Application of new techlonogies in molecular detection and research of Africa Swine Fever virus

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INTRODUCTION

The Africa Swine Fever Pandemic

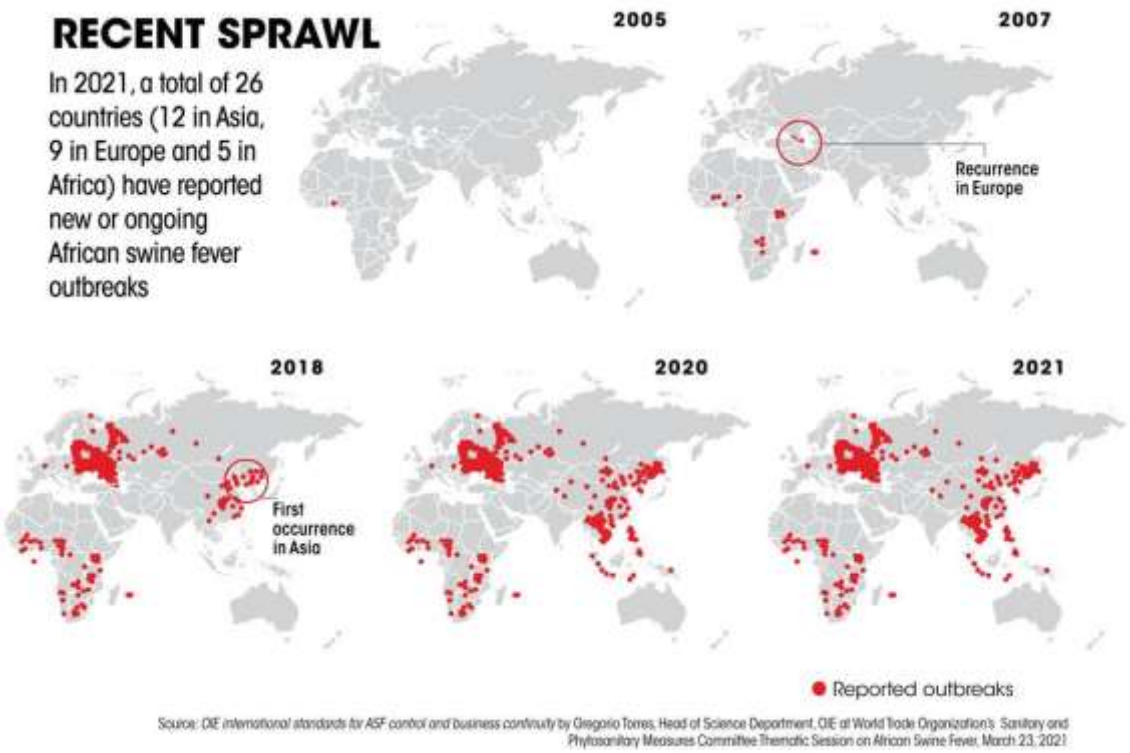
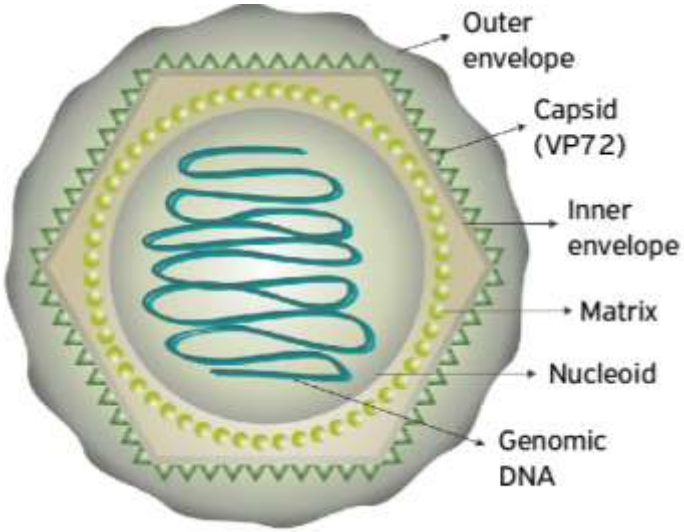
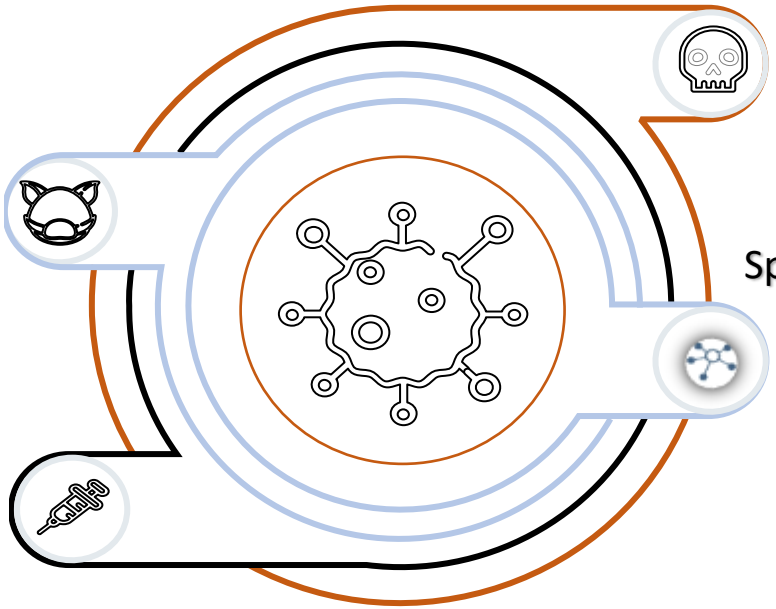


Figure 1. Map of ASF reported worldwide (OIE, 2021)

- Affecting 32 countries (OIE - World Organization for Animal Health)
- Caused huge economic loss

