

**Comparative Expression Profiling of miRNAs in
African Swine Fever Virus infected and
non-infected porcine tissue samples**

Thesis

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Izatnagar - 243 122 (U.P.), India**



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**IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF**

**Master of Veterinary Science
(Animal Genetics and Breeding)**

2025



Dedicated to...

*Respected Guide,
My Beloved Parents,
Friends and Family Members*





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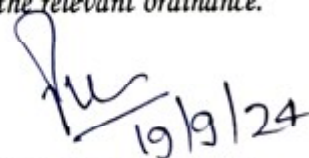
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This is to be certified that the research work embodied in this thesis entitled “Comparative expression profiling of miRNAs in African Swine Fever Virus infected and non-infected porcine tissue samples” submitted by Dr. Nunavath Likhitha, Roll No. M-6397, for the award of Master of Veterinary Science Degree in Animal Genetics and Breeding at ICAR-Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.

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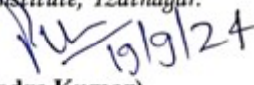
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We the undersigned members of Advisory Committee of Dr. Nunavath Likhitha, Roll No. M-6397, a candidate for the degree of Master of Veterinary Science with the major discipline Animal Genetics and Breeding, agree that the thesis entitled "Comparative expression profiling of miRNAs in African Swine Fever Virus infected and non-infected porcine tissue samples" may be submitted in partial fulfillment of the requirement for the degree.

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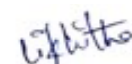
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(Likhitha Nunavath)

ABBREVIATIONS

°C	: Degree centigrade
μL	: micro litre
ASF	: African swine fever
ASFV	: African swine fever virus
<i>C. elegans</i>	: <i>Caenorhabditis elegans</i>
cDNA	: Complementary DNA
CSF	: Classical swine fever
DCs	: Dendritic cells
Dpi	: Days post inoculation
dsDNA	: Double stranded DNA
et al.,	: And others, co-worker
FAO	: Food and agriculture organization
Fig	: Figure
GC	: Germinal centre
i.e	: That is
IFN	: Interferon
IL-1	: Interleukin 1
ISH	: In situ hybridization
Kbp	: kilo basepair
LCMV	: Chronic lymphocytic choriomeningitis virus
miRNA	: micro RNA
MLN	: Mediastinal lymph node
MPS	: Mononuclear phagocyte system
NGS	: Next-generation sequencing
Nm	: nano meter
OIE	: World Organization for Animal Health
ORF	: Open Reading Frames
PABP	: poly(A)-binding protein
PCV2	: Porcine circovirus type 2
PRRSV	: Porcine reproductive and respiratory syndrome virus
RISC	: RNA-induced silencing complex
RNA Pol II	: RNA polymerase II
RT-qPCR	: Quantitative reverse transcriptase Polymerase Chain
SD	: Silencing domain
SYBR	: Cyber green
TNF-α	: Tumour necrosis factor - α

LIST OF TABLES

Table No.	Title	On/After Page No.
Table 3.1	Composition of components for poly-a tailing of RNA	28
Table 3.2	Composition of components for the synthesis of first-strand srcDNA	28
Table 3.3	Composition of components for reverse transcription of RNA	29
Table 3.4	Composition of components for the polymerase chain reaction of the srcDNA	29
Table 3.5	List of microRNA and their forward primers listed below	31
Table 3.6	Composition of components for conventional PCR	32
Table 3.7	Composition of components for qPCR	33
Table 3.8	List of primers of reference gene used for qPCR or miRNAs	34
Table 4.1	Fold change of expression of micrornas involved in African Swine Fever virus infected and non- infected spleen tissue samples	36 37
Table 4.2	Fold change of expression of micrornas involved in African Swine Fever virus infected and non- infected lymph node tissue samples	39

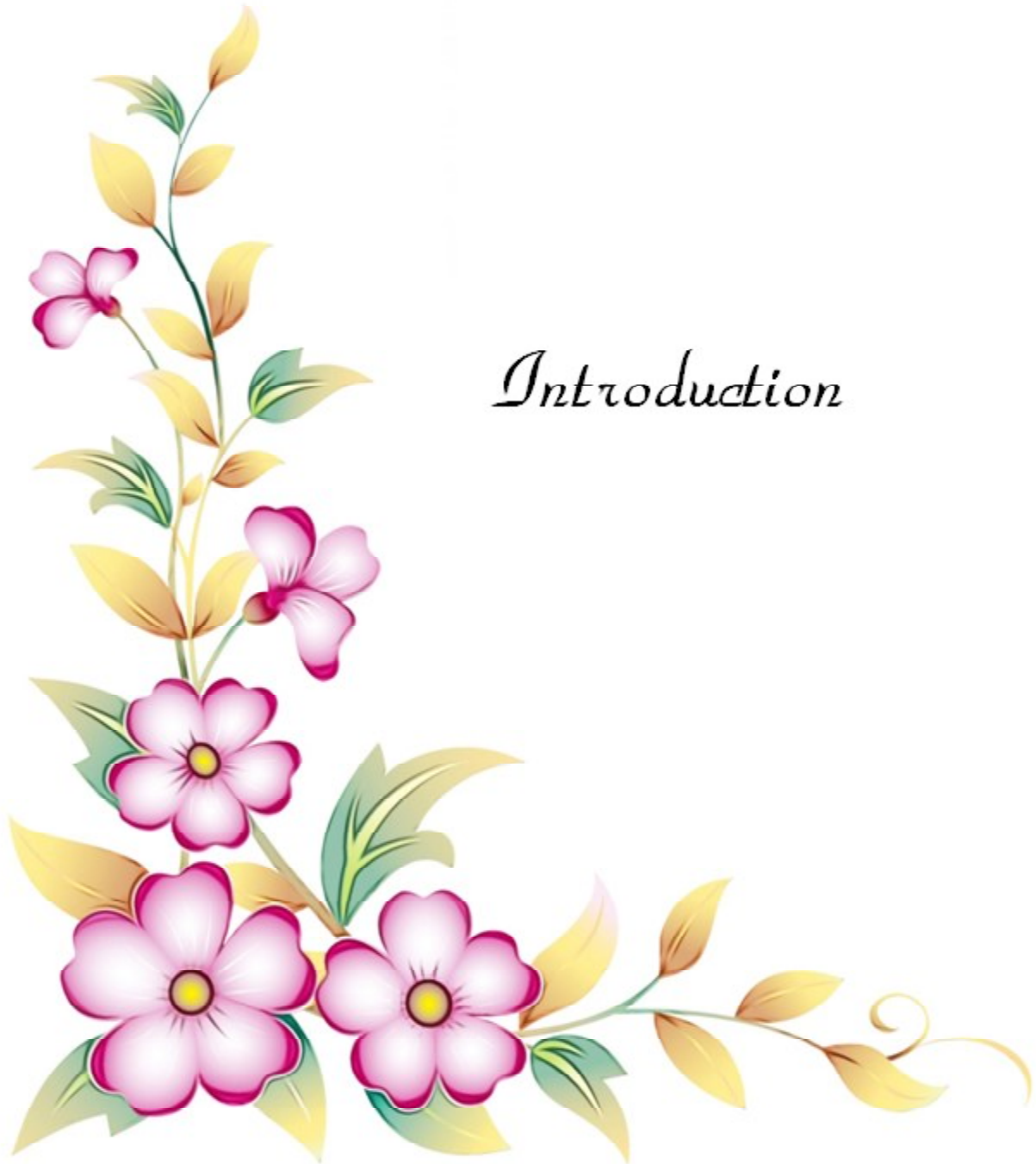
LIST OF FIGURES

Figure No.	Title	On/After Page No.
Fig. 4.1	Expression of miRNAs in the control group of spleen tissue	36
Fig. 4.2	Expression of miRNAs in the control group of Lymph node tissue	36
Fig. 4.3	Expression of miRNAs in the died group of spleen tissue	36
Fig. 4.4	Expression of miRNAs in the died group of Lymph node tissue	36
Fig. 4.5	Expression of miRNAs in Survived group of spleen tissue	36
Fig. 4.6	Expression of miRNAs in Survived group of Lymph node tissue	36
Fig. 4.7	Relative expression of miR-92b-3p	40
Fig. 4.8	Relative expression of miR-30d	40
Fig. 4.9	Relative expression of miR-30e-5p	40
Fig. 4.10	Relative expression of miR-125b	40
Fig. 4.11	Relative expression of miR-122-5p	40
Fig. 4.12	Relative expression of miR-451	40
Fig. 4.13	Relative expression of miR-92b-3p	40
Fig. 4.14	Relative expression of miR-30e-5p	40

CONTENTS

Sl. No.	CHAPTER	PAGE NO.
1.	INTRODUCTION	01-04
2.	REVIEW OF LITERATURE	05-24
3.	MATERIALS AND METHODS	25-34
4.	RESULTS AND DISCUSSION	35-40
6.	SUMMARY AND CONCLUSIONS	41-44
7.	MINI ABSTRACT	45
8.	HINDI ABSTRACT	46
9.	REFERENCES	47-63
10.	APPENDIX	

Introduction



African Swine Fever (ASF) is a deadly viral illness that is now resurrecting and endangering the global pig business (Hernandez *et.al.*,2017). ASF virus (ASFV), is a large and complex virus a member of the *Asfar-viridae* family and genus *Asfi-virus*, is an infectious agent. ASFV structure is characterized by its large double-stranded DNA genome, complex virion morphology, extensive protein composition, and unique strategies for transcription, replication, entry, and egress. ASFV is an enveloped, icosahedral, double-stranded (ds) linear DNA virus that has a genome size of 170–193 kb. Significant economic losses are caused by elevated fatality rates in the impacted areas. Understanding the structure and molecular biology of ASFV is essential for the evolution of effective vaccines and viral suppressants to control this devastating disease. Currently, there are no vaccines or specific treatments available for ASF. This makes controlling the disease more difficult and increases the reliance on prevention measures such as biosecurity and culling infected animals. Controlling the spread of ASF requires strict biosecurity measures on pig farms. However, implementing and maintaining effective biosecurity measures can be challenging for smallholder farmers due to limited resources and infrastructure.

CSF, or Classical Swine Fever : When the World Organization for Animal Health (OIE) designates an animal illness as transboundary, the Food and Agriculture Organization of the United Nations (FAO) does the same. The illness can result in large financial losses for the global swine industry and high rates of morbidity and mortality in both domestic and wild pigs. The causative agent of CSF, known as the classical swine fever virus (CSFV), is a member of the Pestivirus genus within the Flaviviridae family. CSFV has a positive single-strand RNA

genome that is approximately 12.3 kb in size. Clinical symptoms, pathological findings, and the detection of antigens and antibodies are all part of the conventional diagnosis for CSF. Various clinical signs and pathological findings in pigs infected with CSFV are highly variable and frequently resemble other viral diseases of pigs. African Swine Fever, even though unique, clinical and pathological observations, like “button” ulcers in the cecum and large intestine mucosa, may only be found in CSF. Because of immunosuppression, infected animals have non-specific clinical signs. There are three types of CSF: acute, chronic, and permanent (Wang *et al.*, 2020). 8.20 million pigs died between 2016 and 2020, according to the World Organization for Animal Health (Office International des Épizooties, or OIE), with 82% of cases worldwide coming from Asia (World Organization for Animal Health, 2021). According to official and media sources, around 54,150 pigs had passed away as of July 2021 in India, while the precise number of pig deaths attributable to ASF is unknown (Mohan *et al.*, 2021). The first cases of ASF in domestic pigs in India were linked to outbreaks that were documented in 2020 in the northeastern states of Arunachal Pradesh and Assam. Eleven ASF outbreaks across various regions claimed the lives of more than 3700 pigs (Bora *et al.*, 2020).

After the ASF initially struck Mizoram in 2021, there have been numerous reports of the epidemic ever since. The state saw thousands of pigs succumb to the infectious sickness between May 2023 and April 2024. According to AHV Department authorities, the start of the state’s pre-monsoon rains and the beginning of the warmer weather were the main triggers for the ASF outbreak. The ASF epidemic in Mizoram resulted in at least 47,269 pigs and piglets dying between 2021 and 2023, while at least 25,182 pigs were killed over the same time period. An estimated Rs 132.20 crore has been lost as a result of the ASF outbreak in the state, which has affected 19,017 people (Ians *et al.*, 2024).

MicroRNAs (miRNA) are non-coding RNAs with 17–25 nucleotides that primarily regulate gene expression, impacting translation, transcription, and even mRNA stability. They typically derive from longer RNA transcripts and are processed into mature miRNAs. Understanding how these little molecules control genes in our cells has advanced significantly since the 1990s, when miRNAs were first identified. Several subtypes of non-coding RNAs other than ribosomal and transfer RNAs are currently recognized. Among these are microRNAs

(miRNAs), which are small endogenous species of around 22 nucleotides that help an extensive variety of eukaryotes with their meticulously planned post-transcriptional regulation of gene expression. Most miRNAs are synthesized from DNA sequences as primary miRNAs, which are then processed into pre- miRNAs and mature miRNAs. They play important roles in a wide range of biological processes, including cell differentiation, immunity regulation, carcinogenesis, apoptosis and translational repression of mRNAs during viral infections (Hernandez *et al.*,2017).

RNA polymerase II transcribes them into pri- miRNA from the host gene after processing them primarily from introns (Wang, 2023). Primary micro-RNAs are converted to the pre-miRNA and mature miRNA with the help of endonucleases (Drosha and Dicer). The RNA-induced silencing complex (RISC) incorporates the guide miRNA, of which the first 2–8 nucleotides (seed area) are critical for identifying the target mRNA (Du and Zamore,2005; Ketting *et al.*, 2010a). This allows the guide miRNA strand to control gene expression by reducing mRNA stability and/or preventing translation (Sanchez *et al.*,2006). According to many studies done in research, miRNAs that attach to the 3'UTR of mRNAs block translation and mRNA degradation, but those that attach to promoter regions can increase transcription. The miRNAs located in the nucleus regulate transcription and alternative splicing. It is noteworthy that miRNAs are not exclusive to the cells from whence they originated. They can enter the bloodstream and function as disease biomarkers. These circulating miRNAs can elicit behavioural changes in other cells by being absorbed into them. Certain miRNAs can even behave like hormones through interactions with cell surface receptors (O'Brien *et al.*,2018). Several subtypes of non-coding RNAs other than ribosomal and transfer RNAs are currently recognized.

Presently, ASFV is regarded as a primary global biological hazard to pigs, resulting in significant financial losses for impacted nations. Since it originated in several regions of Sub-Saharan Africa, ASFV has been endemic.

However, information about miRNA expression in an ASFV infection is lacking. This study of microRNA related to ASFV will be useful in studying the immune response of the animals infected with the African Swine Fever Virus.

Objectives

- 1. To assess qualitative and quantitative expression profiling of miRNAs in ASFV infected porcine tissue samples (lymph node and spleen)**
- 2. To compare the differential expression pattern of miRNAs among infected and non- infected porcine tissue sample**



*Review
of
Literature*



2.1 African Swine Fever Virus

ASFV is the sole representative of the Asfarviridae family and is a mighty DNA virus that replicates in the cytoplasm. It is a complicated virus with Icosahedral symmetry which causes highly contagious and haemorrhagic disease in pigs incurring huge losses to the farmers rearing pigs as the main source of income. Amidst 150 and 165 proteins are coded by the virus, and these proteins play both “essential” roles in the duplication of the virus and “non-essential” roles in host interactions, such as evading host defences. For instance, many of these proteins inhibit early innate responses, such as type I interferon and Apoptotic pathways (Dixon *et al.*,2013a).

2.2 Structure of ASFV

African Swine Fever Virus, which is indeed an icosahedral, enveloped virus with a double-stranded DNA genome. The size of its genome typically ranges from 170 to 193 kilobases (kb), making it one of the largest known DNA viruses. The causative agent of African Swine Fever (ASF), a highly contagious and often fatal viral disease affecting domestic and wild pigs. That’s the only DNA virus spread by arthropod vectors (Dixon *et al.*,2019). The sole representative of the Asfarviridae family (Alonso *et al.*,2018) is ASFV. The virus with an icosahedral multilayer structure that contains around 50 different protein types. The virus particles have a diameter of 200 nm (Carrascosa *et al.*,1984). Cryo-electron microscopy, however, revealed that the diameter of the pure virus particles in Chinese farms was between 260 and 300 nm (Wang *et al.*,2019). The virus has a 170 to 194 kb dsDNA genome that has

a hairpin structure with inverted repeat sequences at the ends (Alejo *et al.*, 1999). More than 150 open reading frames (Dixon *et al.*, 2013b) and several enzymes involved in DNA replication, gene transcription, and protein modification (Andres *et al.*, 2002a) are encoded in the virus genome. Structural proteins are also encoded.

