small, sealed containers on sea voyages lasting over a month, and for long-term storage in the laboratory for several months without serious loss of virulence*," in 1929, Sawyer reported that yellow fever virus could be preserved for over 155 days in "vacuum-dried" blood stored in sealed containers and refrigerated [36]. Lyophilization consists of freezing samples to immobilize water molecules and then placing them in a vacuum where the frozen water is vaporized, resulting in a dried specimen. This allows for prolonged storage of viruses in biological specimens that otherwise would be unstable in aqueous solutions [37]. In terms of nucleic acid stability, lyophilization is mostly used in vaccine production to preserve viral antigens and adjuvants to extend their shelf lives [38].

- Viral transport medium (VTM). Attempts to improve virus storage have been described since the 1930s. Cook and Hudson [39] compared saline, water, human oral fluid, and serum (rabbit, sheep) and reported that sheep serum optimally preserved St. Louis encephalitis virus stored at 37 °C for 24 h. VTM consists of a mixture typically containing a buffered salt solution to maintain pH, antibiotics to prevent viral contamination, protein stabilizers (e.g., bovine serum albumin), and other additives intended to preserve viral integrity [40]. Although widely used for swab specimens, e.g., oral, nasopharyngeal, oropharyngeal, genital, and fecal swabs, VTM does not suit liquid specimens such as blood, serum, oral fluid, urine, etc. [41].

- Untreated filter paper. The use of untreated filter paper (Guthrie Cards) for the transport and long-term storage of blood and urine began in the 1960s to detect phenylketonuria in infants [42]. Filter paper has long been used for storing and transporting fluid specimens, e.g., blood, saliva, and feces, intended for different assays, e.g., chemical assays, drug monitoring, nucleic acid or antigen detection, and serological markers for disease diagnostics. Nonetheless, filter paper is not typically used in routine viral diagnostics because eluting nucleic acids from specimens dried on the paper can lead to poor recovery and low nucleic acid yield [43].

Since accurate molecular testing is dependent on the quality and quantity of the nucleic acid material in the specimen, delivering intact RNA to the diagnostic laboratory is mandatory if reliable results are to be produced [3][44]. Although specimen stabilization technologies have been researched for over 100 years, the standard approach to RNA preservation remains the cold chain, i.e., chilling or freezing the specimen immediately after collection [34][45][46][47]. However, alternative approaches based on the use of commercial storage matrices emerged in the 1990s [48][49], and numerous commercial products are currently available. The majority of these products are liquids to be combined with samples, but they also include solid surfaces onto which samples are spotted and dried. With some exceptions, these products are virucidal; thus, virus isolation or propagation is no longer an option.

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