Africa Swine Fever virus - ASFV





High Mortality rate
Strongly persistent

Wild boards & domestic swines

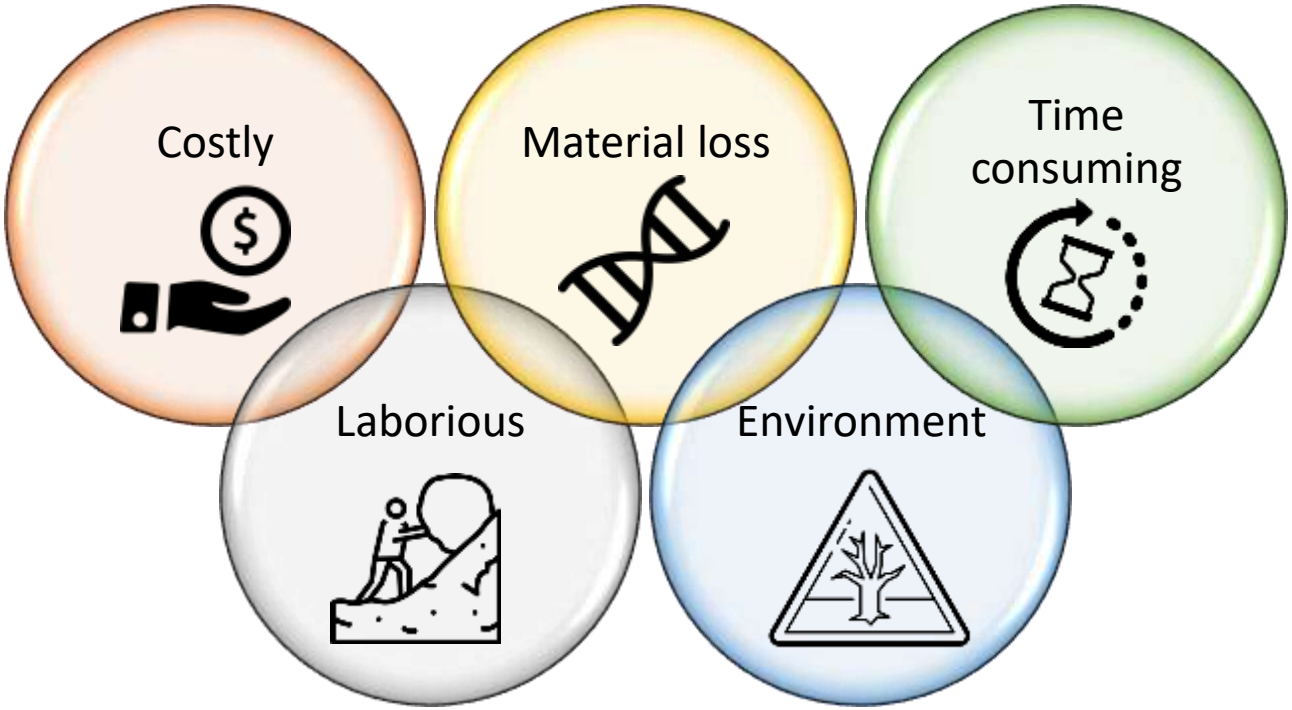
Spread through feed, waste, garbage, parasites
Highly contagious

No ultimate effecive vaccine or treatment

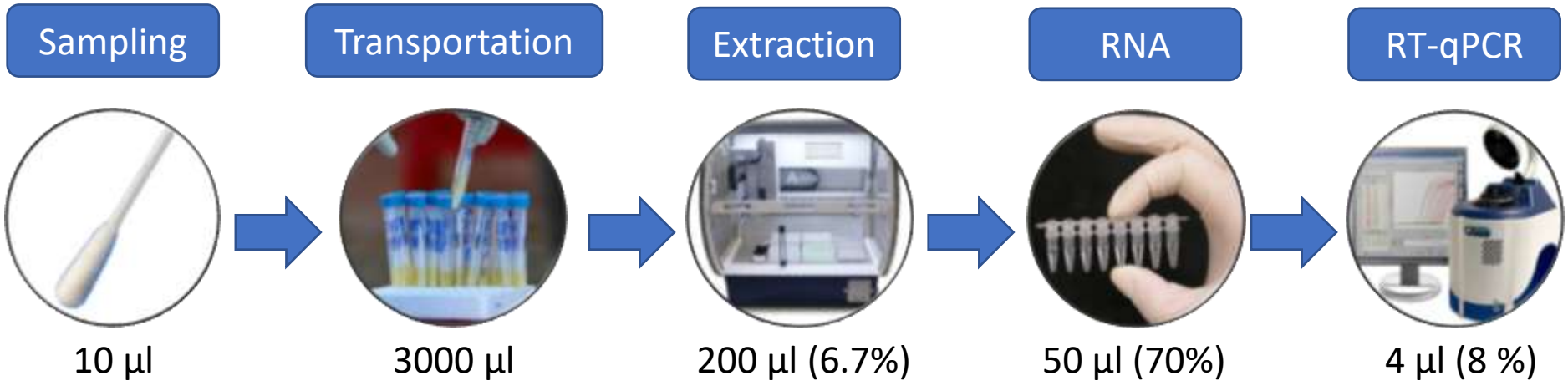
Fast Molecular Detection of ASF virus from whole blood using direct PCR technique

PCR based detection as Gold standard method for ASFV diagnostics (OIE)