The four-layer structure of intracellular ASFV includes a core nucleoid that houses the genome, contained within an icosahedral protein capsid, an inner lipid envelope, and a protein core-shell (Salas and Andres 2013). The outermost coating, known as the envelope, is acquired by mature virus particles as they show up via the cell membrane (Alonso *et al.*, 2018). Infectious viruses can be either mature intracellular particles or enclosed extracellular particles with vesicular membranes (Galindo and Alonso, 2017). With a diameter of roughly 70-100 nm, the ASFV nucleoid is an electron-dense structure that houses the viral genetic material along with related proteins like p10 (Salas and Andres, 2013) pA104R, and portions of the transcriptional mechanism. It is believed that the nucleoid contains proteins and enzymes, such as blocking enzymes, polyadenylate polymerases, multi-subunit RNA polymerases, and early transcription factors, which carry out and aid in gene transcription (Salas and Andres, 2013). The enzyme pS273R is engaged in the processing of viral polyproteins, virus assembly, and protein hydrolysis. (Andres *et al.*, 2002) (Li *et al.*, 2020b). The virus's nucleoid is encircled by the about 30-nm-thick protein layer of the core shell (Andres *et al.*, 2002). The polyproteins pp220 (p150, p37, p34, p14, and p5), pp62 (p35, p15, and p8), and pS273R are present in the core shell protein (Andrés *et al.*, 2001). P15 might be the main protein in this layer. The multiprotein pp220 is essential to core assembly, which includes the processes that lead to genomic wrapping, condensation, and viral nucleoprotein absorption. A single lipid membrane monolayer that originates from the endoplasmic reticulum makes up the inner envelope membrane. The viral attachment protein p12, which was previously thought to be on the outer membrane, is present on this membrane together with the membrane proteins p54, p17, and H248R (Rodríguez *et al.*, 2004).

2.3 Lesions and Clinical Symptoms in ASF Infection

Based on the features of the host, the route, dose, and pathogenicity of viral isolate (Sanchez-Vizcalno *et al.*, 2015). According to their level of virulence, ASFV isolates can be

categorized as extremely, moderately, or low (Pan and Hess, 1984). Per-acute (or hyperacute), acute, subacute, or chronic are the possible clinical courses seen in ASF in domestic pigs.

2.3.1 Peracute form

Characterized by its severe and rapid progression leading to quick fatality, particularly in cases of African Swine Fever (ASF), is often associated with high doses of highly virulent strains. Initial introductions of ASF into previously unaffected areas may manifest as peracute cases, marked by short incubation periods and sudden casualty of animals without evident clinical signs or with pronounced fever and severe illness. Instead, infected animals showed elevated viral levels in their bloodstream, short incubation periods, and rapid progression of clinical symptoms, including high fever, skin inflammation, reduced appetite, and increased respiratory rate (Sierra *et al.*, 1990).

2.3.2 Acute form

Highly or moderately virulent isolates of ASF typically induce acute forms of the disease. The incubation period for ASF in natural conditions generally ranges from 4 to 19 days. In experimental infections, this period may vary between 2 and 7 days, with clinical courses lasting around 1 week (3-8 days). Animals infected with highly virulent isolates usually succumb between 3 to 9 days post-inoculation (dpi), while those infected with moderately virulent isolates may perish later, typically between 11 and 20 dpi. Mortality rates can reach up to 90-100% in herds lacking immunity if the infection remains undetected for an extended period.

Animals exhibiting acute forms of ASF typically experience elevated body temperatures (40.5 - 42°C). They commonly exhibit signs such as loss of appetite, apathy, weakness, recumbency, and reluctance to stand. Abortions, often induced by febrile syndrome, are frequent during all stages of pregnancy and are among the earliest signs observed in ASF outbreaks. Additional clinical signs during acute ASF include dyspnoea, coughing, increased pulse and respiratory rates, nasal discharge, epistaxis, congested ocular mucosa, vomiting, abdominal pain, constipation or diarrhoea, and neurological symptoms such as head tilt and occasional tremors (Moulton and Coggins 1968; Mebus and Dardiri 1979; Carrasco *et al.*, 2002; Gomez *et al.*, 2003; Sanchez *et al.*, 2015)..

2.3.3 Subacute form

This is caused by moderately virulent strains, some pigs may show no clinical symptoms, while others exhibit mild symptoms resembling those seen in acute cases, albeit less severe and persisting for up to four weeks. The incubation period ranges from 2 to 7 days, with clinical symptoms lasting between 7 to 20 days. Mortality typically occurs within 14 to 30 days after infection, although some pigs may succumb up to 45 days post-infection. Recovered pigs can still shed viral DNA in secretions for several weeks. Mortality rates are usually lower in adult pigs (20-70%) compared to young pigs (70-100%), which also tend to have more severe symptoms.

Pyrexia may vary, with temperatures reaching as high as 42°C, and affected pigs may huddle together, similar to acute cases. Thrombocytopenia and leukopenia typically appear later than in acute cases and may be transient but more severe, leading to bleeding from injection sites. Other symptoms may include laboured breathing, diarrhoea with blood-stained mucus, or melaena. Neurological signs may be more prevalent and severe compared to acute cases (Villeda *et al.*, 1993; Gomez *et al.*, 1998; Gomez *et al.*, 2013).

2.3.4 Chronic form

These chronic cases are typically associated with strains of the ASF virus (ASFV) that are less virulent. As a result, they may exhibit a milder clinical course compared to acute or subacute forms of ASF. In chronic ASF, the disease progresses over a prolonged period, lasting more than a month, and typically has a lower mortality rate, usually less than 30%. The symptoms observed in chronic ASF include a range of nonspecific signs such as recurrent low-grade fever, poor body condition, loss of appetite, depression, stunting, emaciation, and joint swelling. One of the characteristic features of chronic ASF is the presence of severe skin lesions. These lesions often manifest as cutaneous ulcers and, reddened or raised necrotic skin foci, particularly in areas that overlay bony protuberances, as well as in other areas such as the ears, nose, neck, flanks, abdomen, and limbs (Neilan *et al.*, 1993; Yanez *et al.*, 1995; Brun *et al.*, 1996; Afonso *et al.*, 1996; Oura *et al.*, 1998).

2.4 Pathogenesis

When an ASFV infection progresses, the lymphoid system experiences the most dramatic histopathological lesions. Depletion of lymphocytes, tissue damage, and the quantity

of ASFV-infected cells are typically greater in lymphoid organs. In acute stages of infections with moderately virulent isolates, these histological alterations are less noticeable than in infections with extremely virulent ASFV isolates. According to the (Oura *et al.*, 1998) quantity of macrophages infected with ASFV is directly correlated with the extent of lymphocyte destruction

2.4.1 Spleen

One of the primary targets of ASFV, the network of smooth muscle cells that make up the structure of pig splenic cords is encircled by a static macrophage population (Carrasco *et al.*, 1995). The cytopathic effect of ASFV replication in resident macrophage populations is followed by a gradual separation of resident macrophages from muscle cells as a result of the breakdown of intercellular connections, and ultimately, macrophage necrosis. The basal lamina of smooth muscle's collagen is exposed to blood components, which causes these alterations. The coagulation cascade, platelet activation and aggregation, and fibrin deposit development are all facilitated by this exposure, which results in the buildup of erythrocytes within splenic cords.

2.4.2 Lymphoid tissue

In the early stages following the African Swine Fever Virus (ASFV) infection, lymphoid tissue in primary and secondary lymphoid organs such as tonsils, lymph nodes, spleen, and thymus are the main sites for viral replication. This leads to significant histopathological changes characterized by both direct viral action on specific cell populations and indirect destructive mechanisms triggered by the virus. Highly virulent ASFV isolates cause extensive destruction of lymphoid tissue with widespread karyorrhexis, while less virulent isolates result in milder tissue damage but severe vascular changes and haemorrhages affecting multiple lymphoid organs.

The destruction of lymphoid tissue leads to a reduction in the total leukocyte count, particularly in acute forms of ASF. Lymphopenia, affecting both B and T lymphocytes, along with a decrease in monocyte numbers, are typically observed. In pigs that survive longer, leukocyte counts normalize, likely due to increased haematopoiesis (Blome *et al.*, 2013; Gómez-Villamandos *et al.*, 2013).

The primary target cells for ASFV replication are mononuclear phagocyte system (MPS) cells, particularly monocytes and fixed-tissue macrophages, which are critical in innate and adaptive immune responses. ASFV genome encodes proteins that inhibit apoptosis in infected cells, initially promoting virus replication and later aiding virus persistence through apoptosis of MPS cells, facilitating virus spread while evading immune responses (Sierra *et al.*, 1989; Gómez-Villamandos *et al.*, 1995; Carrasco *et al.*, 1996).

In acute ASF forms, the virus induces apoptosis of uninfected cells, particularly B and T lymphocytes, through indirect mechanisms from early stages post-infection. Studies suggest that ASFV replication in macrophages activates neighbouring non-infected macrophages through an autocrine effect, leading to the secretion of proinflammatory cytokines like TNF- α and IL-1. These cytokines, along with chemokine CXCL10 expressed in macrophages, contribute to a “cytokine storm,” inducing apoptosis of nearby lymphocytes (Salguero *et al.*, 2002, 2004, 2005; Fernández de Marco *et al.*, 2007; Fishbourne *et al.*, 2013).

2.5 microRNAs

According to (Wang *et al.*, 2016), microRNAs are small non-coding RNAs that vary in length from 17 to 25 nucleotides and are involved in the regulation of gene expression in several biological processes. miRNAs were first reported to downregulate LIN-14, a critical protein in the nematode *Caenorhabditis elegans* (Lee *et al.*, 1993; Bhaskaran and Mohan, 2014). Subsequent investigations have uncovered the role that miRNAs play in controlling both physiological and pathological processes in humans and animals. The discipline of molecular biology was completely transformed in 1993 when the Ambros and Ruvkun groups discovered the first microRNA (miRNA), lin-4, in *Caenorhabditis elegans*. The groundbreaking discovery of lin-4 and its role in regulating the temporal development of *C. elegans* larvae, a significant contribution to developmental biology and gene regulation, was conducted by H. Robert Horvitz’s lab and published in a seminal paper in 1993. The lin-4 gene produces a microRNA that serves as a short RNA sequence involved in post-transcriptional regulation, specifically negatively regulating the expression of the developmental timing regulator lin-14 in *C. elegans* by binding to its mRNA, thereby inhibiting translation and causing a delay in larval development.

The lin-4/lin-14 pathway in *C. elegans* has since served as a model system for studying the roles of microRNAs in gene regulation and developmental timing across various organisms, including humans. Our knowledge of the intricate molecular networks regulating development and cellular processes has been greatly enhanced by the use of the *C. elegans* lin-4/lin-14 pathway as a model system for studying the roles of microRNA in gene regulation and developmental timing across a variety of organisms, including humans.

Every animal model system has identified miRNAs, some of which have been demonstrated to be substantially conserved across species.

2.6 Biosynthesis of microRNA

According to Ketting (2010b), the production of mature miRNAs is the outcome of a complex and dynamic process known as microRNA biogenesis, which involves numerous pathways. According to the physiological functions regulated by the impaired miRNA, any perturbing event on this pathway may result in an increase or decrease in the production of miRNAs in the targeted tissue, which may cause a variety of diseases like neoplasia, ischemic heart disease, haematological diseases, muscular dystrophies, neurodegenerative diseases, psychiatric disorders, brain tumours, kidney disease, etc (Sayed and Abdellatif, 2011; Garofalo *et al.*, 2014; Trionfini *et al.*, 2015; Barwari *et al.*, 2016; Luoni and Riva, 2016).

The RNA polymerase II (Pol II) transcription of the miRNA genes initiates the process of miRNA production in the nucleus, producing a main transcript with a “hairpin” structure that codes for the miRNA sequences (Ha and Kim, 2014). RNA Pol II-associated transcription factors, such as p53, ZEB1 and ZEB2, MYC, as well as epigenetic modulators, like DNA methylation and histone modification, can either positively or negatively regulate this step (Lee *et al.*, 2004; Davis-Dusenbery and Hata, 2010; Krol *et al.*, 2010; Ha and Kim, 2014). Later on, the nucleus hosts the first of several maturation processes that the primary miRNA (pri-miRNA) undergoes. RNase III Drosha and cofactor DGCR8 now combine to create the Microprocessor complex, which crops the loop end of pri-miRNA to produce precursor miRNA and pre-miRNA, which resembles a “hairpin” structure (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004). According to Ha and Kim (2014), the final product is exported

into the cytoplasm by Exportin-5, where it goes through the subsequent stages of maturation. There, another RNase known as Dicer crops the pre-miRNA close to the loop end, producing a short RNA duplex (Ketting *et al.*, 2001; Knight and Bass, 2001; Hutvagner *et al.*, 2001). The resultant product then forms the RNA-induced silencing complex (RISC) in conjunction with an argonaute (AGO) protein (Hammond *et al.*, 2001).

2.7 Functions of microRNA

According to O'Brien *et al.*, (2018), the two most significant functions of miRNAs are in intercellular signalling and gene control. Regarding the first, there are two ways that the miRISC can operate via: the canonical mechanism and the most commonly employed non-canonical mechanism. The canonical mode of action of miRISC is binding to the targeted mRNA's 3'-untranslated region (3'-UTR), which causes translation to either decrease or stop when the two strains are nearly completely complementary. Such strong complementarity is not necessary for the non-canonical pathway (Helwak *et al.*, 2013).

Early research found that a functional miRNA-targeted mRNA association may be obtained within the seed region with just 6 nt matches (Bartel, 2009). Consequently, the following was the definition of the canonical sites: (Lee *et al.*, 1993), (Wightman *et al.*, 1993), and (Poy *et al.*, 2004), there are three potential canonical sites for 6-mers matches to positions 1-6, 2-7, and 3-8, two potential canonical sites for 7-mers matches to positions 2-8 and 1-7, and one potential canonical site for 8-mers matches to position 1-8 (Bartel, 2009; Helwak *et al.*, 2013; Chevillet *et al.*, 2014; Eichhorn *et al.*, 2014; Kai *et al.*, 2018). According to (Reinhart *et al.*, 2000), Dalmay (2008), and Sand (2014). Nevertheless, it has been discovered that miRISC complexes, which target locations other than the seed area and have less complementarity, do have some minor regulatory effects (Helwak *et al.*, 2013). Non-canonical locations include “nucleation bulges,” which are composed of five consecutive nucleotides and are found at positions 2–6. According to the “pivot pairing rule” put forward by Chi *et al.*, 2012, these nucleotides pair themselves at position 6 of the miRNA, which is referred to as the “pivot” (Chi *et al.*, 2012; Seok *et al.*, 2016).

A pivot nucleotide that can bind a nucleation bulge is present in several of the miRNAs under study. As an illustration, let-7 has a U pivot nucleotide, miR-708 has a G pivot nucleotide,

and miR-124 has a C nucleotide at position 6 that would bind a complementary G-bulge (Chi *et al.*, 2012). Targeted mRNA also contains non-canonical binding sites known as “seed-like motifs” (Seok *et al.*, 2016). According to (Seok *et al.*, 2016), these sites appear to contain some mismatches or deletions that require an extra thirty interactions to maintain the relationship. The precise roles of the noncanonical routes have not yet been established, though they have not been studied in great detail. It has also been demonstrated that miRNAs play a part in viral infections. Viruses are intracellular organisms that can only reproduce in their host environment, as we already know (Girardi *et al.*, 2018). Various host miRNAs have been reported to be up- or down-regulated by these pathogens in an attempt to evade the host’s immune system (Scheel *et al.*, 2016; Girardi *et al.*, 2018; Shimakami *et al.*, 2012; Trobaugh *et al.*, 2014).

On the other hand, certain miRNAs that have been researched may have an antiviral impact, which would allow the body’s defensive mechanisms to combat the infection, or they might cause the pathogen to enter a latent state (Guo *et al.*, 2013; O’Connor *et al.*, 2014; Pan *et al.*, 2014; Girardi *et al.*, 2018).

2.8 Techniques for microRNA Identification

The development of a low-cost, effective technique for identifying miRNAs implicated in viral infections will help with both the diagnosis of more serious infectious disorders and the search for novel miRNAs linked to this condition that were previously unidentified.

Finding such a detection method is difficult, though, in part because miRNAs are a relatively new field of study. As a result, many of their properties such as concentration in biological samples, detection levels, and up- or down-regulation in healthy and pathological samples remain unknown (Gillespie *et al.*, 2018).

