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Identification of small non-coding RNA classes expressed in swine whole blood during HP-PRRSV infection

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ABSTRACT

It has been established that reduced susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV) has a genetic component. This genetic component may take the form of small non-coding RNAs (sncRNA), which are molecules that function as regulators of gene expression. Various sncRNAs have emerged as having an important role in the immune system in humans. The study uses transcriptomic read counts to profile the type and quantity of both well and lesser characterized sncRNAs, such as microRNAs and small nucleolar RNAs to identify and quantify the classes of sncRNA expressed in whole blood between healthy and highly pathogenic PRRSV-infected pigs. Our results returned evidence on nine classes of sncRNA, four of which were consistently statistically significantly different based on Fisher's Exact Test, that can be detected and possibly interrogated for their effect on host dysregulation during PRRSV infections.

1. Introduction

For decades, researchers have been exploring the many complications to swine health caused by porcine reproductive and respiratory syndrome virus (PRRSV) in order to find ways of promoting resistance and tolerance that lessen losses in commercial pig populations (Goyal, 1993; Lunney et al., 2010; Nieuwenhuis et al., 2012; Holtkamp et al., 2013). The virus itself is a single strand positive RNA virus of the Arteriviridae family within the Nidovirales order. The virus, which attacks and replicates in lung monocytic cells, (Lunney et al., 2010, 2016; Gomez-Laguna et al., 2013; Wang et al., 2001) has exhibited a dual ability to affect both the respiratory and reproductive function of infected pigs. The PRRS virus itself has multiple strains that can be classified into the categories of low or high pathogenicity based on virulence potential and are usually referred to as either type 1 or type 2. The less studied of the two diseases is the highly pathogenic type 2 strain (HP-PRRSV) which can be characterized by its Chinese isolates (An et al., 2011; Dietze et al., 2011; Miller et al., 2012). The increased pathogenicity of these strains causes extreme insults to the porcine metabolic and immunological systems that move away from the persistent infection caused by the low pathogenic strains and towards acute symptoms ending in death of the animal. The increase in strain diversity has been linked to characteristic deletions in the non-structural protein 2 (NSP2) of the PRRSV genome (Faaberg et al., 2010; Kappes and Faaberg, 2015) found in the Chinese isolates. Research into the host response to HP-PRRSV has focused on the transcriptomic response of genes related to the immune system processes (Lunney et al., 2016; Miller et al., 2012, 2017, 2014; Albina et al., 1998; Koltes et al., 2015; Kommadath et al., 2017, 2009; Schroyen et al., 2015; Wang et al., 2016a). However, much of the research into the host response to HP-PRRSV has been concentrated on mRNA expression that transcribes genes related to the innate immune functions, especially those of monocyte-derived cells (MDCs) of the lung, the key route of infection (Miller et al., 2012, 2017). This interest is fueled by the intriguing ability of PRRSV to circumvent inflammatory cytokines and neutralizing antibody biogenesis in the host (Gomez-Laguna et al., 2013; Lunney et al., 2016; Albina et al., 1998; Xiao et al., 2010). The actions that allow PRRSV infections to evade immune processes and become persistent likely involves dysregulation of multiple cellular and humoral mediated immune pathways (Miller et al., 2017; Schroyen et al., 2015; Patel et al., 2010; Sun et al., 2012; Yang et al., 2017). The phenomenon of PRRSV's evasiveness of host immune functions hints at possible epigenetic regulation taking place during viral infections (Abernathy and Glaunsinger, 2015; Bowie and Unterholzner, 2008; Morales et al., 2017). Epigenetic regulation can arise through post-transcriptional modification of genes by various small non-coding RNA (sncRNA) and, although it is used by invading pathogens, it also represents an avenue of anti-viral defense by the host (Bushell and Sarnow, 2002; Hiscox, 2007; Moazed, 2009; Costa, 2010; Ouellet and Provost, 2010; Kaikkonen et al., 2011; Michaux et al., 2014; Ma et al.,