Nucleic acid extraction drawbacks



Material loss during nucleic acid extraction



Loss: 100 % - (6.7 % x 70 % x 8 %) = 100 % - 3.7 %

Laborious – Laziness

Direct PCR approach

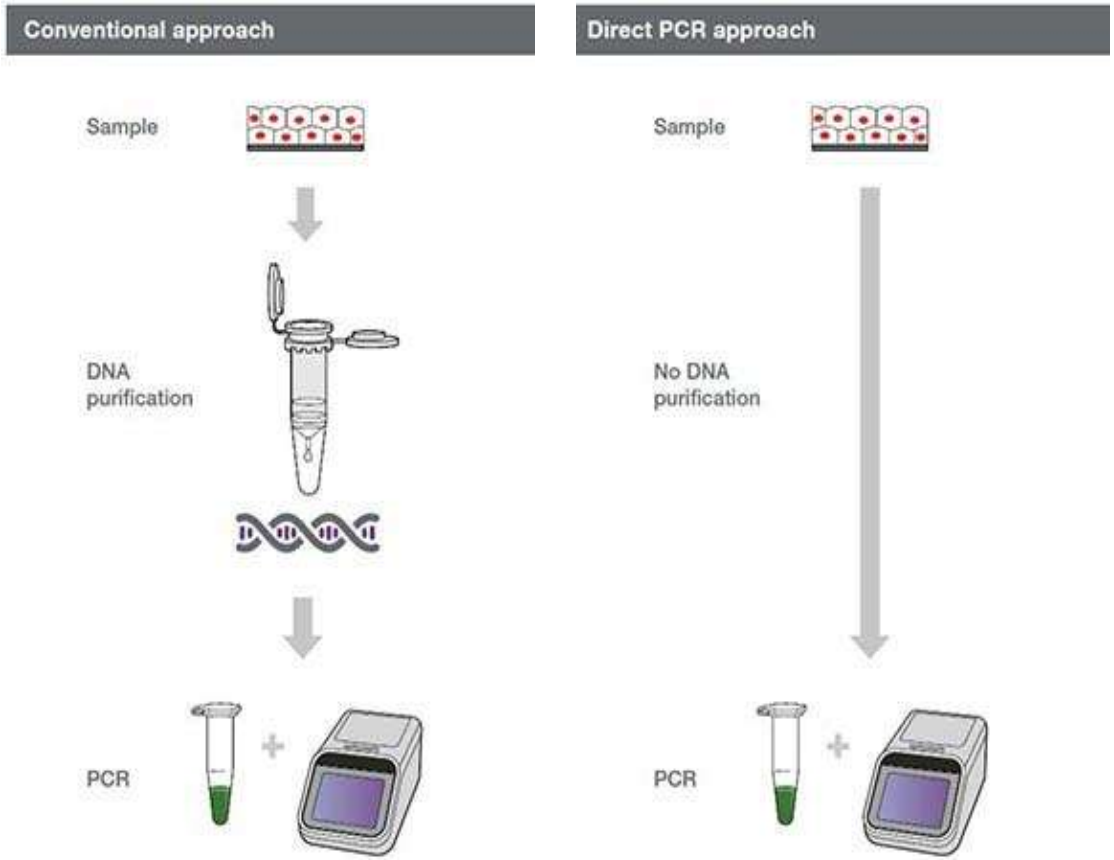


Fig 3. Comparison of conventional approach to PCR vs. direct PCR approach (Thermo Fisher Scientific).

FDA Approved nucleic acid based tests: direct tests

On this page:

- List of Human Genetic Tests
- List of Microbial Tests

Mycobacterium tuberculosis	Xpert MTB/RIF Assay	Cepheid	K131706
	BDProbetec ET Mycobacterium tuberculosis complex culture identification kit	Becton, Dickinson, & Co.	K000884
	Amplified Mycobacterium tuberculosis Direct Test	Gen-Probe, Inc.	P940034
Mycoplasma pneumoniae	illumigene Mycoplasma Direct DNA Amplification Assay	Meridian Bioscience, Inc.	K160829
	illumigene Mycoplasma DNA Amplification Assay	Meridian Bioscience, Inc.	K152800, K123423

Why Blood?