Quantitative reverse transcriptase Polymerase Chain Reaction-PCR (RT-qPCR) is by far the most used technique for identifying miRNAs (Chen *et al.*, 2018; Gillespie *et al.*, 2018; Yuan *et al.*, 2018). Stem-loop reverse transcription (RT)-based TaqMan MicroRNA test is the most widely used PCR quantification method due to its high sensitivity and specificity (Chen *et al.*, 2011). According to its schematic, this technique consists of two steps: first, stem-loop reverse transcription, in which primers bind the 3’ end of miRNA molecules that are

going to be reverse transcribed; second, real-time PCR is used to quantify microRNAs (Chen *et al.*, 2005). Other PCR methods available are poly (A) tailing-based and direct RT-based SYBR miRNA tests (Chen *et al.*, 2011). Nevertheless, there are drawbacks to the RT-qPCR approach as well, as there is a significant chance of contamination during the amplification processes and sample sensing mistakes (Gillespie *et al.*, 2018).

Northern blot hybridization is another widely used quantitative method for the detection of miRNAs. It involves separating the total RNA on a polyacrylamide gel with denaturation properties, transferring it to a nylon membrane, UV-cross-linking it, and then hybridizing it with the aid of a probe labelled with a radioactive substance (Kruszka *et al.*, 2014; Bariszewska-Pacak *et al.*, 2015; Pacak *et al.*, 2016; Smoczynska *et al.*, 2019). However, it is a time-consuming, arduous method that needs a lot of RNA and occasionally fails to identify uncommon miRNAs (Smoczynska *et al.*, 2019). New procedures have been created recently to improve the process; as a result, smaller levels of RNA are being used, and the procedure can be completed in less time (Varallyay *et al.*, 2007; Pall and Hamilton, 2008; Wang *et al.*, 2010).

The discovery of miRNAs may also be accomplished via next-generation sequencing (NGS) and in situ hybridization (ISH). ISH makes it possible to see RNA in fixed tissue samples and compare the expression levels of miRNA in various cell types by binding the targeted RNA with fluorescent, dioxigenin, or radioactive probes (Timmermans, 2012). But this method is also difficult, time-consuming, and error-prone at every stage (Timmermans, 2012). According to Hu *et al.*, (2017), NGS is essentially a second-generation sequencing approach that builds on the widely used Sanger method. Kits for quantifying miRNAs are also offered by several NGS systems.

The fundamental idea behind all of them is the amplification and sequencing of DNA fragments, with extra steps involved in the sequencing of miRNAs. These steps begin with miRNA extraction, and then the miRNAs are reverse - transcribed into cDNA (Bar *et al.*, 2008; Creighton *et al.*, 2009; Hu *et al.*, 2017). The high resolution of one nucleotide that NGS provides for identifying single miRNA makes it less accessible than other methods due to

its expensive cost (Smoczynska *et al.*, 2019). But when selecting a quantification method, there are a lot of other important features to keep in mind. For example, this technique's high throughput, means that several researchers' samples would be sequenced simultaneously. (Hu *et al.*, 2017). Furthermore, NGS has a high accuracy and the potential to find new miRNAs, which is a benefit not offered by a hybridization method (Hu *et al.*, 2017).

2.9 Signalling pathways involved during viral infection

2.9.1 The Wnt signalling pathway plays a crucial role in regulating cellular processes such as proliferation and differentiation. In the context of viral infection, some viruses exploit this pathway to enhance their replication and manipulate host cell behaviour. By modulating Wnt signalling, viruses can create a microenvironment that facilitates their survival and evasion of host defences (Yang *et al.*, 2010; Zhu *et al.*, 2011; Hack *et al.*, 2012; Baril *et al.*, 2013; Hillesheim *et al.*, 2014; Khan *et al.*, 2015).

2.9.2 The Type I Interferon signalling pathway is a key component of the innate immune response, inducing an antiviral state in cells. Viruses often target this pathway to evade the host's immune response, inhibiting interferon production or interfering with downstream antiviral effects. By disrupting Type I Interferon signalling, viruses can establish successful infection and impede the host's ability to mount an effective antiviral defence (Pichiorri *et al.*, 2008; Jiang *et al.*, 2010; Wang *et al.*, 2010; Yao *et al.*, 2012; Li *et al.*, 2013).

2.9.3 The NF- κ B signalling pathway regulates inflammation, immune responses, and cell survival. In viral infections, viruses can either activate or inhibit NF- κ B to manipulate host cell survival, evade immune responses, or promote inflammation, depending on their strategies. This manipulation allows viruses to create a cellular environment conducive to their replication and persistence (Janeway, 1989; Ryseck *et al.*, 1995; Karin *et al.*, 2002; Karin, 2006; Ma *et al.*, 2011; Androulidaki *et al.*, 2009; Bhaumik *et al.*, 2008; Houzet *et al.*, 2008).

2.9.4 The PI3K/Akt signalling pathway, which controls cell growth, survival, and metabolism, is also targeted by viruses. Some viruses activate this pathway to enhance their replication, modulate host cell metabolism, or inhibit apoptosis, ensuring their survival within the host cell (Ji and Liu, 2008; Deregibus *et al.*, 2002; Kim *et al.*, 2006; Shen *et al.*, 2006; Guo *et al.*, 2007; Alisi *et al.*, 2008; Chugh *et al.*, 2008).

2.9.5 The MAPK signalling pathway, involved in cell proliferation and differentiation, is manipulated by viruses to regulate host cell cycle progression, apoptosis, or immune evasion. By interfering with MAPK signalling, viruses can promote their own replication and alter the cellular environment to their advantage (Pearson *et al.*, 2001; Dong *et al.*, 2002; Ludwig *et al.*, 2001; Ludwig *et al.*, 2006; Gaur *et al.*, 2011; Johnson *et al.*, 2000; He *et al.*, 2019; McCaskill *et al.*, 2017).

2.9.6 The Notch signalling pathway, which regulates cell fate determination and immune responses, is exploited by certain viruses to manipulate host cell differentiation and modulate immune responses. This manipulation contributes to the successful establishment of viral infection within the host. In summary, viruses have evolved intricate strategies to interact with these signalling pathways, highlighting the importance of understanding these interactions for the development of antiviral therapeutics (Kopan, 2012; Hayward, 2004; Sarma *et al.*, 2012).

2.10 MicroRNA signalling in viral infection

Viruses are microorganisms reliant on host cells for survival and reproduction. They have evolved mechanisms to evade the host's immune system, ensuring their ability to proliferate and complete their life cycle. Whatever the virus type, the first stage of any infection is the microorganism's entry into a vulnerable host cell through the binding of viral proteins present in the virion and host cell surface molecules, which can also affect the infection's tropism (Marsh and Helenius, 2006; Greber and Puntener, 2009). Once the virus has attached itself to the cell's surface, it must cross the membrane and enter the cytoplasm. Many methods, including the fusion of the host and viral membranes (Sodeik *et al.*, 1997; Maurer *et al.*, 2008), clathrin-dependent pathways, or endocytosis (Marsh and Helenius, 2006; Greber and Puntener, 2009), can accomplish this. The kind of virus that is trying to infect the organism will now affect the next phases. For instance, with DNA viruses, the genome contained in the nucleocapsid must travel from the cytoplasm to the nucleus, where the viral genes are transcribed. However, large viruses exploit the cytoskeleton and motor proteins to reach this desired size because the maximal size of free molecules permitted in the cytoplasm is limited to roughly 500 k Da

(Sodeik, 2000, 2002). The majority of RNA viruses, on the other hand, replicate only in the cytoplasm (Greber and Way, 2006). The only exception to this rule is retroviruses, which use reverse transcription in the host cell nucleus to produce a DNA provirus that is then integrated into the host's genome, resulting in a persistent and resistant infection (Cullen, 2001). MicroRNAs interact with a wide range of viruses because they are essential to the host's modulation systems (Umbach and Cullen, 2009).

The literature describes a variety of possible interactions between the two entities. First off, by binding to the viral RNA or mRNA, the host miRNA can control many stages of the viral life cycle, including translation. Additionally, the virus may influence the host's miRNA, changing the targets of that miRNA's expression. In addition, the microbe might create its miRNAs, which would control the host or viral RNA targets even more (Roberts *et al.*, 2011). On the other hand, miRNAs have also been reported to have indirect functions in viral infections. A case in point is the function of miRNAs in regulating different signalling pathways (Bruscella *et al.*, 2017).

2.11 microRNAs expression of gene regulation

Generally speaking, the RISC complex targets transcripts through base complementation, which is facilitated by the seed sequence, which is the 2–7 nucleotide sequences of the mature single-stranded miRNA's 5' end (Chendrimada *et al.*, 2007; Eichhorn *et al.*, 2014). As stated by (Zhang *et al.*, 2017; Jiang *et al.*, 2020; Agarwal *et al.*, 2015; Carrasco *et al.*, 1996; Zhang *et al.*, 2005), (Zheng *et al.*, 2013), and other studies, the other bases on some miRNAs can aid the RISC complex in finding mRNA. The AGO proteins of the miRISC and the degree of complementarity between the targeted mRNA sequence and the miRNA core sequence determine the miRNAs' methods after the miRNAs form into miRISC complexes (Tang, 2005; Loeb *et al.*, 2012). However, in animals, the transcript targets rarely provide complete complementary sequences to the core sequence of the miRNA, and not all AGO proteins have cleavage activity, which precludes mRNA cleavage by AGO proteins. Tang (2005); Ipsaro and Joshua-Tor, (2015) state that when the transcript target and core sequence of the miRNA are perfectly complementary for cleaving miRISC, miRISC

performs the slicing of the mRNA and efficiently degrades the mRNA. MiRISC uses the GW182 protein as a platform to recruit other proteins because AGO proteins are insufficient to effect silencing on their own (Takimoto *et al.*, 2009; Kobayashi and Tomari, 2016; Sheu-Gruttadauria and MacRae, 2018). The two unique structures of the GW182 proteins are the silencing domain (SD), which interacts with silencing effectors like cytoplasmic poly(A)-binding protein (PABP), the cytoplasmic deadenylase complexes PAN2-PAN3, and CCR4 NOT, and the amino-terminal AGO-binding domain (ABD), which binds to AGO (Guo *et al.*, 2010; Braun *et al.*, 2013). According to some researchers, these proteins' interactions with GW182 proteins disrupt the PABPC1-eIF4G interaction, lowering translational efficiency and altering transfer RNA conformation. This, in turn, makes the mRNA's poly(A) tail and 5' cap more accessible for mRNA degradation (Elkayam *et al.*, 2017; Niaz and Hussain, 2018). However, another theory holds that the miRNA-mediated de-adenylation of target transcripts is catalysed by the interactions between PABPC and GW182 (Meijer *et al.*, 2013; Niaz and Hussain, 2018). A growing body of research indicates that host miRNAs bind directly to the RNA of many RNA viruses, thereby taking a role in the pathogenesis and replication of the viruses. miRNA can then target RNA virus genes during infection in a manner akin to how they target host genes (Teterina *et al.*, 2014; Bakre *et al.*, 2017). Although evidence has been gathered to support the existence of miRNA-binding sites in the RNA virus protein open reading frame (ORF), the majority of natural binding sites for miRNA are found in the 5'UTR and 3'UTR of the RNA virus gene. While the positive-strand intermediate of dsRNA and negative RNA viruses function similarly, the genome of a positive-strand RNA virus can bind directly to the host's miRNAs like host mRNA binding. Two strategies are involved in the direct targeting of RNA viruses by miRNA reducing viral replication through suppression of viral RNA translation, and altering the stability of the viral RNA secondary structure.

2.12 miRNA involved in infectious diseases

Numerous functional investigations have elucidated the noteworthy significance of miR-155 in the host's defence against microbiological illnesses. When the miR-155 gene was absent, effector CD8⁺ T cell activity was compromised in cases of acute or chronic lymphocytic choriomeningitis virus (LCMV) infection, and memory cell differentiation was affected in cases

of *Listeria monocytogenes* infection (Dudda *et al.*, 2013; Gracias *et al.*, 2013; Lind *et al.*, 2013). The direct involvement of miRNAs in the interplay between invading pathogens and host immune responses is an important finding. The expression of both host and viral genes is regulated by miRNAs produced by viruses, highlighting the critical function of miRNAs in immune responses to infection (Sullivan and Ganem, 2005). miRNAs also affect the course of infectious disorders (Sullivan *et al.*, 2005) reported that the expression of viral T-antigens is downregulated when viral miRNA is expressed late in SV40 infection, as a means of avoiding cytotoxic T cells. By controlling the expression of miRNAs in host cells, on the other hand some of which are expected to directly target viral genes the immune system, on the other hand, carries out antiviral activity (Pedersen *et al.*, 2007). miRNAs affect the course of infectious illnesses as mediators of host-pathogen interaction.

2.13 miRNAs involved in innate-immunity

It is also well established that individual miRNAs regulate the growth and operation of innate immune cells. MiR-17-92, for instance, has been demonstrated to play a significant role in monocyte differentiation. Runx1 suppresses miR-17-92 by binding to its promoter region, whereas miR-17-92 targets Runx1, which promotes Monocytopoiesis (Fontana *et al.*, 2007). Research on pattern recognition receptor pathways has further elucidated the breadth and intricacy of miRNA participation in innate immunity. It was found that several miRNAs, including miR-155, miR-146a, and miR-21, are expressed in response to toll-like receptor (TLR) signalling (Taganov *et al.*, 2006; O'Connell *et al.*, 2007; Sheedy *et al.*, 2010). TLR ligand-induced miR-155 in murine macrophages represses TLR signaling negative regulators such as SHIP1 and SOCS1 (Androulidaki *et al.*, 2009; O'Connell *et al.*, 2009). On the other hand, autoimmunity and myeloid malignancy are caused by a shortage in miR-146a, which functions as a negative regulator of NF- κ B through IRAK1 and TRAF6 (Taganov *et al.*, 2006; Boldin *et al.*, 2011; Zhao *et al.*, 2010). According to (Hou *et al.*, 2009), miR-146a targets IRAK2 and is involved in the RIG-I-dependent type 1 interferon (IFN) generation by macrophages following viral infection. This further supports the role of miR-146a as a negative regulator of inflammation. The identification of these relationships shows the dynamic

nature of miRNA activity: inflammatory stimuli can affect the expression of miRNA, and individual miRNAs closely control innate immunity by focusing on particular mRNAs involved in the initiation and resolution of immune responses.

2.14 miRNAs involved in adaptive immunity

Both growth and loss of functionality Certain features of the formation and function of adaptive immune cells can be influenced by a particular miRNA, according to research on specific miRNAs. An important increase in B cells is observed when miR-181 is expressed ectopically, as it is a positive regulator of B cell development (Chen *et al.*, 2004). According to (Ventura *et al.* 2008), miR-17<“92 has also been demonstrated to play a crucial role in promoting the development of early B cells, since its removal results in increased Bim expression and death at the pro-B cell to pre-B cell line. However, forced expression of miR-150, which targets c-Myb, hinders the transition from pro-B cells to pre-B cells, which limits early B cell development (Xiao *et al.*, 2007). Numerous investigations have discovered that miR-155 is crucial in regulating B cell biology on the periphery. Defects in antibody production and class switching are seen in mice lacking miR-155, which is produced during germinal centre (GC) reaction in vivo (Thai *et al.*, 2007; Vigorito *et al.*, 2007). Repression of several genes, such as PU.1 and AID, an enzyme essential for somatic hypermutation and antibody class switching, appears to be the cause of the phenotype (Vigorito *et al.*, 2007; Teng *et al.*, 2008). On the other hand, animals lacking miR-150 exhibit diminished T cell-dependent antigen immunization but increased follicular B cell activation and improved antibody production (Xiao *et al.*, 2007).

There is growing evidence that miRNAs in T cells also control the maturation and operation of distinct T cell subsets necessary for the adaptive immune response. (Rodriguez *et al.*, 2007; O’Connell *et al.*, 2010) Loss of miR-155 promoted Th2 but hindered Th1 and Th17 differentiation. MiR-326 was also demonstrated to influence Th17 differentiation (Du *et al.*, 2009). Also, follicular T (T_{fh}) cells a subgroup of T cells that aids in B cell activation during humoral response have been the focus of recent research on miRNA regulation.

miR-10a has been found to modify T_{fh} cell plasticity (Takahashi *et al.*, 2012), and Bcl-6, a crucial transcription factor for T_{fh} differentiation, has been demonstrated to control

the expression of many miRNAs (Yu *et al.*, 2009). According to recent studies (Baumjohann *et al.*, 2013; Kang *et al.*, 2013), miR-17⁹² promotes Tfh differentiation through several mechanisms that entail targeting ROR α and phosphatase, PHLPP2. Similar to this, studies on Treg cells may provide light on the cell-extrinsic function of miRNAs. According to reports, by affecting SOCS1 and Stat1, respectively, miR-155 and miR-146a can affect Treg homeostasis and suppressor activity (Lu *et al.*, 2009, 2010).