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2016; Samir and Pessler, 2016). Therefore, more needs to be done to understand the relationship of the drivers of host epigenetic regulation and viral infections such as PRRSV. Small (18 bp - < 200 bp) non-coding RNAs (sncRNAs) can be defined as multiple classes of short nucleotide sequences that do not transcribe into functional proteins, but do have epigenetic potential in their ability to perform post-translational modifications of expressed gene functions (Moazed, 2009; Costa, 2010; Kaikkonen et al., 2011; Michaux et al., 2014; Massirer and Pasquinelli, 2006; Mattick, 2009; Tuck and Tollervey, 2011). Studies of gene expression during viral infections and cancer progression have been able to link both positive and negative outcomes to changes in the type or level of certain classes of sncRNA (Moazed, 2009; Costa, 2010; Kaikkonen et al., 2011; Michaux et al., 2014; Ma et al., 2016; Massirer and Pasquinelli, 2006; Mattick, 2009; Tuck and Tollervey, 2011; Amaral et al., 2013; Martens-Uzunova et al., 2013; Gu et al., 2014; Holoch and Moazed, 2015; Stepanov et al., 2015; Herbert and Nag, 2016). As stated, the sncRNAs fall into many distinct classes based upon function.

Micro RNAs and their precursor pri-miRNAs are sncRNAs that can range from ~24 bp in their mature form to ~70 bp in their premature stem-loop form (Mattick, 2009; Bartel, 2004). They have been shown to be potent modulators of posttranscriptional regulation of gene expression, giving miRNAs the ability to generate epigenetic modifications (Massirer and Pasquinelli, 2006; Bartel, 2004). Over the past decade and a half, much data has come out about the roles and functions of miRNA involvement in host defense against viral infections, (Wang et al., 2016a; Morales et al., 2017; Samir and Pessler, 2016; Martens-Uzunova et al., 2013) as well as, it's potential as a double-edged sword that can possibly aid invading pathogens to subvert host immune machinery (Hiscox, 2007; Ouellet and Provost, 2010; Samir and Pessler, 2016; Martens-Uzunova et al., 2013; Herbert and Nag, 2016).

Another large group sncRNAs are the transfer RNAs (tRNA) that function to transport amino acids as part of mRNA translation in the creation of proteins (Mattick, 2009; Phizicky and Hopper, 2010; Randau and Soll, 2008). Transfer RNAs are ~70–100 bp long structural non-coding RNAs that contain several internal stem-loops referred to as the D, T, and anticodon loop and as a molecule is usually highly modified by methyltransferases to guide tRNA functions in vivo (Hori, 2014). The tRNAs genes also exist as precursors of smaller ~16–40 bp versions of themselves, called transfer RNA fragments (tRFs) that can participate in epigenetic modifications of gene expression (Michaux et al., 2014; Gu et al., 2014; Gebetsberger and Polacek, 2013). The tRFs fall into 5 groups based upon the portion of the mature tRNA in which they are derived (Randau and Soll, 2008; Keam and Hutvagner, 2015; Kumar et al., 2016)and represent a growing area of study for host –viral interactions in humans and livestock (Casas et al., 2015; Ivanov, 2015).

Another group of small non-coding regulatory RNAs are the ~30–400 bp small nucleolar RNAs (snoRNAs). The biogenesis of snoRNAs takes place within the nucleolus of host cells with their classical functions being as guides to rRNA modifications (Costa, 2010; Tuck and Tollervey, 2011; Reichow et al., 2007), but have also been shown to be involved in additional epigenetic nucleotide modifications of small nuclear RNAs (snRNA). The snoRNAs form part of larger ribonucleoprotein units termed snoRNPs that they help guide to specific modifications based on their sequence motif. These sequence motifs define the 2 main classes of snoRNA; the SNORDs and SNORAs. The SNORDs are the C/D Box-small nucleolar RNAs, that guide 2′-O-ribose methylation, while the SNORAs are the H/ACA-small nucleolar RNAs responsible for pseudouridylation (Reichow et al., 2007; Dieci et al., 2009; Watkins and Bohnsack, 2012). There is also a third smaller group of snoRNAs referred to as small cajal body specific RNAs (scaRNAs), that contain motifs allowing it to perform either methylation or pseudouridylation of spliceosomal RNA (Mattick, 2009; Reichow et al., 2007; Dieci et al., 2009).