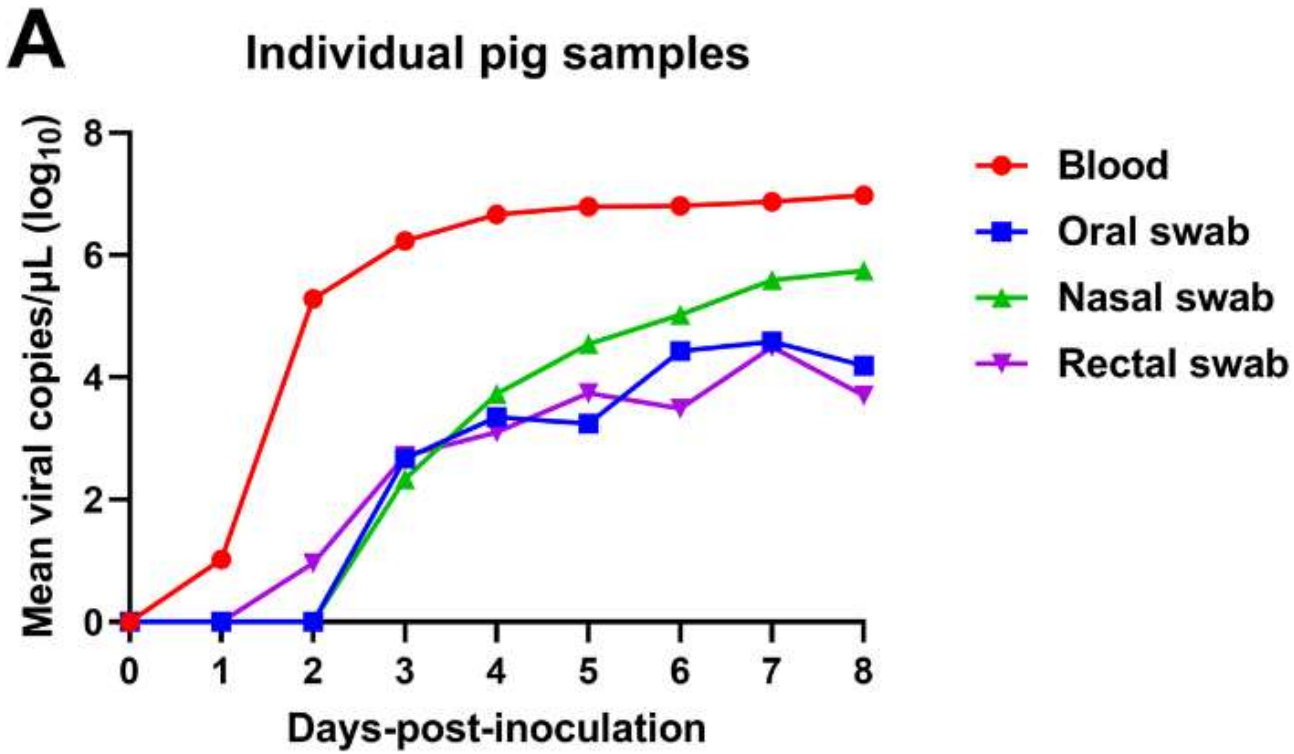


Fig 4. Mean viral copy of ASFV detected in different samples from inoculated pig (Lee et al., 2021)

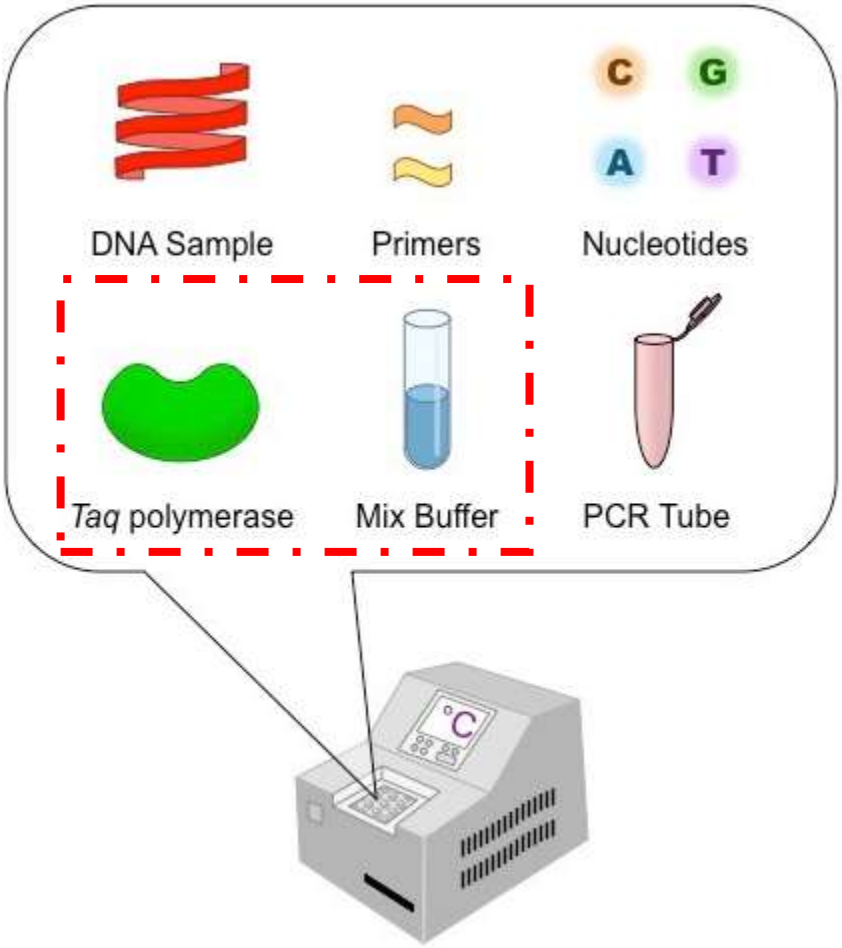
PCR inhibitors are main obstackles

Matrix	Contained inhibitors	References
Clinical specimens (e.g. blood; muscle tissues)	Antiviral substances (e.g. acyclovir), Haemoglobin, Heparin, Hormones, IgG, Lactoferrin, Myoglobin	Al-Soud and Rådström (2001); Burkardt (2000); Rådström *et al.* (2004); Yedidag *et al.* (1996)
Stool	Complex polysaccharides, Bile salts, Lipids, Urate	Kreader (1996); Monteiro *et al.* (1997); Rådström *et al.* (2004); Chaturvedi *et al.* (2008)
Seafood, bivalves, oysters	Algae, Glycogen, Polysaccharides	Atmar *et al.* (1993, 1995); Richards (1999)
Berries	Phenols, Polysaccharides	Seeram *et al.* (2006); Wei *et al.* (2008)
Plants	Pectin, Polyphenols, Polysaccharides, Xylan	Demeke and Adams (1992); Henson and French (1993); John (1992); Sipahioglu *et al.* (2006); Su and Gibor (1988); Wan and Wilkins (1994); Wei *et al.* (2008); Wilkins and Smart 1996
Cheese, milk	Proteases (e.g. plasmin), Calcium ions,	Bickley *et al.* (1996); Powell *et al.* (1994); Rossen *et al.* (1992)
Water, environment	Debris, Fulmic acids, Humic acids, Humic material Metal ions, Polyphenol	Abbaszadegan *et al.* (1993); Ijzerman *et al.* (1997)
Palaeobiology, archaeology, forensic	Bone dust, Coprolite Peat extract, Clay-rich soil	Baar *et al.* (2011)

Adapted from Schrader et al, 2012

But How? Hack it and Beat it

- Change the platform (Crispr-Cas, LAMP, RPA, LFA, ...)
- Device investigation
- Sample quick treatment
- **Hack PCR components**