2.15 Work done on miRNAs in different diseases

Numerous candidates' expression analyses identified microRNAs that may be significant for the innate immune response. The expression of miR-155, a recognized mediator of inflammation, was discovered in infected lung and non-infected lung tissue. Furthermore, miR-664-5p, miR-451, and miR-15a seem to be highly intriguing candidates for miRNAs that play a role in the immune system's reaction to pathogen infection. This is the first investigation that shows notable variations in the miRNA expression profiles and composition in bacterially infected lung tissue. Their findings broaden the annotation of pig miRNA and shed light on the function of several microRNAs in controlling the inflammatory and immunological responses brought on by bacteria in the pig lung (Podolska *et al.*, 2012).

When compared to the control pigs, the expression of the following miRNAs varied during one or more time periods: miR-15a, miR-21, miR-146, miR-206, miR-223, and miR-451. Compared to the control group, lung tissue protein levels of IL-6, IL-12, and IFN- α were considerably higher at day 1 pi. while haptoglobin and C-reactive protein were significantly higher at day 3 pi. Their findings imply that miRNAs may have a role in regulating acute influenza infection in pigs, in addition to a variety of innate immune responses. Their findings show that during acute influenza infection, there is a notable regulation of many miRNAs, their targeted mRNA, and the resultant protein in the lung tissue of pigs. Functional studies of carefully chosen miRNAs are intended to elucidate their significance in regulating and fine-tuning the innate immune response during influenza infection, both locally and systematically (Skovgaard *et al.*, 2013).

Porcine circovirus type 2 (PCV2) is the primary infectious agent responsible for PCV2-systemic disease and is linked to various swine illnesses collectively referred to as porcine circovirus diseases. By Comparing sub-clinically infected PCV2 pigs to non-infected pigs, the objective of this work was to determine the miRNA expression patterns. Infected and non-infected pigs' MLNs showed different miRNA expression levels according to high throughput sequencing, however the tonsils showed a substantially conserved pattern. mir-129a, let-7d-3p, miRNAs 126-3p, 126-5p, and mir-let-7b-3p were down-regulated in the MLN, while mir-193a-5p, mir-574-5p, and mir-34a were up-regulated. The Functional analysis predictions indicated that these miRNAs might be associated with immune system pathways and PCV2 pathogenesis-related processes; however, functional experiments are required to validate these hypotheses. This study is the first to use a high throughput sequencing method to examine the expression of miRNA genes in PCV2-infected pigs, and it has identified numerous host miRNAs that were differentially expressed in response to PCV2 infection (Hernandez *et al.*, 2015).

Between the uninfected and infected cells by porcine parvovirus, there was a difference in the expression of miRNAs: 128 miRNAs were downregulated and 65 miRNAs were upregulated. The expression of previously reported immune-related miRNAs, including miR-10b, miR-20a, miR-19b, miR-181a, miR-146b, and miR-18a, was found. The host target genes' KEGG function annotations and Gene Ontology analysis revealed that the miRNAs are connected to a variety of intricate cellular pathways, such as immune system functions, cellular metabolism, and gene expression. Numerous microRNAs are crucial in controlling the expression of genes linked to the immune system (Li *et al.*, 2015).

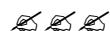
miRNA let-7 is a crucial modulator of the differentiation and operation of both innate and adaptive immune cells. Let-7 has been linked to multiple human disorders, such as viral infections and cancer. Multiple viral infections have developed strategies to dysregulate miRNA expression. let-7 family of miRNAs control several essential biological processes. Viral pathogenesis may be impacted by let-7 miRNAs. Let-7 miRNAs have demonstrated the ability to decrease the expression levels of multiple target genes, such as H1N1 M1, MAP4K4, STAT3, IL-10, ORF1ab, SARS-CoV-2, and IL-10 (Letafati *et al.*, 2022).

miR-22-5p and miR-27b-5p were found to be differentially expressed at 7 and 21 days, post-vaccination, respectively, in the first report of genome-wide identification of differentially expressed miRNA in response to live attenuated vaccine virus of classical swine fever. Two DE miRNAs, ssc-miR-22-5p and ssc-miR-27b-5p, were chosen for study based on the roles of their target genes in the immune system and viral infection pathway, as well as their log2 fold change. miR-22-5p and miR-27b-5p have the potential to be useful as biomarkers, therapeutic agents, and preventative measures against CSFV infections (Sailo *et al.*, 2019)

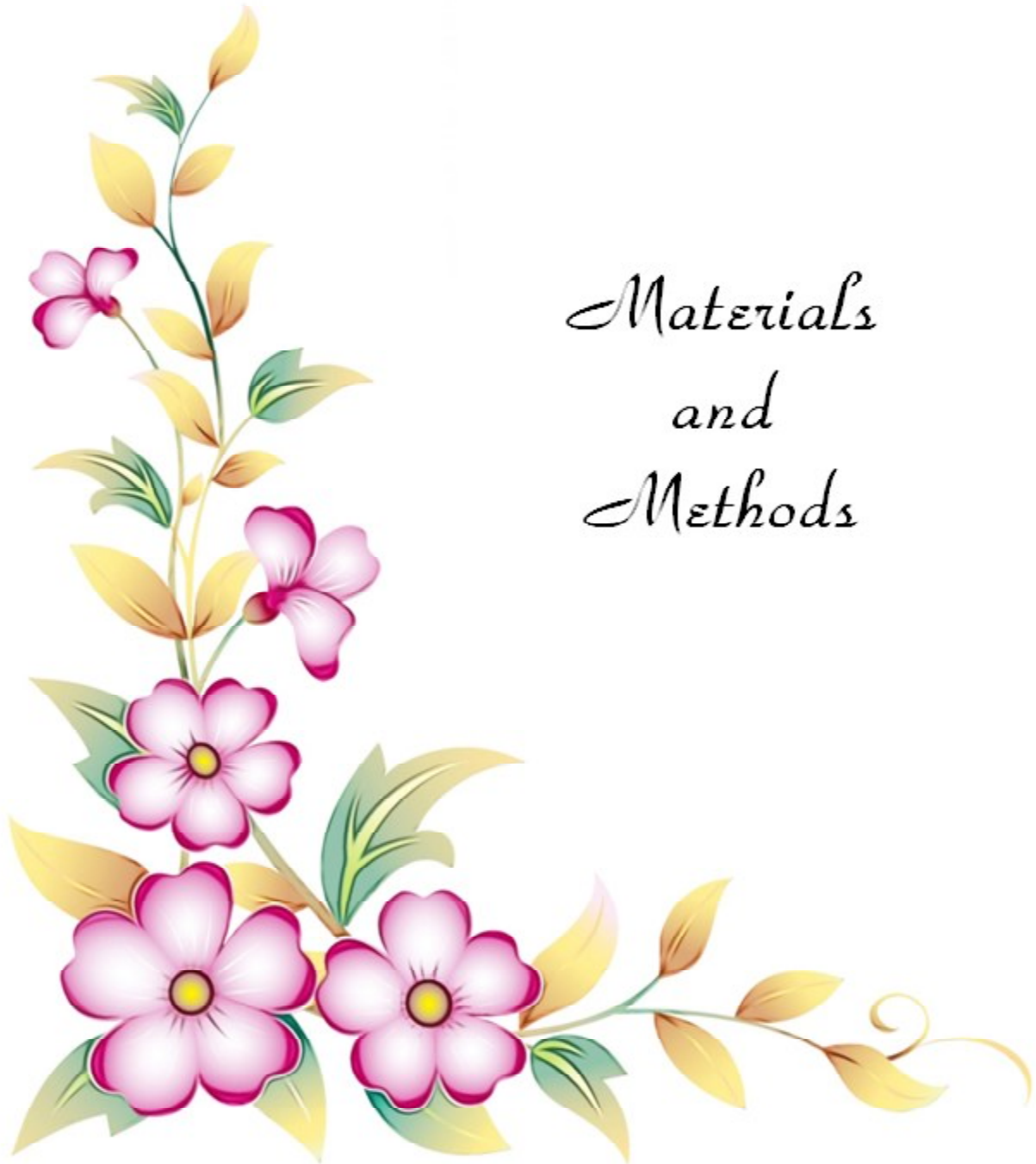
Dendritic cells (DCs) are subject to the regulation of microRNA expression by viruses, which affects immune responses. Murine DCs differently expressed 20 microRNAs (miRNAs) in response to the RNA agonist poly (I:C), with live virus infection specifically increasing miR-451. MiR-451 inhibits pro-inflammatory cytokine responses by lowering YWHAZ/14-3-3 ζ protein levels, which consequently lowers ZFP36 expression, a protein that destabilizes cytokines. TNF, CCL5, CCL3, IL-6, and TNF production were increased when miR-451 was inhibited. A regulatory feedback loop in the generation of cytokines during viral infection is also suggested by the elevation of miR-451 levels in response to IL-6 and type I interferon (Carrie M. Rosenberger *et al.*, 2012).

The swine industry has been severely impacted by the porcine reproductive and respiratory syndrome virus (PRRSV), an Arterivirus that is inhibited by miR-125b, a cellular microRNA with antiviral properties. The study screened ten conserved miRNAs and found that miR-125b inhibits PRRSV replication in MARC-145 cells and primary porcine alveolar macrophages, where it reduces viral replication and gene expression in a dose-dependent manner. Unlike other miRNAs, miR-125b does not directly target the PRRSV genome, instead it inhibits NF- κ B activation, a pathway necessary for PRRSV replication; moreover, PRRSV downregulates miR-125b post-infection to enhance its replication, highlighting a novel mechanism of viral manipulation of host miRNAs. This finding highlights the potential of targeting host factors like miR-125b for developing new antiviral strategies against PRRSV (Wang *et al.*, 2013).

With a recently identified function in hepatocyte innate immunity, microRNA-122 (miR-122) is important in liver biology and pathology. Interferon (IFN) activation in response to viral RNA, mainly hepatitis C virus RNA and poly (I), is markedly increased in hepatoma cells with miR-122 restoration. In order to do this, miR-122 downregulates STAT3 phosphorylation (Tyr705), which lifts STAT3's inhibitory effect on IFN signalling. By decreasing IRF1, STAT3 prevents IFN production, whereas miR-122 targets RTKs that encourage STAT3 phosphorylation, such as MERTK, FGFR1, and IGF1R. With possible therapeutic implications for hepatitis viruses, this miR-122, RTKs/STAT3, IRF1-IFNs circuit is essential for controlling hepatocyte innate immunity.



*Materials
and
Methods*



3.1 Design of Experiment

3.1.1 In-silico search of miRNAs related to African Swine Fever virus infection

A literature search was conducted using keywords such as microRNA, African swine fever virus, viral infections, immune response etc. in pig species to find publications published up until 2023 on Google Scholar and PubMed. According to their role in the viral infections, 15 miRNAs linked to the ASFV infection were identified and were selected for the study.

3.1.2 Source of sample

The current study was conducted at the National Research Center on Pig, Rani, Guwahati's Animal Genetics and Breeding Lab of ICAR.

3.1.3 Selection of Experimental groups

The tissue samples that were gathered during the outbreak, preserved at -80°C, and kept under these circumstances at the ICAR-National Research Center on Pig, Rani, Guwahati, were used in this investigation. For the study, three groups of animals—Survived, Died, and Control were chosen, each group includes 5 animals. The samples were collected after slaughtering the animals.

3.1.4 Selection of the biological samples

Tissue samples from the three animal groups were obtained during the ASF outbreak and preserved in RNA Later at -80°C, at the Animal Genetics and Breeding Lab of ICAR-National Research Center on Pig, Rani, Guwahati.

3.2 Characterization of microRNAs in lymphoid tissues associated with ASFV infection

3.2.1 Isolation of small RNA molecules

Isolation and Extraction of miRNA from the lymphoid organs like the spleen and lymph node was carried out using Pure Link™ miRNA Isolation kit (Invitrogen) as per the manufacturer's protocol described below

1. Minced tissue of 5mg was placed in a sterile microcentrifuge tube
2. Binding buffer (L3) of 300µL was added to the tube and ensured that the tissue was completely immersed in the buffer
3. With the help of a tissue homogenizer, tissue was homogenized
4. The lysate was centrifuged at 12000×g for 5 minutes at room temperature
5. The supernatant was transferred to a new sterile micro centrifuge tube
6. 300µl of 70% ethanol was added to it
7. Thoroughly mixed by vortexing
8. The sample was then transferred to a spin cartridge
9. Centrifuged at 12000×g for 1 minute. Do not discard the flow through
10. 700µl of 96-100% Ethanol was added to the flow through
11. Vortexed and mixed well
12. 700µl of the sample was transferred to a new spin cartridge
13. Centrifuged at 12000×g for 1 minute
14. The Remaining sample was transferred to the same spin cartridge
15. Centrifuged at 12000×g for 1 minute
16. Flow through was discarded and placed the spin cartridge into the collection tube which is sterile
17. 500 µl of wash buffer (W5) was added to the spin cartridge
18. Centrifuged at 12000×g for 1 minute
19. Repeat the wash step with the wash buffer of 500 µl
20. Flow through was discarded then placed the spin cartridge into the wash tube

21. Centrifuged for 2-3 minutes at maximum speed
22. The Wash tube was then discarded
23. Spin cartridge was then placed into a clean 1.7ml recovery tube 50-100 µl of sterile RNase- free water was added to the centre of the spin cartridge
24. Incubated for 1 minute at room temperature
25. Centrifuged at maximum speed for 1 minute
26. Spin cartridge was then removed and discarded

The recovery tube containing the purified RNA was stored at -80°C for further use

3.2.2 Determination of miRNA quality and quantity

Using a Thermo Scientific™ NanoDrop™ Lite Spectrophotometer, the number and quality of the tiny RNA molecules that were separated was determined.

3.2.3 Analysis of miRNA quality

Measurement were made of the optical density values of RNA samples blanked against RNase-free water at 260 and 280 nm. If the OD260/280 of RNA samples is greater than 1.9, it signifies that the RNA is devoid of proteins and other UV chromophores that could potentially disrupt the subsequent applications.

3.2.4 Estimation of miRNA quantity

Using a spectrophotometer blanked against RNase-free water, the solution's OD260 was determined. The following formula was used to determine the total amount of RNA.

Total RNA (µg) = OD260 × [40µg/(OD260 × 1mL)] × dilution factor × total sample volume (mL)

3.2.5 poly-A tailing

Using a poly(A) tailing kit (Invitrogen), the tiny RNA molecules that were isolated from the tissue were 3' extended with the poly(A) tail.

A master mix was made for polyadenylation in order to achieve the required number of reactions. A mixture of reagents containing 2 µL of 5x EAP Buffer, 1 µL of 2.5 mM MnCl₂,

0.1 μL of 10 mM ATP, and 0.2 μL of Poly-A Polymerase comprised the 3.3 μL of polyadenylation mix in each experiment. Following that, 10 μL was the final reaction volume after 6.7 μL of RNA was added, as indicated in the table 3.1 below. The PCR was run for 60 minutes at 37 °C after the reaction tubes were quickly spun.

Table 3.1 Composition of components for poly-a tailing of RNA

Sl No.	Components	Volume
1	5X E-PAP Buffer	2 μL
2	25 mM MnCl_2	1 μL
3	10 mM ATP	0.1 μL
4	E-PAP poly A	0.2 μL
5	RNA	6.7 μL
Total		10 μL

3.2.6 Reverse Transcription of miRNA

The polyadenylated small RNA samples were reverse-transcribed using oligo-dT primers that had adapters attached to create small RNA complementary DNA (srcDNA). The poly-A RNA strand was first prepared for reverse transcription. To do this, a reaction mixture was made to have a final volume of 10 μL , comprising 3 μL of 25 μM RT primer, 1 μL of 10 mM dNTPs, and 6 μL of Poly-A RNA. After quickly combining the reaction tubes, the Poly-A RNA strands were annealed onto the RT primers by running PCR for five minutes at 65 °C.

Table 3.2 Composition of components for the synthesis of first-strand srcDNA

Sl No.	Components	Volume
1	25 μM Long RT primer	3 μL
2	10 mM dNTPs	1 μL
3	poly-A RNA	6 μL
Total		10 μL

Using the SuperScript™ IV First-Strand Synthesis System, srcDNA was synthesized. 1 µl of 10 mM dNTP mix (10 mM each), 1 µl of DEPC-treated water, 10 µl of the initial reverse transcription mix, and 1 µl of 50 µM Oligo dT primers were all included in each reaction mix. As a result, the total reaction volume of each mixture was 13 µL. The reaction tubes were rapidly centrifuged, and the RNA-primer mixture was then heated in a thermocycler for five minutes at 65 °C to enable the primer to anneal to the template RNA. Next, the combination was allowed to incubate on ice for a minimum of one minute.