Spliceosomal RNAs, also known as small nuclear RNAs (snRNAs) are a group of sncRNAs that along with small nuclear ribonucleoproteins (snRNP) form components of the spliceosome machinery involved in the removal of intronic sequences from pre-mature mRNA that give rise to functional gene products and alternatively spliced isoforms (Hernandez, 2001; Fischer et al., 2011; Valadkhan and Gunawardane, 2013). Spliceosomal RNAs are numerous structural sncRNAs that include a major group of highly conserved molecules labeled U1, U2, U4, U5, and U6, there is also a secondary category termed as minor spliceosomal RNA, that includes the sncRNAs U11, U12, U4atac, and U6atac (Hernandez, 2001; Fischer et al., 2011; Turunen et al., 2013; Wahl et al., 2009). Spliceosomal RNAs are key to mRNA maturation and diversity through alternative splicing events, making them critical to the proper functioning of a gene needed to perform a task such as antiviral defense. The last two groups of sncRNAs examined in our study were the vault RNAs and the Y-RNA, two groups of less characterized epigenetic modifiers. Vault RNAs are large cytosolic ribonucleoprotein complexes that have been associated with drug resistant cellular activity (van Zon et al., 2001, 2003; Gopinath et al., 2005). The Y-RNAs are considered to be initiators of DNA replication and that perturbations in Y-RNA can suppress DNA replication (Christov et al., 2006; Krude et al., 2009).

Understanding the nature and role of the different encoded functions of the immune system during PRRSV infection has focused mostly on host mRNA expression creating a paucity of information on the actions of sncRNA during infections. Because of this, our study examined the expression profile of both well and lesser characterized sncRNAs in order to identify and quantify the classes of sncRNA expressed in whole blood between healthy and highly pathogenic PRRSV-infected pigs. Overall, the results will serve to bring researchers closer to elucidating how gene function in the pig can become dysregulated due to PRRSV by presenting another class of molecules that can be identified and interrogated during infection.

2. Materials and methods

2.1. Animals and sample preparation

The experimental design called for the collection of whole blood samples (~2.5 ml/pig) by jugular venipuncture from twenty-eight 9-week old anesthetized pigs. Animals were given either a sham inoculation for the controls (N = 12) (2 ml/pig) or challenged (N = 12) with HP-PRRSV isolate rJXwn06 (10^4 $TCID_{50}$/ml, 2 ml/pig). Whole blood samples were taken from the pigs at 1, 3, and 8 dpi and cryopreserved.

2.2. Sequencing and mapping

Samples were chosen for total RNA extraction and small non-coding RNA library creation producing a total of 24 samples for analysis. Samples were subjected to an additional extraction step using the mirVana miRNA isolation kit™ (Thermo Scientific, Wilmington, DE, USA) per in-house protocol to maximize the recoverable number of small RNA transcripts. All RNA was globin depleted to account for high levels of globin transcripts using porcine specific hemoglobin A and B (HBA and HBB) oligonucleotides based on the procedure from Choi et al., 2014 (Choi et al., 2014). Small RNA libraries were not size selected to allow for the capture of multiple sncRNAs between 18nt-200nt. Sequencing was carried out on the Illumina Hiseq. 3000™ at the Iowa State University genomic sequencing center in Ames, IA to produce a total of 24 100 bp single-end reads.

2.3. Statistical analysis of sncRNA classes

The sequenced reads were mapped to the *S.scrofa* 10.2 reference genome using the Hisat2 (Kim et al., 2015) package and annotated using FeatureCounts (Liao et al., 2014) and an in-house created sncRNA GTF file produced from data within mIRBase (Kozomara and Griffiths-