Materials and Methods

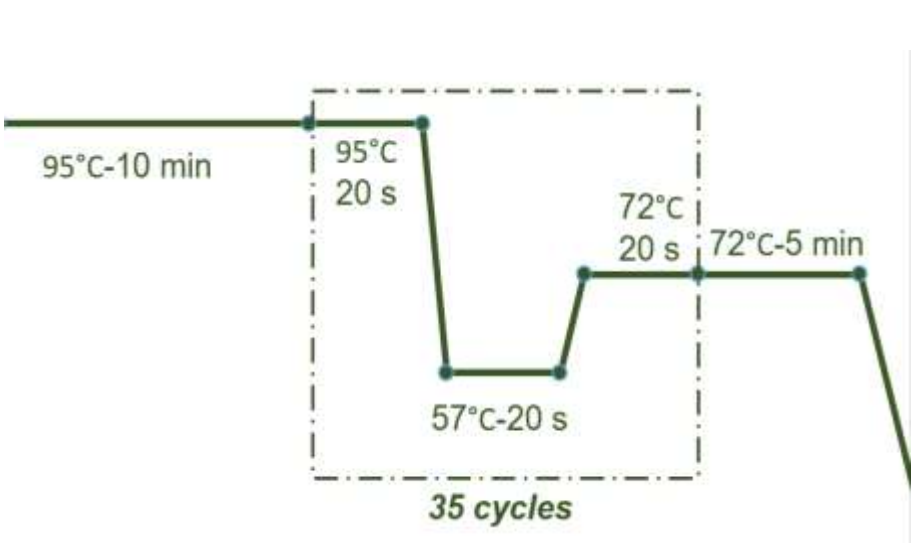
- Samples:
  - Infected DNA
  - Whole blood from uninfected pig
  - Inactivated infected blood
  - p72 recombinant plasmid DNA
- Primer:
  - ASFV P72 (*Ballester et al., 2004*),
  - Pig Estrogen Receptor(*Vega, R. S. et al, 2018*)

Table 1: The PPA-1/2 and GCG primer sets .

Primer	Sequence	Target gene	Product size
PPA-1 forward	5'-AGTTATGGGAAACCCGACCC-3'	p72 (ASFV)	257
PPA-2 reverse	5'-CCCTGAATCGGAGCATCCT-3'		
ESR forward	5′ CCTGTTTTTACAGTGACTTTTACAGAG 3′	Estrogen (control)	120
ESR reverse	5′ CACTTCGAGGGTCAGTCCAATTAG 3′		

RESULTS

Initial optimization



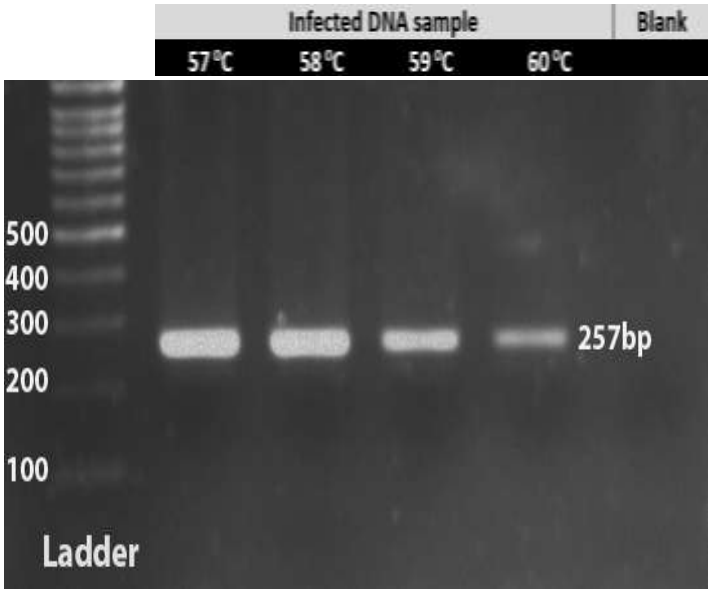


Fig. 5: PCR performance on ASFV amplification with annealing temperature vary from 57oC to 60oC.