Table 3.3 Composition of components for reverse transcription of RNA

Sl No.	Components	Volume
1	50 µM Oligo dT (20)	1 µL
2	10 mM dNTP mix (10 mM each.)	1 µL
3	DEPC-treated water	1 µL
4	Template RNA	10 µL
Total		13 µL

After that, an RT reaction mix was made by combining the following components: A total of 7 µL is made up of 4 µL of 5× SSIV Buffer, 1 µL of 100 mM DTT, 1 µL of Ribonuclease Inhibitor, and 1 µL of SuperScript™ IV Reverse Transcriptase (200 U/µL). After mixing, a quick centrifugation was performed on the contents.

Table 3.4 Composition of components for the polymerase chain reaction of the srcDNA

Sl No.	Components	Volume
1	5x SSIV Buffer	4 µL
2	100 mM DTT	1 µL
3	Ribonuclease Inhibitor	1 µL
4	SuperScript™ IV Reverse Transcriptase (200 U/µL)	1 µL
Total		7µL

After that, the annealed RNA (13 μ L) was mixed with 7 μ L of the RT reaction mix, making a total volume of 20 μ L. Following that, the reaction mixture as a whole was incubated at 50–55 °C for 10 minutes. After being incubated at 80 °C for 10 minutes, the reaction was eventually rendered inactive.

3.2.7 Removal of RNA

In order to eliminate all the RNAs, 1 μ L of *E. coli* RNase H was added to the freshly produced srcDNA sample, which was then incubated for 20 minutes at 37°C and subsequently for 10 minutes at 85°C. The concentration of srcDNA was measured with a NanoDrop™ Lite Spectrophotometer. Next, the concentration of each srcDNA sample was brought to the same level.

3.2.8 Polymerase chain reaction of miRNA

For the current experiment, a total of 15 miRNAs related to ASF infection in swine species were chosen based on prior literature. Polymerase chain reaction (PCR) of the miRNAs was done using miRNA-specific forward primers and universal reverse primers.

3.2.9 Designing of primers for miRNA

The miRBase microRNA database (<http://www.mirbase.org/>), where a miRNA functions as a forward primer on its own, is where the miRNA sequences were chosen. Since most miRNAs are only 19–23 bp long, replicating them with traditional PCR is less likely to yield a unique result. In order to design using two sets of primers, the miRNA must elongate sufficiently to create a template long enough. This has the extra benefit of allowing for the use of two distinct oligos, which increase the flexibility and specificity of primer creation. Using, two miRNA-specific primers, a universal reverse primer with 3–8 specific nucleotides at the 3' end and an extension that is complementary to a universal tag added to the template during cDNA synthesis, and a forward primer that is 20–24 bp long, the miRNAs were amplified in the current effort. In the following step, the product size is increased to 70–80 bp long by polyadenylation, adaptor ligation, and sufficient specificity enhancement along with a universal reverse primer. The pool of short RNA cDNAs (srcDNA) on the agarose gel plate can therefore

be more easily divided into individual miRNAs to be isolated and seen. primers utilized in the current study along with their corresponding sequences are listed in Table 3.5

Table 3.5. List of microRNAs and their forward primers are described below

Sl. no	miRNA	Primers	bp
1	miR-451	AAACCGTTACCATTACTGAGTT	22
2	miR-145-5p	GTCCAGTTTTCCCAGGAATCCCTT	24
3	miR-181a	AACATTCAACGCTGTCGGTGAGTT	24
4	miR-122-5p	TGGAGTGTGACAATGGTGTTTGT	23
5	miR-122-3p	AACGCCATTATCACACTAAAT	21
6	miR-92b-3p	TATTGCACTCGTCCCGGCCTCC	22
7	miR-126-3p	TCGTACCGTGAGTAATAATGCG	22
8	miR-30d	TGTAAACATCCCCGACTGGAAGCT	24
9	MiR-92a	TATTGCACTTGTCCCGGCCTGT	22
10	miR-30e-5p	TGTAAACATCCTTGACTGGAAGCT	24
11	miR-500a-5p	TAATCCTTGCTACCTGGGTGAGA	23
12	miR-125b	TCCCTGAGACCCTAACTTGTGA	22
13	miR-125a	TCCCTGAGACCCTTTAACCTGTG	23
14	miR-140-5p	AGTGGTTTTACCCTATGGTAG	21
15	miR-140-3p	TACCACAGGGTAGAACCACGGAC	23
16	Universal Reverse primer	ATCCAGTGCAGGGTCCGAGG	20
17	Long RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTTTAGCTTTTTTTTTTTT TTTTTTTTTTTTTTT	

Primers unique to miRNA were used for the forward and universal reverse of the srcDNA polymerase chain reaction (PCR). Using the following ingredients, a PCR reaction mix was made: One microliter of 10 mM universal reverse primer, five microliters of 2x PCR master mix, and one microliter of nuclease-free water. For every tube, 7 μ L of the reaction mix was aliquoted. A final volume of 10 μ L was achieved by adding 1 μ L primer and 2 μ L src DNA to each designated tube. Following a quick centrifugation, the contents were combined.

Table 3.6 Composition of components for conventional PCR

Sl No.	Components	Volume
1	2x PCR master mix	5 µL
2	10 mM universal reverse primer	1 µL
3	Nuclease Free Water	1 µL
Total		7 µL

Then PCR was run for 30 cycles

- a. 94°C for 30 seconds
- b. 94°C for 1 minute
- c. 59°C for 30 seconds
- d. 72°C for 30 seconds
- e. 72°C for 10 minutes

Following PCR, gel electrophoresis was used to run the samples, and a qualitative analysis based on the sample's gene expression was conducted.

3.2.10 Agarose gel electrophoresis

The amplified product was checked for quality and quantity by agarose gel electrophoresis using the protocol briefed below.

1. After dissolving and melting 3 g of agarose in 100 ml of 1x TBE and letting it cool to 60 °C, Ethidium Bromide of 2 ml (10 mg/ml stock at a final concentration of 0.5 µg/ml) was added and well mixed.
2. After filling a gel-casting tray with an acrylic comb, pour the mixture into it and let it to solidify.
3. Following the gel's solidification, a small amount of TBE buffer was added, the comb was gently taken out, and the gel was submerged in the electrophoresis tank that held one doubling of TBE buffer.

4. 10 µl of PCR product was loaded into the wells along with 50 bp DNA marker in a separate lane.
5. Electrophoresis was carried out @ 80 volts. The bands were visualized in Gel Doc XR+ System (BIO-RAD).

3.2.11 Expression Profiling of miRNAs using qPCR

The PCR-positive tests miRNAs were further validated by qRT-PCR. Agilent Aria Mx real-time PCR system was used to perform the quantitative analysis using Fast SYBR green PCR Master Mix (Thermo Fisher) keeping miR-16 as reference gene. These tests were carried out in duplicates. Ct values were generated using fluorescent data. Two, PCR replicates were used to calculate the mean Ct values.

Table 3.7 Composition of components for qPCR

Sl No.	Components	Volume
1	Fast SYBR Green PCR Master Mix	5 µL
2	Forward Primer	0.5 µL
3	Reverse Primer	0.5 µL
4	Template srcDNA	2 µL
5	Nucleus Free water	2 µL
Total		10 µL

The reaction was performed using 5 µL Fast SYBR green PCR Master Mix, 1 µL forward and reverse primers, nucleus free water of 2 µL and 2 µL srcDNA as a template in a 10 µL total reaction volume. The qPCR cycling conditions were as follows:

- a. 94°C for 30 seconds
- b. 94°C for 1 minute
- c. 60 °C for 15 seconds
- d. 72°C for 15 seconds
- e. 72°C for 10 minutes

miR-16 was utilized as the reference gene for qPCR analysis. The details of primers of reference gene are provided in Table 3.8

Table 3.8: List of primers of reference gene used for qPCR or miRNAs

Gene	Primer sequence	bps
1.miR-16F	TAGCAGCACGTAAATATTGGCG	22
2.miR-16R	ATCCAGTGCAGGGTCCGAGG	20

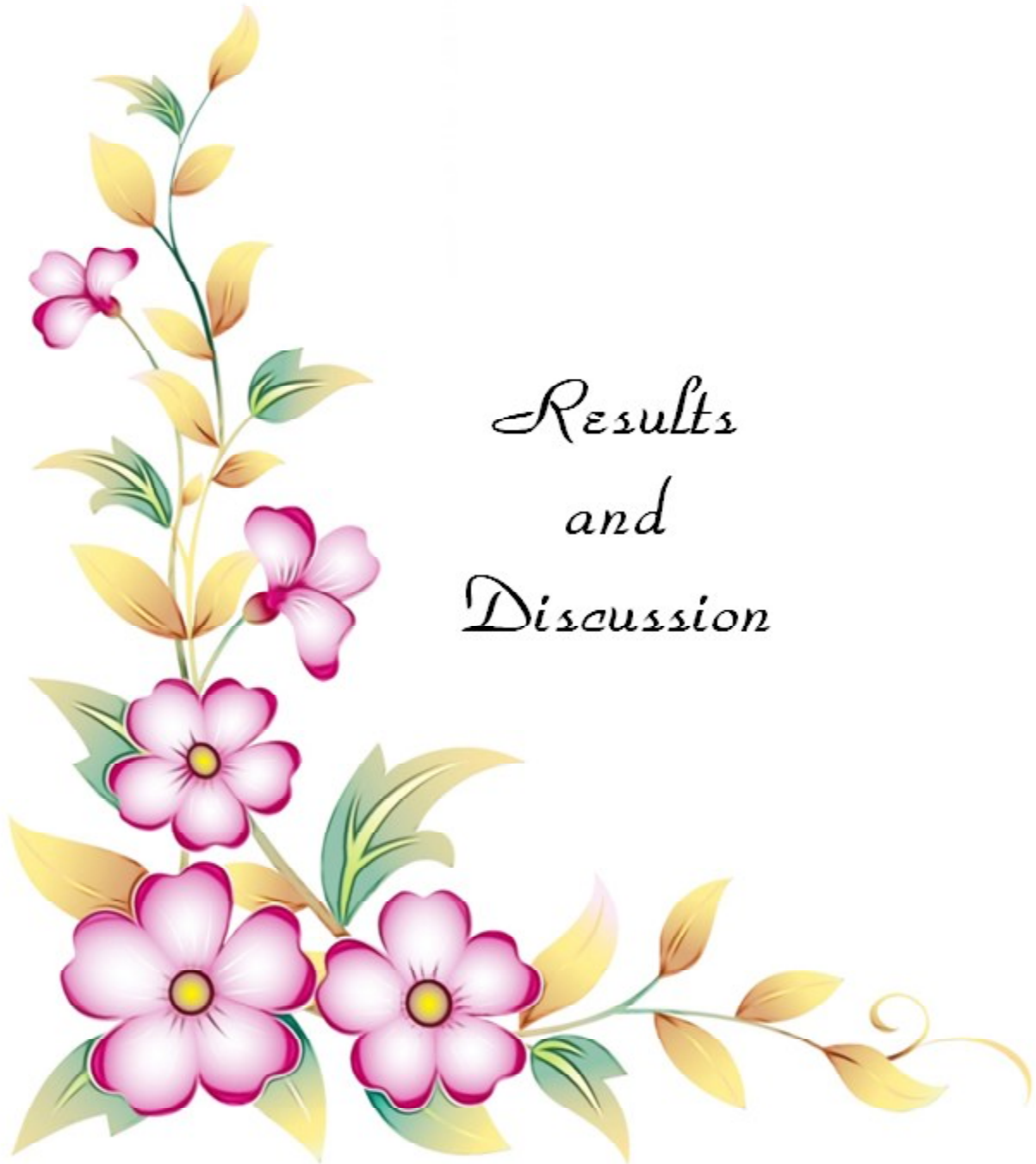
3.2.12 Analysis of the Results

To determine if there was a difference in miRNA expression between the ASF virus-infected pigs who died and survived, with control (healthy pigs), the data from quantitative PCR was analyzed using the comparative CT method, also referred to as the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The melt curve analysis was performed to confirm the non-specific amplification.

The CT values obtained from real-time PCR instrumentation were used to find “CT, which is equal to the difference in threshold cycles for the target and reference gene assuming that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene. The miR-16 gene served as an internal control. The relative expressions were analyzed by calculating $2^{-\Delta\Delta CT}$. The results were expressed in fold change and compared to the control group. T test was used to examine changes in the relative expression of miRNAs in ASF infected tissues in relation to miR- 16 as the reference gene and A p-value < 0.05 was considered statistically significant (*) and p - value < 0.01 was considered highly statistically significant (**).



*Results
and
Discussion*



The present study on comparative expression profiling of microRNAs in African swine fever infected and non-infected porcine tissue samples is focused on In-silico searched miRNAs related to ASF infection, The identification of differentially expressed miRNAs in tissue samples of 3 groups (control, infected died, infected survived) of pigs and Quantification of miRNAs using real-time PCR. The study's major findings and a discussion are presented below.

4.1 *In-silico* research of miRNAs related to ASFV infection in lymphoid tissues

The miRNAs in lymphoid tissues were In-silico searched by literature search using the keywords miR, microRNA, ASFV, ASF infection, spleen, lymph node, viral infection and immune response. All research outputs published up to 2023 in PubMed, Google Scholar and other search engines for scientific literature were used. The corresponding functional activity and roles, especially concerning ASF infection and viral infections, were identified. A total of 15 miRNAs associated with ASF infection were listed and selected in accordance with their functional roles. The OD ratio 260/280 of the samples was almost 1.98 and the concentration of the samples was nearly 110 ng/μl in all the three groups.

4.2 Expression Profiles of Selected miRNAs in Lymphoid Tissue

In-silico searched miRNAs were subsequently screened for their target gene having a definitive role in immune pathways and viral diseases. A total of 15 miRNAs were selected for their differential expression study in infected and non-infected samples. All of the 15 miRNAs displayed specific amplification in qualitative PCR, which was later confirmed by quantitative PCR. The results of the polymerase chain reaction of the miRNAs in Died, survived and control groups of animals in spleen and lymph node tissue are shown in the gel images below in figures (4.1,4.2,4.3,4.4,4.5,4.6).

Table 4.1. Fold change of expression of miRNAs involved in African Swine Fever Virus infected and non- infected spleen tissue samples

miRNAs	Fold change average			Standard Error		
	Died	Survived	Control	Died	Survived	Control
miR-451	2.423	0.027	1.0004	0.5302	0.0128	3.2043
miR-92b-3p	0.0005	0.019	1.0004	0.0050	0.0403	0.0360
miR-30d	0.014	0.072	1.0002	0.0311	0.0493	0.1156
miR-30e-5p	0.046	0.197	1.0064	0.0524	0.0530	0.1181
miR-125b	0.003	0.398	1.0008	1.42E-06	0.0161	0.0003
miR-122-5p	0.002	0.002	1.0006	0.0070	0.0153	0.0170

The relative expression profiling of miR-451 showed 0.027 fold lower expression in survived group compared to the control group where as it showed 2.42 fold higher expression in died group in comparison to the control group, miR-451 is upregulated in the died group of animals compared to the survived group of animals miR-451 has a role in immune response, it was found to reduce the production of several cytokines (like IL-6, TNF, CCL5/RANTES and CCL3/MIP1 α), signalling molecules that promote inflammation. miR-451 controls the levels of a protein called YWHAZ that influences the activity of two other proteins, FOXO3 and ZFP36, which are known to suppress cytokine production.

The relative expression profiling of miR-92b-3p showed 0.0005-fold lower expression in the Died group of animals than a control group of animals. miR-92b-3p showed 0.019-fold lower expression in survived samples in comparison to control samples. miR-92b-3p is up-

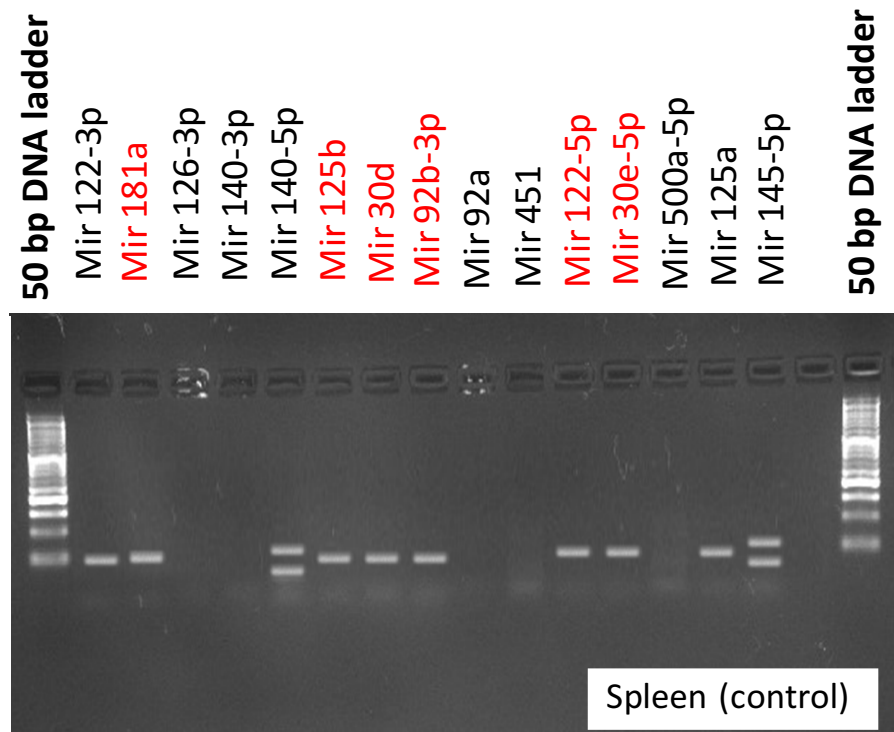


Fig. 4.1: Expression of miRNAs in the control group of spleen tissue

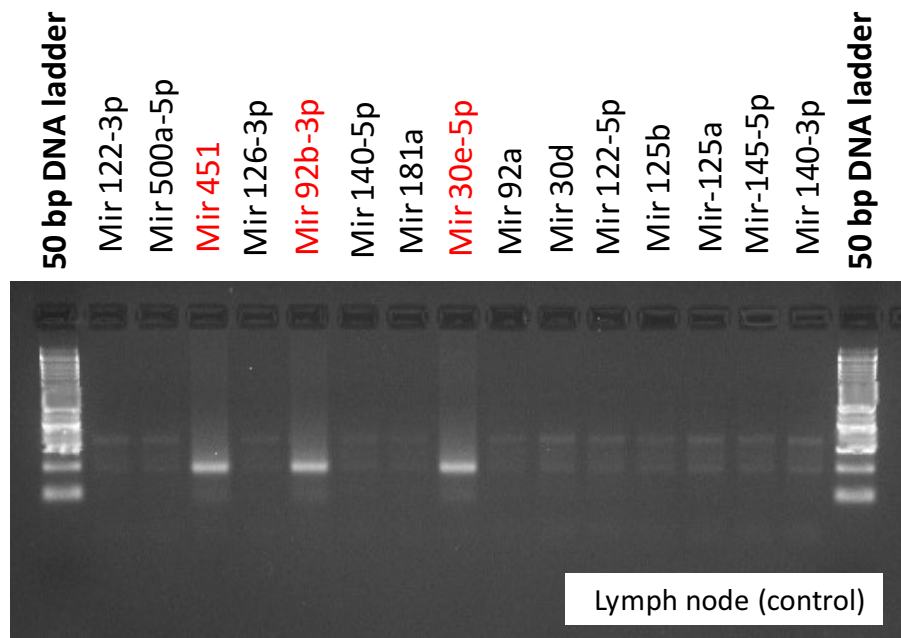


Fig. 4.2: Expression of miRNAs in the control group of Lymph node tissue

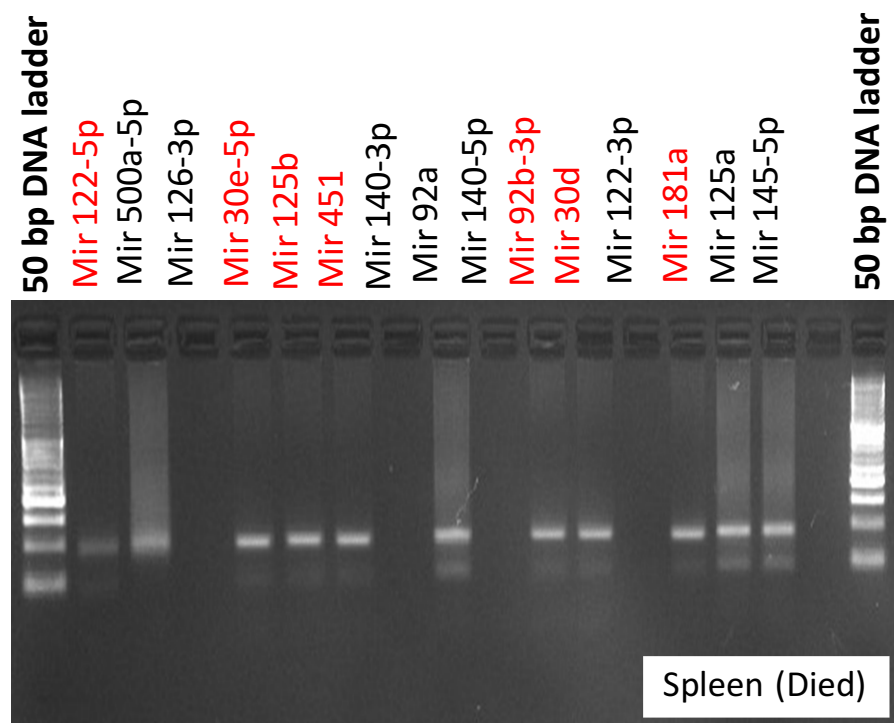


Fig. 4.3: Expression of miRNAs in died group of spleen tissue

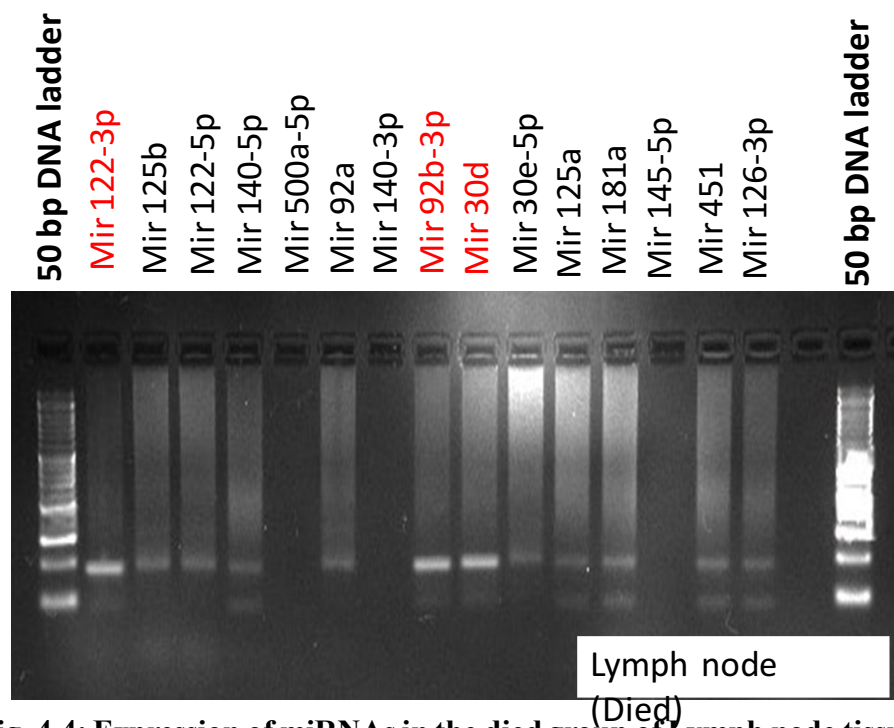


Fig. 4.4: Expression of miRNAs in the died group of Lymph node tissue

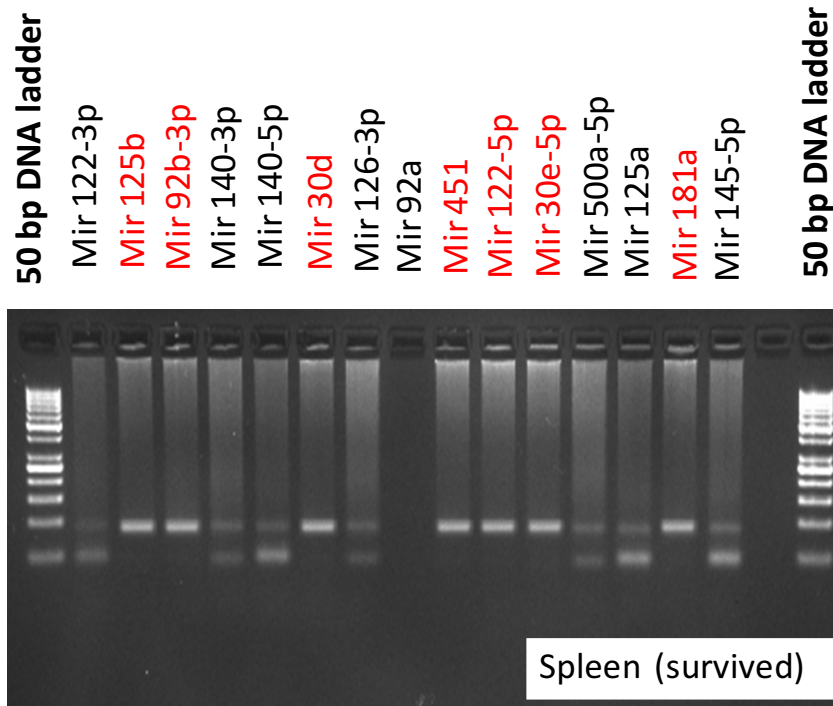


Fig. 4.5: Expression of miRNAs in Survived group of spleen tissue

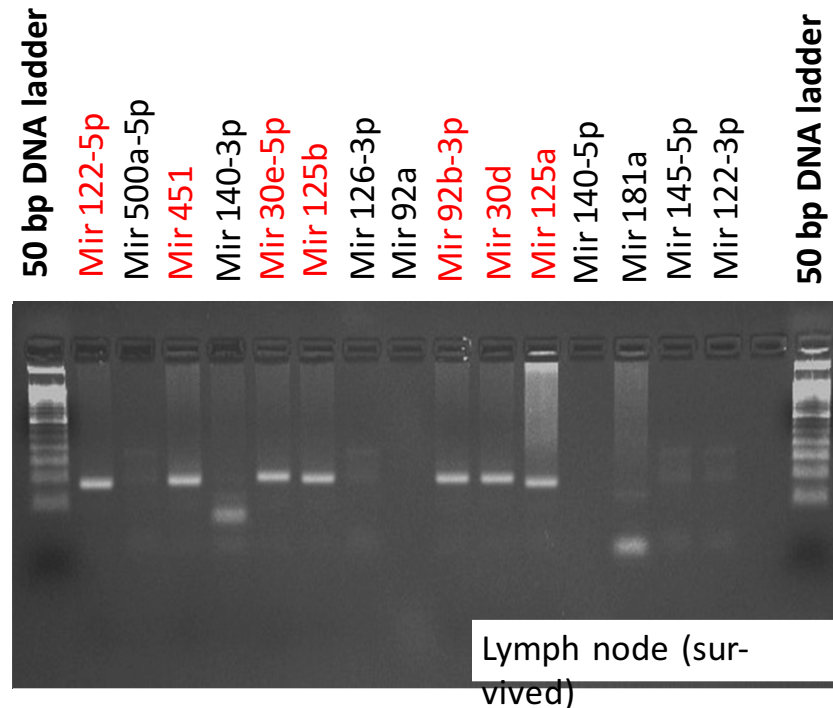


Fig. 4.6: Expression of miRNAs in Survived group of Lymph node tissue

regulated in the survived group of animals compared to the died group of animals in the Fig. 4.7. Relative expression of miR-92b-3p is highly significant at a 1% level of significance in both Died and Survived groups of animals

The function of miR-92b-3p is, it reduces inflammation and autophagy. miR-92b-3p directly targets TRAF3, meaning that miR-92b-3p can bind to TRAF3 and reduce its levels. By doing so, miR-92b-3p helps to control the inflammatory response and autophagy. MKK3-p38 Pathway activity which is also associated with the inflammatory response was reduced by blocking the TRAF3 gene according to (Sun *et al.*, 2020).

The miR-92b-3p which is involved in reducing the inflammatory response is upregulated in survived group compared to died group indicating that it may further prevent the death of healthy tissue due to inflammation.

The relative expression profiling of miR-30d showed 0.014-fold lower expression in the died group of animals than control group of animals. In survived group of animals, miR-30d was 0.07-fold less expressed than control samples. miR-30d is up-regulated in the survived group of animals compared to the died group of animals was shown in the (Fig. 4.8). Relative expression of miR-30d is highly significant at a 1% level of significance in both the died and survived group of animals.

miR-30d targets a specific gene called USP47, which produces an enzyme involved in De-ubiquitination (removing certain tags from proteins) which regulates viral replication, increased levels of mir-30d reduce the replication of the virus and vice versa (Li *et al.*, 2020).

The miR-30d was upregulated in the survived group compared to the died group in the present study suggesting that it prevents the replication of virus and might be responsible for the survival of that group.

The relative expression profiling of miR-30e-5p showed 0.19 fold lower expression in a survived group of animals than in a control group of animals. In the died group of animals, miR-30e-5p was 0.046 fold less expressed than in control samples as shown in the Fig. 4.9. miR-30e-5p is upregulated in the survived group of animals compared to the Died group of animals indicating that this miRNA might be responsible for the death of the animals. Relative

expression of miR-30e-5p is highly significant at a 1% level of significance in both the died and survived groups of animals

miR-30e-5p seems to play a role in inflammation. MiR-30e-5p normally decreases the amount of a protein called AtI2. The AtI2 seems to help reduce inflammation, so when miR-30e-5p lowers AtI2, it contributes to inflammation. miR-30e-5p expression was negatively correlated with AtI2 expression.

This study shows that this miR-30e-5p which is upregulated in the survived group might responsible in preventing further damage of the cells due to inflammation.

The relative expression profiling of miR-125b showed 0.4 fold lower expression in survived group of animals than control group of animals. miR-145 showed 0.003 negligible fold of expression in died samples in comparison to control samples as shown in the Fig. 4.10. Relative expression of miR-125b is highly significant at 1% level of significance in both the died and survived groups of animals.

miR-125b, a microRNA known for its role in immunity, can inhibit the replication of the virus. miR-125b does this by suppressing the activation of a protein complex called NF-kB. NF-kB is crucial for various immune responses, but it also seems necessary for PRRSV replication. When miR-125b is active, it reduces NF-kB activation, which in turn hinders the virus's ability to replicate (Wang D *et al.*, 2013).

Since the miR-125b is upregulated in the survived group compared to the Died group. This microRNA miR-125b could be responsible for the survivability of the animals in this group of animals.

The relative expression profiling of miR-122-5p showed 0.0025 fold negligible expression levels in the Died and 0.0026 fold in survived group as compared to control group of animals as shown in the Fig. 4.11. Relative expression of miR-122-3p is highly significant at 1% level of significance in both Died and Survived groups of animals.

The role of miR-122-5p increases the production of interferons (IFNs), which are proteins that help the immune system recognize and fight off viral infections by blocking the activation of a protein called STAT3 by stopping its phosphorylation (Xu *et al.*, 2019).

miR-122-5p which was upregulated in this study suggests that this miRNA might be responsible for survivability of the animals by preventing the viral replication by increasing the interferon levels.

Table 4.2. Fold change of expression of miRNAs involved in African Swine Fever Virus infected and non- infected lymph node tissue samples

miRNAs	Fold change average			Standard Error		
	Died	Survived	Control	Died	Survived	Control
miR-451	0.027	1.094	1.0003	0.050	0.144	0.047
miR-92b-3p	3.061	0.266	1.029	0.641	0.037	1.243
miR-30e-5p	3.718	0.332	1.0004	0.028	0.142	0.048

The relative expression profiling of miR-451 showed significantly 0.027-fold lower expression in the died group of animals than a control group of animals. miR-451 shows 1.09-fold higher expression in survived group of animals, in comparison to control samples as shown in the Fig. 4.12. Relative expression of miR-451 is highly significant at a 1% level of significance in the died group of animals and significant at a 5% level of significance Survived group of animals.

miR-451 was up-regulated in the survived group of animals compared to the died and control group of animals.

miR-451 has a role in immune response, it was found to reduce the production of several cytokines (like IL-6, TNF, CCL5/RANTES, and CCL3/MIP1 α), signalling molecules that promote inflammation. miR-451 controls the levels of a protein called YWHAZ that influences the activity of two other proteins, FOXO3 and ZFP36, which are known to suppress cytokine production (Rosenberger *et al.*, 2012).

The miR-451 upregulated in the survived group in this study suggests that this miRNA by reducing the levels of cytokines reduces the inflammation and prevents the damage of the cells due to inflammation.