Direct amplification of ASFV using whole blood

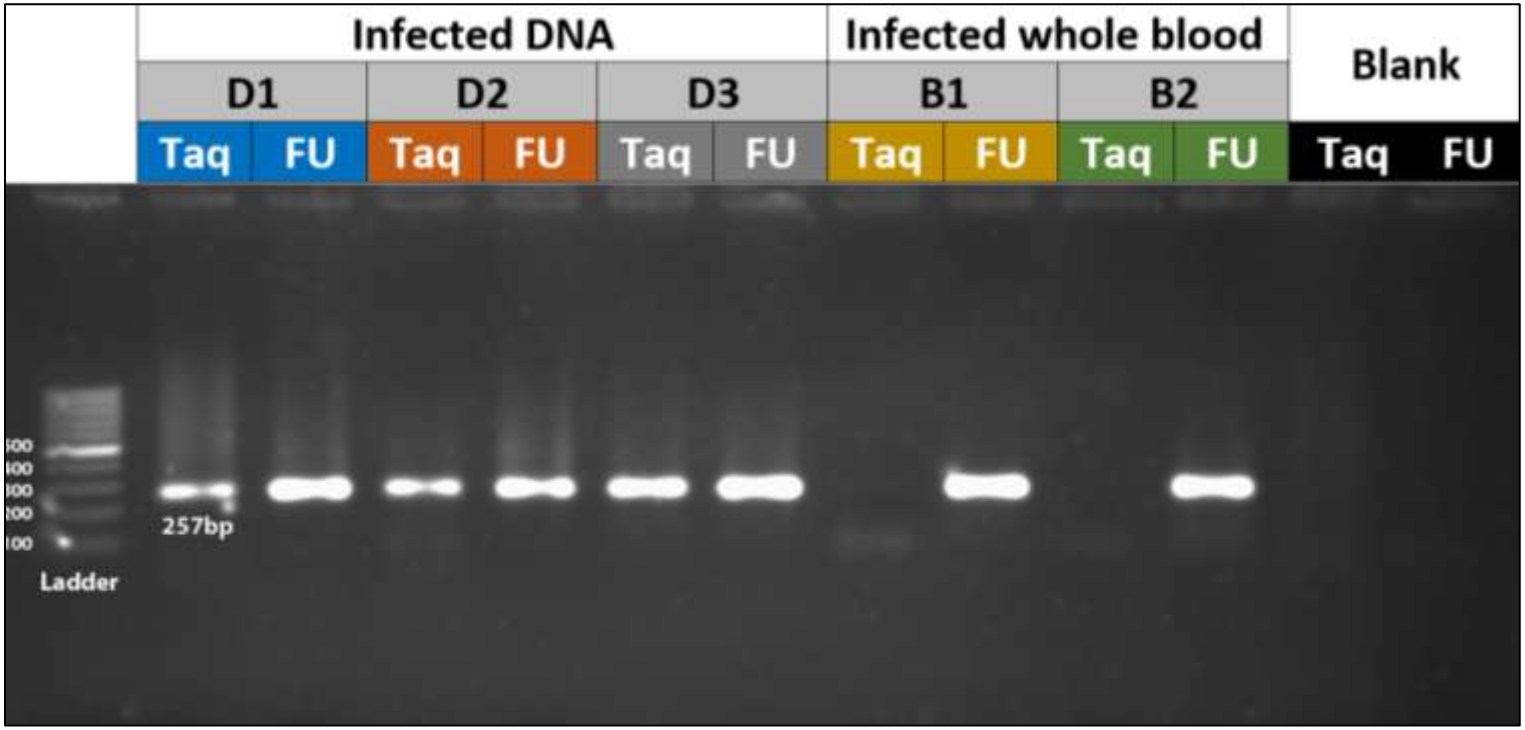
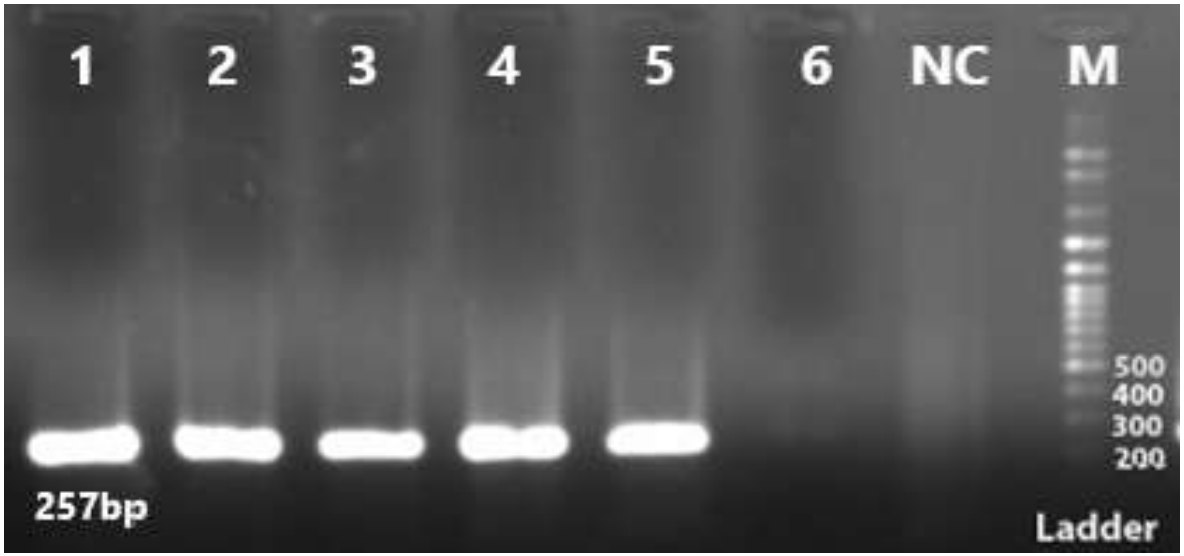


Fig. 6: Comparison of PCR performance of 2 enzymes, Taq and FU in the presence of 5 % whole blood components and anticoagulants EDTA

Amplification of ASFV with increasing whole blood content



Lane 1: 8 µL infected blood
Lane 2: 9 µL infected blood
Lane 3: 10 µL infected blood
Lane 4: 11 µL infected blood
Lane 5: 12 µL infected blood
Lane 6: 8 µL uninfected blood
Lane NC: nuclease-free water

Figure 7: Amplification of ASFV using with increased whole blood content

Direct multiplex PCR for ASFV and an internal control

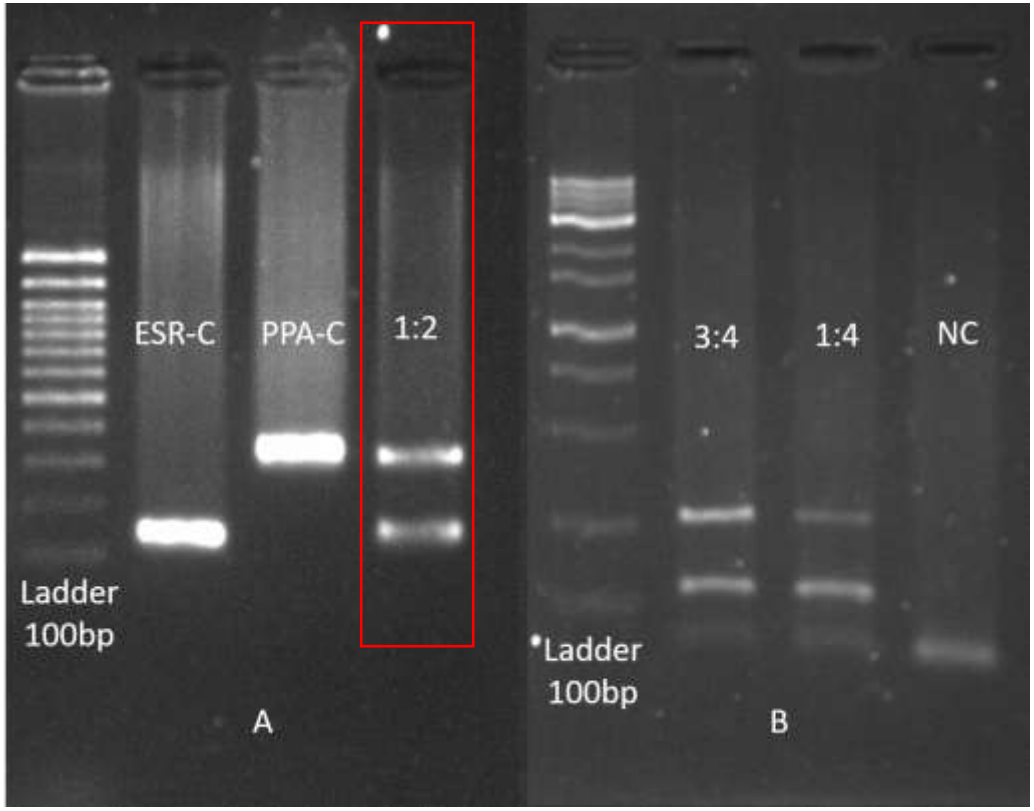


Fig 8. Whole blood direct-multiplex PCR with internal trontrol ESR-R/F and PPA-1/2

Initial acessment of Limit of detection

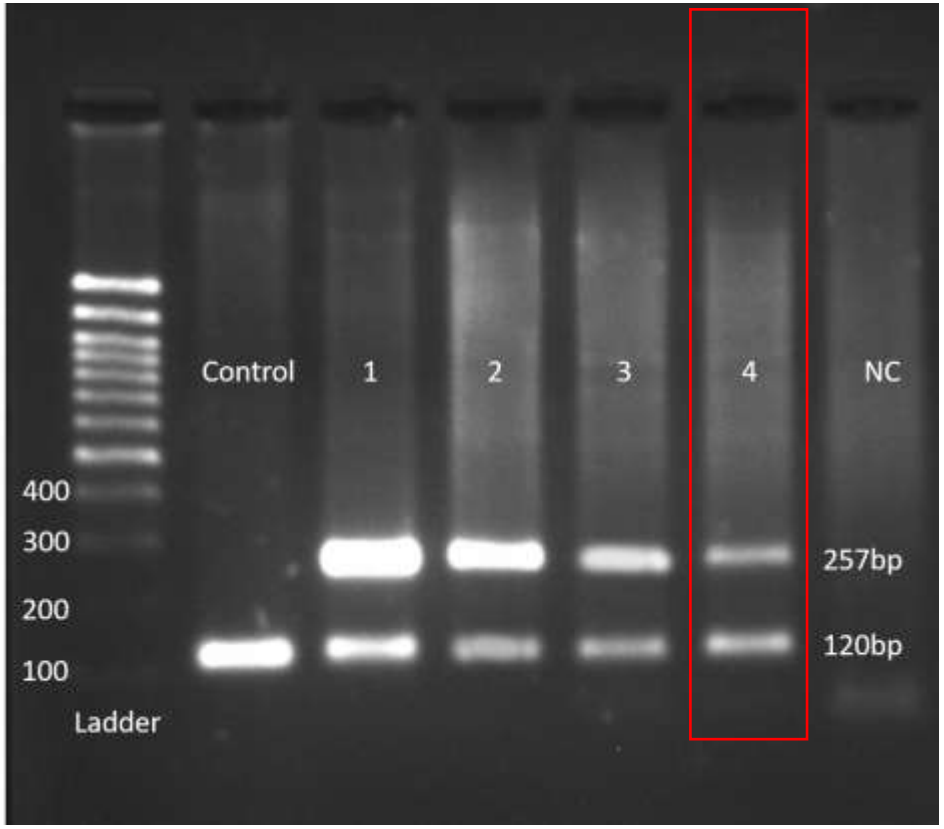


Fig 9. Whole blood direct-multiplex PCR with vary P72 plasmid copies

Lane Control
Lane 1: 1 billion copies/µL
Lane 2: 10 milion copies/µL
Lane 3: 100,000 copies/µL
Lane 4: 1000 copies/µL
Lane NC: Negative control

CONCLUSIONS and FUTURE WORKS

- *Direct multiplex PCR using whole blood may become potential alternative for ASFV detection compare with traditional procedure*
- *Need investigation for LOD, sensitivity and specificity*
- *Validation on other sample types*
- *Direct multiplex realtime PCR?*
- *Mastermix for LOD of 10^1 copies per ml*
- *Light weight PCR device for point-of-care application*

Whole genome sequencing of ASF virus using Long Amplification and Nanopore sequencing

Native Dificulties in Combat with the ASFV

- *Environmental stability*
  - *Transmission without the live host*
- *Variation in vaccine efficiency*
  - *Vaccine can not cover all genotypes*
- *Complex genome*
  - *170 – 193 kb of duplex DNA with ~ 200 proteins*
- *High genetic variability*
  - *24 genotypes by B646L – p72 sequencing*