The relative expression profiling of miR-92b-3p showed significantly 3.06-fold higher expression in the died group of animals than a control group of animals. miR-92b-3p shows 0.26-fold lower expression in the survived group of animals in comparison to control samples as shown in the Fig. 4.13. Relative expression of miR-92b-3p is significant at 5% level of significance in both the group of animals.

MiR-92b-3p helps to reduce inflammation (the body's response to injury or infection, which can cause pain, redness, and swelling) and autophagy (a process where cells clean out damaged parts to keep themselves healthy). The MKK3-p38 pathway and TRAF3 is a gene that plays a role in inflammation and immune responses. miR-92b-3p targets the TRAF3 and MKK3- p38 pathways and blocks them, which helps to reduce inflammation (Sun *et al.*, 2020).

miR-92b-3p is upregulated in the Died group of animals compared to the survived group of animals indicating that this miRNA might be responsible for the death of the animals in the Lymph node.

The relative expression profiling of miR-30e-5p showed significantly 3.7-fold higher expression in Died group of animals than control group of animals. miR-30e-5p had 0.33-fold low expression in survived samples in comparison to a control samples. miR-30e-5p is up-regulated in the dead group of animals compared to the survived group of animals as shown in the Fig. 4.14. Relative expression of miR-30e-5p is highly significant at a 1% level of significance in both died and survived groups of animals.

miR-30e-5p seems to play a role in inflammation. miR-30e-5p normally decreases the amount of a protein called AtI2. AtI2 seems to help reduce inflammation, so when miR-30e-5p lowers AtI2, it contributes to inflammation. miR-30e-5p expression was negatively correlated with AtI2 expression (Liu *et al.*, 2022).

Both the miRNAs, miR-92b-3p and miR-30e-5p were upregulated in the died group compared to the survived group in the Lymph node.



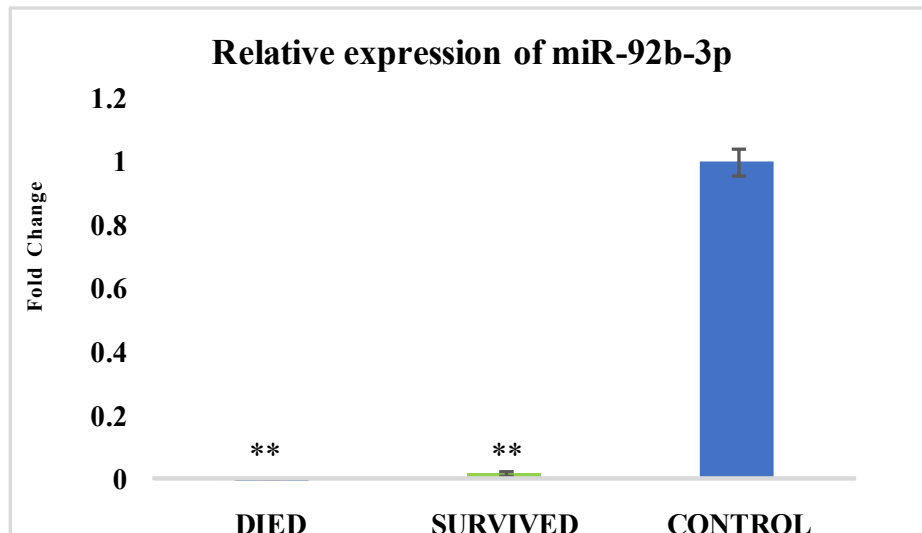


Fig. 4.7: RELATIVE EXPRESSION OF miR-92b-3p-Spleen

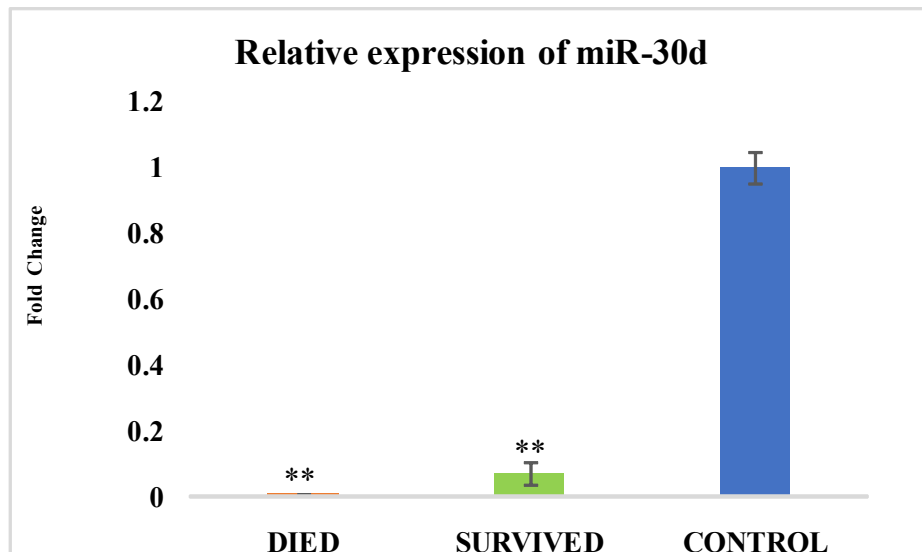


Fig. 4.8: RELATIVE EXPRESSION OF miR-30d-Spleen

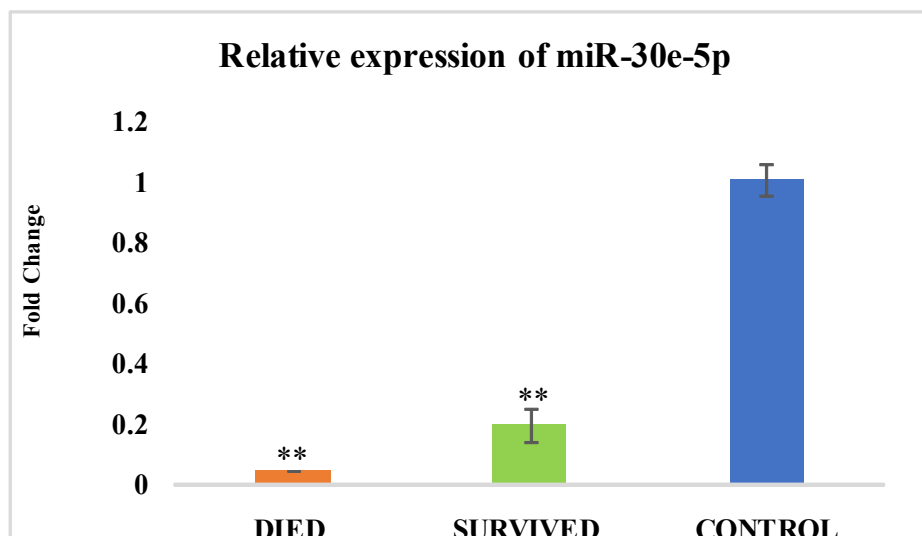


Fig. 4.9: RELATIVE EXPRESSION OF miR-30e-5p-Spleen

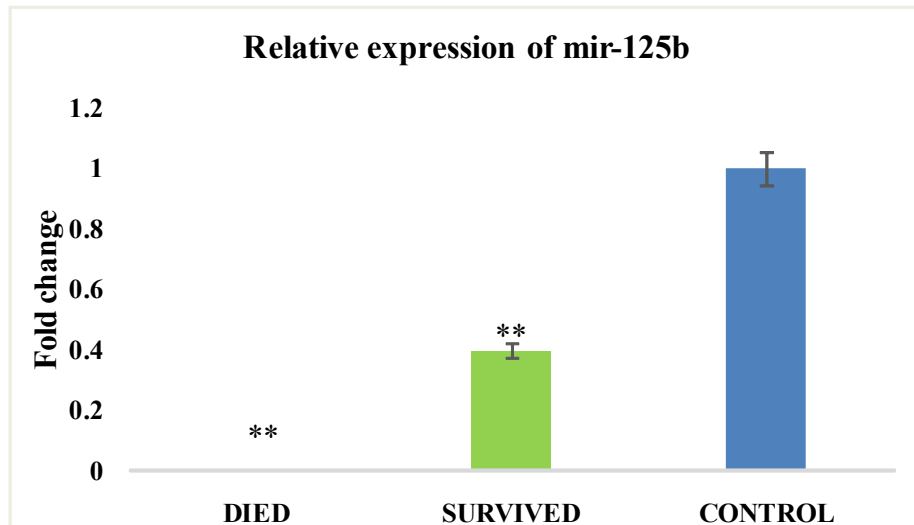


Fig. 4.10: RELATIVE EXPRESSION OF miR-125b-Spleen

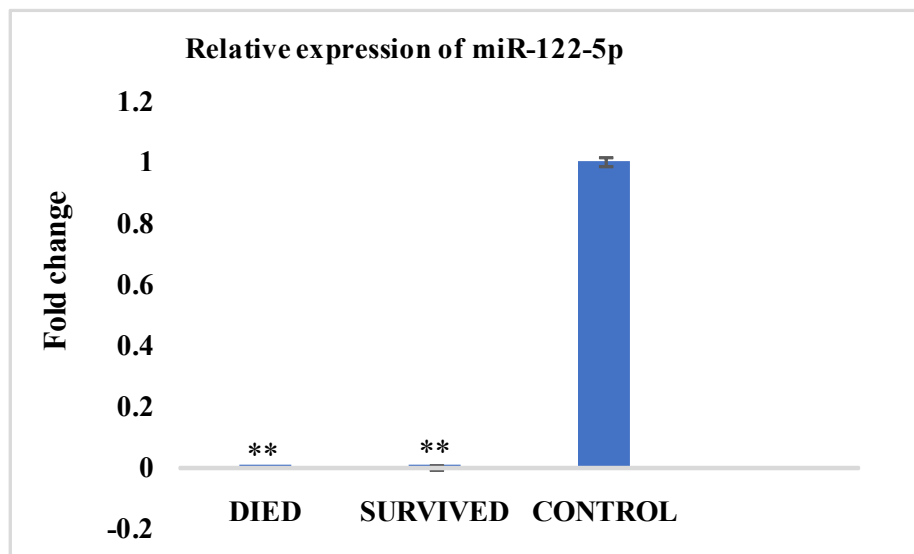


Fig. 4.11: RELATIVE EXPRESSION OF miR-122-5p-Spleen

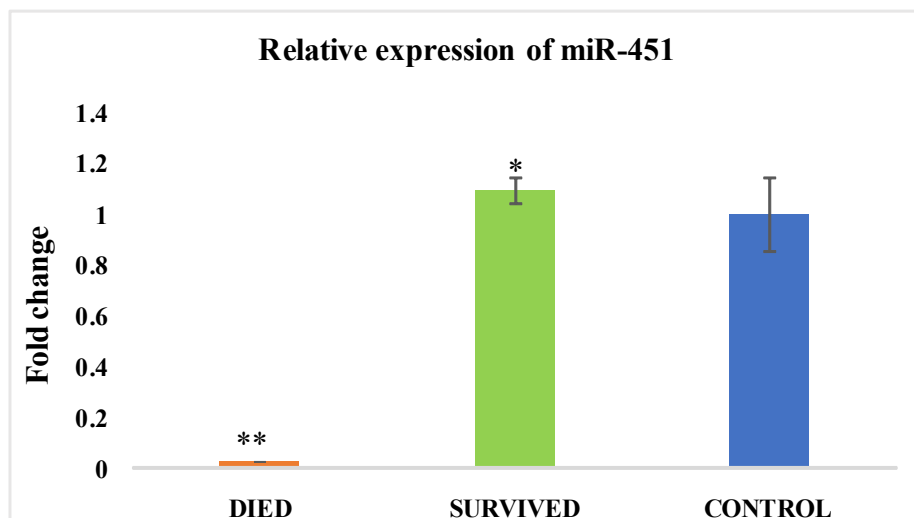


Fig. 4.12: RELATIVE EXPRESSION OF miR-451-Lymph node

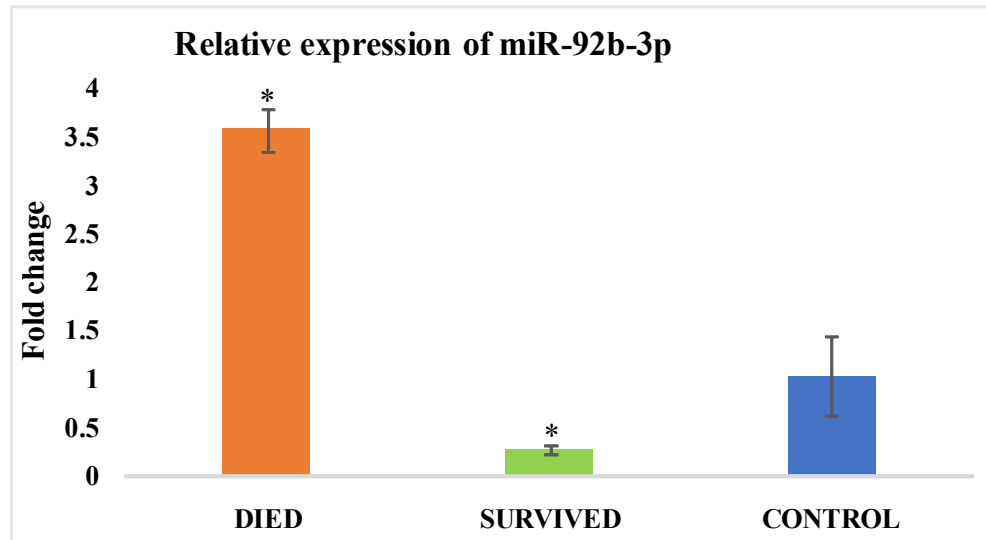


Fig. 4.13: RELATIVE EXPRESSION OF miR-92b-3p-Lymph node

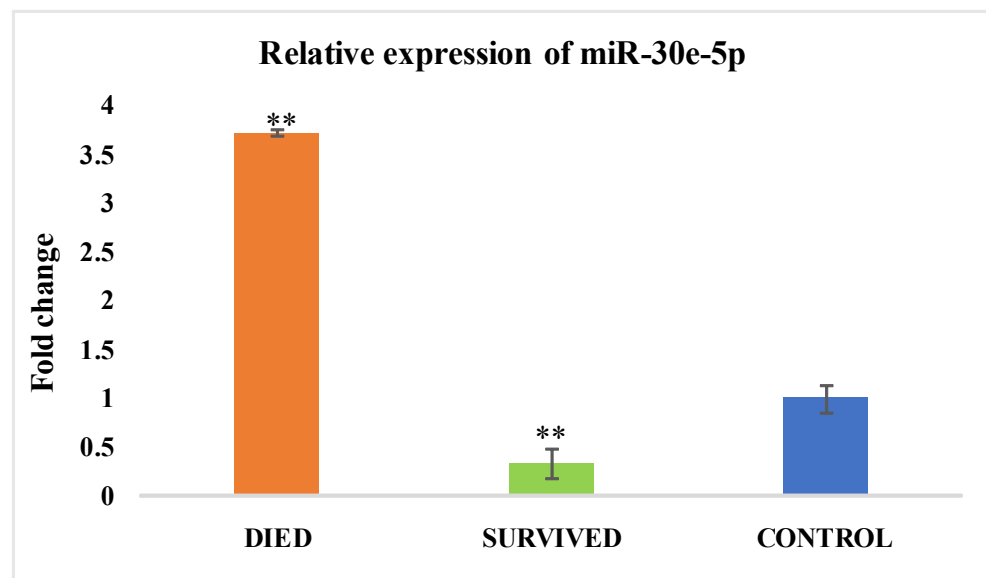
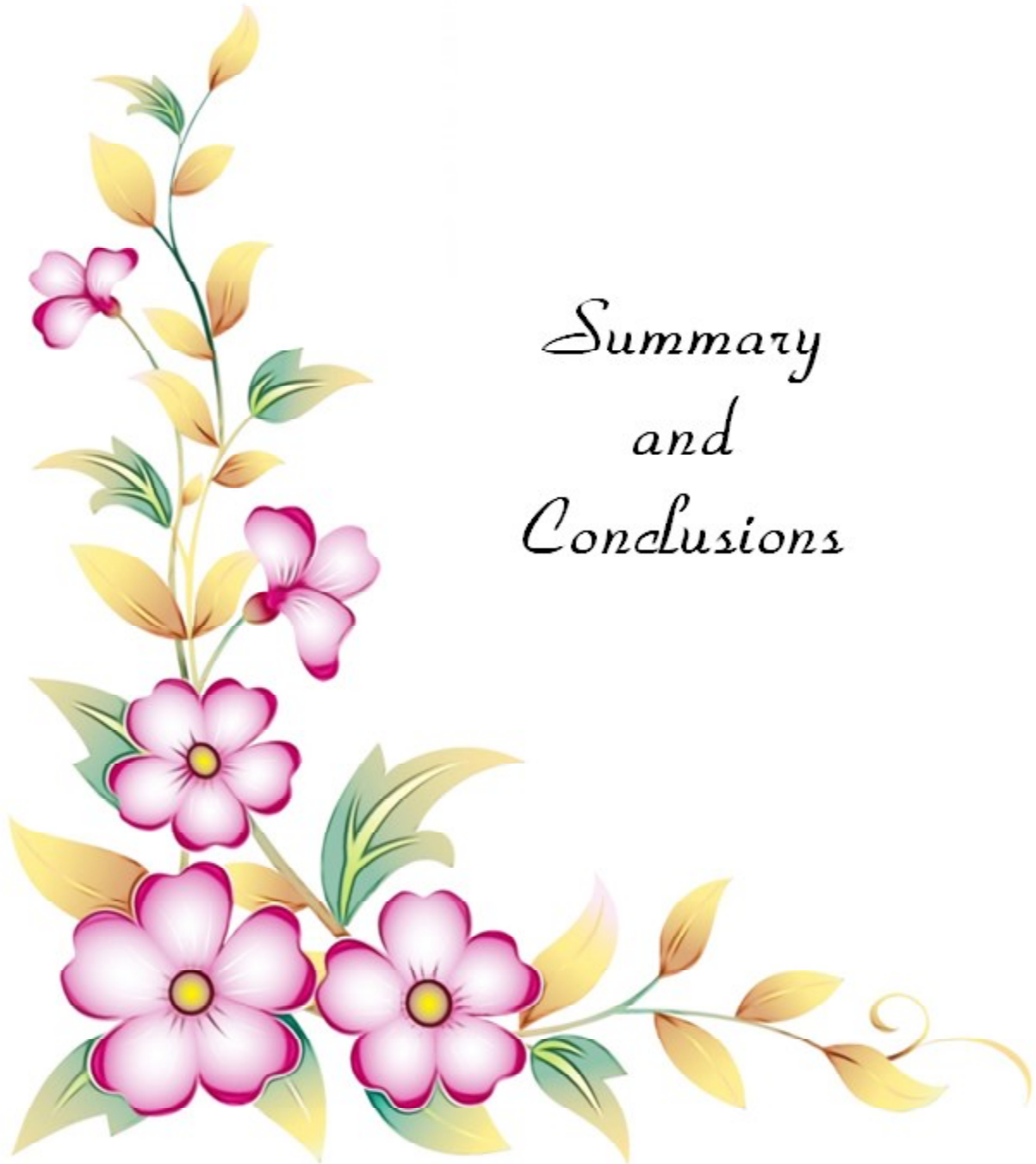


Fig. 4.14: RELATIVE EXPRESSION OF miR-30e-5p-Lymph node

*Summary
and
Conclusions*



Recent scientific advances have shown that small non-coding RNAs (sncRNA) such as microRNAs (miRNAs) can act as predictive markers for disease diagnosis in viral infections. In this direction, the present research work focused on the comparative expression profiling of microRNAs (miRNAs) in African swine fever virus infected and non-infected tissue samples. The study was conducted with the objectives of qualitative and quantitative expression profiling of miRNAs in infected and non-infected tissue samples and their comparison of expression between infected and non-infected groups of animals.

For the In-silico search of miRNAs associated with ASF infection, literature search in PubMed a Google Scholar and other search engines for articles published up to 2023 was done using the following keywords: miR, microRNA, ASFV, ASF infection, spleen, and lymph node, immune response, viral infections. A total of 15 miRNAs related to ASF infection were listed which were basically involved in ASF infection. All - the miRNAs were checked for the expression by using conventional PCR. miRNAs expressed in qualitative studies were taken for their quantification using real time PCR. 15 such miRNAs had been identified in the present study which were directly involved in immune pathways and ASF infection and therefore chosen based on previous literature.

The spleen and lymph node tissue samples of three groups of animals (Died, Survived and control) were collected during the outbreak- and which were stored at -80°C to maintain the integrity of the tissue samples at the pig farm of ICAR-National Research Centre on Pig, Rani, Guwahati. It was then processed for gene expression profiling. For this, miRNAs were

isolated from spleen and lymph node tissue and were 3'-extended with a poly (A) tail using a poly(A) tailing kit in order to increase the length of miRNAs since these are short sequences with 17-25nt. The polyadenylated small RNA samples were reverse-transcribed into small RNA complementary DNA. Polymerase chain reaction (PCR) of the miRNAs was done using miRNA-specific forward primer and universal reverse primer. The PCR-positive spleen and lymph node miRNAs were further validated by qRT-PCR keeping miR-16 as the reference gene.

All 15 miRNAs underwent testing in the three distinct groups of animals. Each of the tested miRNAs exhibited distinct amplification in qualitative PCR, a result that was subsequently validated through quantitative PCR and identified differentially expressed miRNAs between control, died and survived groups of animals. Out of the 15 miRNAs analysed, 1 miRNA was upregulated in the died group of animals and 5 miRNAs up-regulated in survived group of animals in lymphoid tissues (spleen and lymph node). The miRNAs that were upregulated in the died group of animals include miR-451, which has a role in the regulation of gene involved in inflammation during viral infection. The 5 miRNAs that were upregulated in a survived group of animals include miR-92b-3p, miR-30e-5p that regulate the inflammation and miR-30d, miR-125b, miR-122-3p those have a function in inhibiting the viral replication to fight off viral infection.

Overall, this study concluded that miRNAs can be used to study host-pathogen interactions and have important implications for modulating the expression of genes involved in the host's immune response. For instance, they can enhance or suppress the production of interferons and other cytokines that are critical for antiviral defence. Further studies are needed to validate the roles of the differentially expressed miRNAs in viral infection in pigs and to explore the potential applications of these small RNAs as biomarkers for therapeutic interventions and in vaccine development.

Furthermore, the results of our study could be used to develop strategies for vaccine development and therapeutic approaches for controlling the diseases in pigs. Moreover, the identified small RNAs could be used as potential biomarkers for controlling the disease by finding out the gene which is involved in suppressing or increasing the infection. The identified

small RNAs could also serve as potential biomarkers for viral diseases in pigs, which could help farmers to overcome the economic loss by the death of the pigs. Putative miRNA biomarkers will pave the way to rapid advances in future selection methodologies. Targeted modulation of specific miRNA functions may be used as a novel approach for therapeutic intervention in managing viral infections.

In conclusion, our study identified immune related miRNAs in the lymphoid tissue of infected and non-infected groups of animals. We found differences in the expression of these small RNAs in three groups of animals and also differences in expression in different tissue samples. Our study provides new insights into the molecular mechanisms that regulate the immune response in pigs in response to ASF infection and could be useful in enhancing the health performance of the pig herd.

Future prospects

After completing differential expression studies of miRNAs in ASFV infection, there are several advanced studies you can pursue to further understand the role of these miRNAs and their potential therapeutic applications. Here are some suggestions:

- In Vitro/In Vivo Knockdown or Overexpression: Use of antisense oligonucleotides (ASOs), siRNAs, or miRNA mimics in cell lines and pigs to identify miRNA roles in viral replication, immune response, and cell survival.
- Transcriptomic Profiling (RNA-Seq): Perform RNA-Seq to integrate miRNA data and identify miRNA-target gene interactions involved in ASFV pathogenesis. Conduct pathway and network analysis on differentially expressed genes.
- Gene Therapy Approaches: Develop miRNA inhibitors or mimics to modulate immune response and reduce ASFV replication. Explore CRISPR-Cas9 to edit miRNA-regulated genes.
- Vaccine Development; miRNA as Vaccine Adjuvants to Investigate the potential of using miRNAs as vaccine adjuvants to enhance immune responses against ASFV. This could involve designing vaccines that modulate miRNA expression to boost immunity.

- O Vaccination Response Studies: Analysing miRNA expression profiles in vaccinated pigs to identify biomarkers of vaccine efficacy and immune response.

Each of these studies will help us to understand the pathogenesis of ASFV, host responses, and potential therapeutic or diagnostic applications, potentially leading to significant advancements in controlling and treating ASFV.





This study was designed to explore the differential expression of miRNAs in response to ASFV infection across two key immune-related tissues: the spleen and lymph nodes. These tissues are critical components of the porcine immune system, playing central roles in the detection and response to pathogens. We have selected miRNAs that were hypothesized to be involved in ASFV infection based on earlier studies and bioinformatics predictions were used in this study. The study included samples from three distinct groups of animals: those that succumbed to ASFV infection (died), those that survived the infection (survived), and a control group of uninfected animals (control). Our research approach was divided into two main phases. The first phase involved qualitative expression studies using conventional polymerase chain reaction (PCR) techniques. This phase was crucial for identifying miRNAs that were differentially expressed in the infected versus uninfected groups, and in the animals that survived the infection compared to those that did not. By comparing miRNA expression profiles across these groups, the candidate miRNAs that might be associated with the progression or resolution of ASFV infection were identified. Following the qualitative analysis, the second phase of the study involved quantitative real-time PCR (qPCR) to measure the exact expression levels of the identified miRNAs. qPCR allowed precise quantification of miRNA expression, enabled to assess the extent to which these molecules were upregulated or downregulated in response to ASFV infection. This quantitative data provided a deeper understanding of the miRNA-mediated regulatory networks involved in ASFV pathogenesis and the host's immune response. In this study, 15 miRNAs related to ASF infection were initially selected for qualitative expression analysis. Of these, 8 miRNAs were found to be expressed and were further chosen for quantitative profiling to determine the levels of expression in the died, survived, and control groups of animals. A comparative analysis of the expression values revealed that in spleen tissue, miR-92b-3p, miR-30d, miR-30e-5p, miR-125b, and miR-122-5p were upregulated in the survived group compared to the died group. However, these miRNAs were downregulated when compared to the control group. In lymph node tissue, miR-451 was upregulated in the survived group compared to the control group. Meanwhile, miR-92b-3p and miR-30e-5p were upregulated in the died group compared to the control group. These findings suggested that these miRNAs may play a protective role during ASFV infection. Understanding the miRNA-mediated regulatory networks involved in ASFV pathogenesis may contribute to the development of novel strategies for prevention, diagnosis, and treatment. By understanding the role of miRNAs in ASFV pathogenesis, we aimed to contribute to the development of novel strategies for the prevention, diagnosis, and treatment of ASFV. Additionally, this study may provide broader insights into the role of miRNAs in viral infections, potentially extending its relevance beyond ASFV to other viral diseases affecting livestock and other animals. In conclusion, this research addresses a critical gap in the understanding of ASFV infection by focusing on the miRNA expression profiles in key immune tissues. The findings from this study have the potential to advance our knowledge of ASFV biology and to inform the development of effective counter measures against this devastating disease. The ultimate goal of this research was to identify specific miRNAs that could serve as biomarkers for ASFV infection or as potential therapeutic targets.



यह अध्ययन प्रतिरक्षा से संबंधित शूकरों की दो प्रमुख ऊतकों (प्लीहा, तिल्ली और लिम्फनोड्स) में एएसएफवी संक्रमण के जवाब में miRNAs की विभेदक अभिव्यक्ति का पता लगाने के लिए डिजाइन किया गया। यह ऊतक पोर्सिन प्रतिरक्षा प्रणाली के महत्वपूर्ण घटक हैं, जो रोगजनकों का पता लगाने और इसके विरुद्ध प्रतिक्रिया में मुख्य भूमिका निभाते हैं। इस अध्ययन में उन miRNAs का चयन किया गया जिन्हें जैव सूचना विज्ञान की अनुमान एवं पूर्व प्रकाशित अध्ययनों के आधार पर एएसएफवी संक्रमण में शामिल होने की परिकल्पना की गई थी। इस अध्ययन में तीन अलग-अलग समूहों के शूकरों के नमने शामिल थे। एएसएफबी संक्रमण से मृत शूकर, (मृत), संक्रमण के पश्चात जीवित (जीवित), और असंक्रमित शूकरों का नियंत्रण समूह (नियंत्रक)। हमारे शोध दृष्टिकोण को दो मुख्य चरणों में विभाजित किया गया था। पहले चरण में पारंपरिक पोलीमरेज चेन रिएक्शन (पीसीआर) तकनीकों का उपयोग करके गुणात्मक अभिव्यक्ति अध्ययन शामिल थे। यह चरण उन miRNAs की पहचान करने के लिए महत्वपूर्ण था जो मृत बनाम नियंत्रक समूहों तथा जीवित बनाम नियंत्रक समूहों में अलग-अलग व्यक्त किए गए थे। इन समूहों में miRNA अभिव्यक्ति प्रोफाइल की तुलना करके, उम्मीदवार miRNAs की पहचान कर रहे थे जो एएसएफवी संक्रमण की प्रगति या समाधान के साथ जुड़े हो सकते हैं। गुणात्मक विश्लेषण के बाद, अध्ययन के दूसरे चरण में पहचाने गए miRNAs के सटीक अभिव्यक्ति स्तरों को मापने के लिए मात्रात्मक रियल टाइम पीसीआर (qPCR) शामिल था। qPCR, miRNA अभिव्यक्ति की सटीक मात्रात्मकता की अनुमति देता है जिससे हमें यह आकलन करने में सहायता मिलती है कि एएसएफवी संक्रमण के जवाब में यह अणु कितने स्तर तक अपरेगुलेट या डाउन रेगुलेट किया होते हैं। इस अध्ययन में, एएसएफ संक्रमण से संबंधित 15 miRNAs शुरू में गुणात्मक अभिव्यक्ति विश्लेषण के लिए चुना गया था, इनमें से 8 miRNA गुणात्मक अभिव्यक्ति में व्यक्त करने के लिए पाया गया और आगे मृत, जीवित एवं नियंत्रण समूहों में अभिव्यक्ति के स्तर का निर्धारण करने के लिए मात्रात्मक रूपरेखा के लिए चुना गया। अभिव्यक्ति मूल्यों के एक तुलनात्मक विश्लेषण से पता चला है कि तिल्ली ऊतक में miR-92b-3p, miR-30d, miR-30e-5p, miR-125b और miR-122-5p को मृत समूह की तुलना में जीवित समूह में ज्यादा अभिव्यक्त किया गया था। हालांकि यह miRNAs नियंत्रण समूह की तुलना में कम अभिव्यक्त थे। लिम्फनोड ऊतक में miR-451 को नियंत्रण समूह की तुलना में जीवित समूह में ज्यादा अभिव्यक्त पाया गया था। इस बीच, miR-92b-3p और miR-30e-5p को नियंत्रण समूह की तुलना में मृत समूह में ज्यादा अभिव्यक्त पाया गया था। इस मात्रात्मक डेटा ने एएसएफवी रोगजनन और मेजबान की प्रतिरक्षा प्रतिक्रिया में शामिल miRNA-मध्यस्थता नियामक नेटवर्क की गहरी समझ प्रदान की। इन निष्कर्षों ने सुझाव दिया कि यह miRNAs एएसएफवी संक्रमण के दौरान एक सुरक्षात्मक भूमिका निभा सकते हैं। एएसएफवी रोग जनन में शामिल miRNA मध्यस्थता नियामक नेटवर्क को समझने में रोकथाम, निदान और उपचार के लिए उपन्यास रणनीतियों के विकास में योगदान कर सकता है। एएसएफवी रोगजनन में miRNAs की भूमिका को समझकर, एएसएफवी के रोकथाम, निदान और उपचार के लिए रणनीतियों का विकास किया जा सकता है। इसके अतिरिक्त, यह अध्ययन विषाणु जनित संक्रमण में miRNAs की भूमिका में व्यापक अंतर्दृष्टि प्रदान कर सकता है जिससे इस की प्रासंगिकता संभावित रूप से एएसएफवी के अलावा अन्य विषाणु जनित रोगों, जो पशुधन और अन्य जानवरों को प्रभावित करते हैं, तक भी बढ़ सकती है। अंत में, इस शोध प्रमुख प्रतिरक्षा ऊतकों में miRNA अभिव्यक्ति प्रोफाइल पर ध्यान केन्द्रित करके एएसएफवी संक्रमण की समझ में एक महत्वपूर्ण कमी को संबोधित करता है। इस अध्ययन से प्राप्त निष्कर्षों से हमें एएसएफवी विषाणु के बारे में हमारे ज्ञान को आगे बढ़ाने और इस विनाशकारी बीमारी के खिलाफ प्रभावी रक्षा उपायों के विकास को सूचित करने में मदद मिलेगी। इस शोध से प्राप्त विशिष्ट miRNAs एएसएफवी संक्रमण के लिए बायोमार्कर के रूप में या संभावित चिकित्सीय लक्ष्यों के रूप में काम कर सकते हैं।



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