Meaning of research on ASFV on Genome level

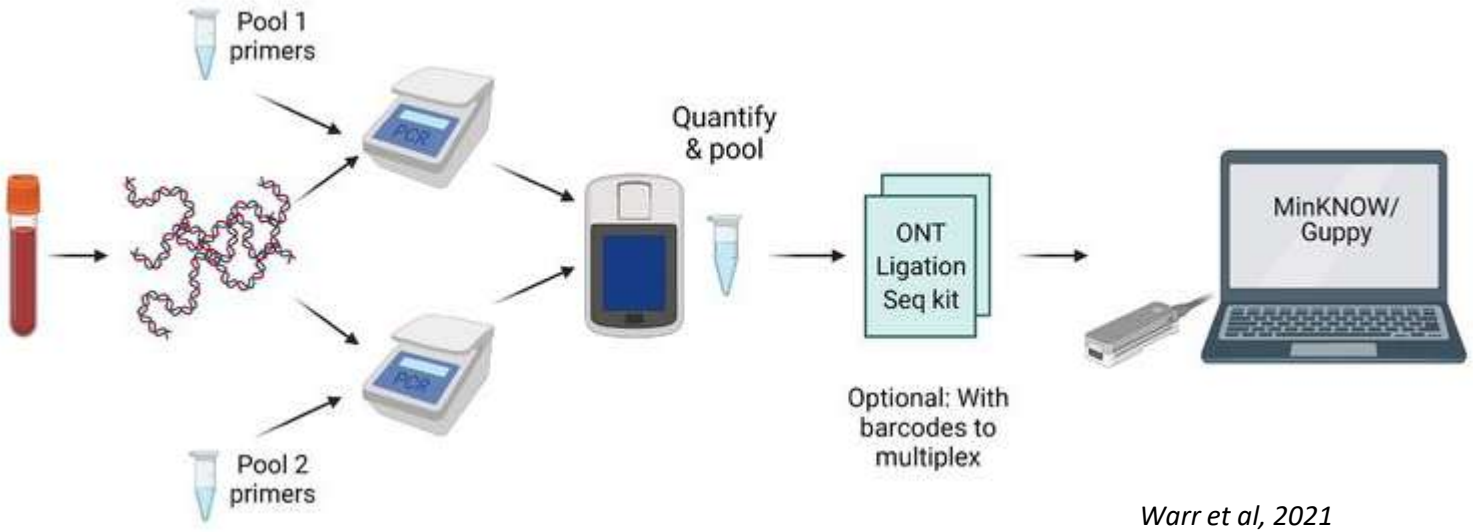
- *Monitoring viral diversity and evolution*
- *Tracking the epidemic and infectious origin of the outbreaks*
- *Understanding of host immune evasion*
- *Development of molecular detection*
- *Development of effective vaccine*

Challenges in ASFV whole genome sequencing

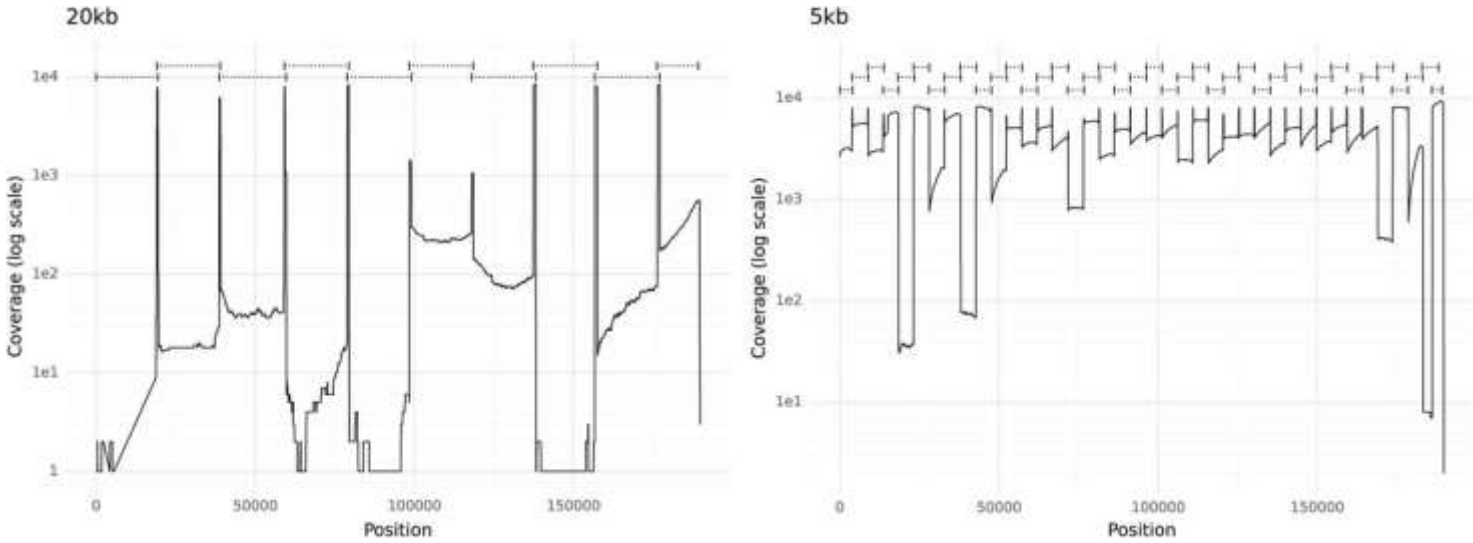
- *Large and complex genome*
  - *~ 190 kb*
  - *Repeat sequence*
  - *Low CG content regions*
- *Containment of high fraction of host DNA (low viral DNA)*

Sample	Group	PL	No Reads	Mapped Reads
SAMN22028792	ASFV	ILLUMINA	4,446,389	19,337 (0.43%)
SAMN22028794	ASFV	ILLUMINA	4,801,869	10,571 (0.22%)
SAMN29762774	ASFV	ILLUMINA	3,917,863	6,534 (0.17%)
SAMN22028798	ASFV	ONT	535,304	1,435 (0.27%)
SAMN22028809	ASFV	ONT	100,729	343 (0.34%)
SAMN22028812	ASFV	ONT	202,385	240 (0.12%)

Strategy: Viral DNA Enrichment by Long range PCR and Longread sequencing (ONT)

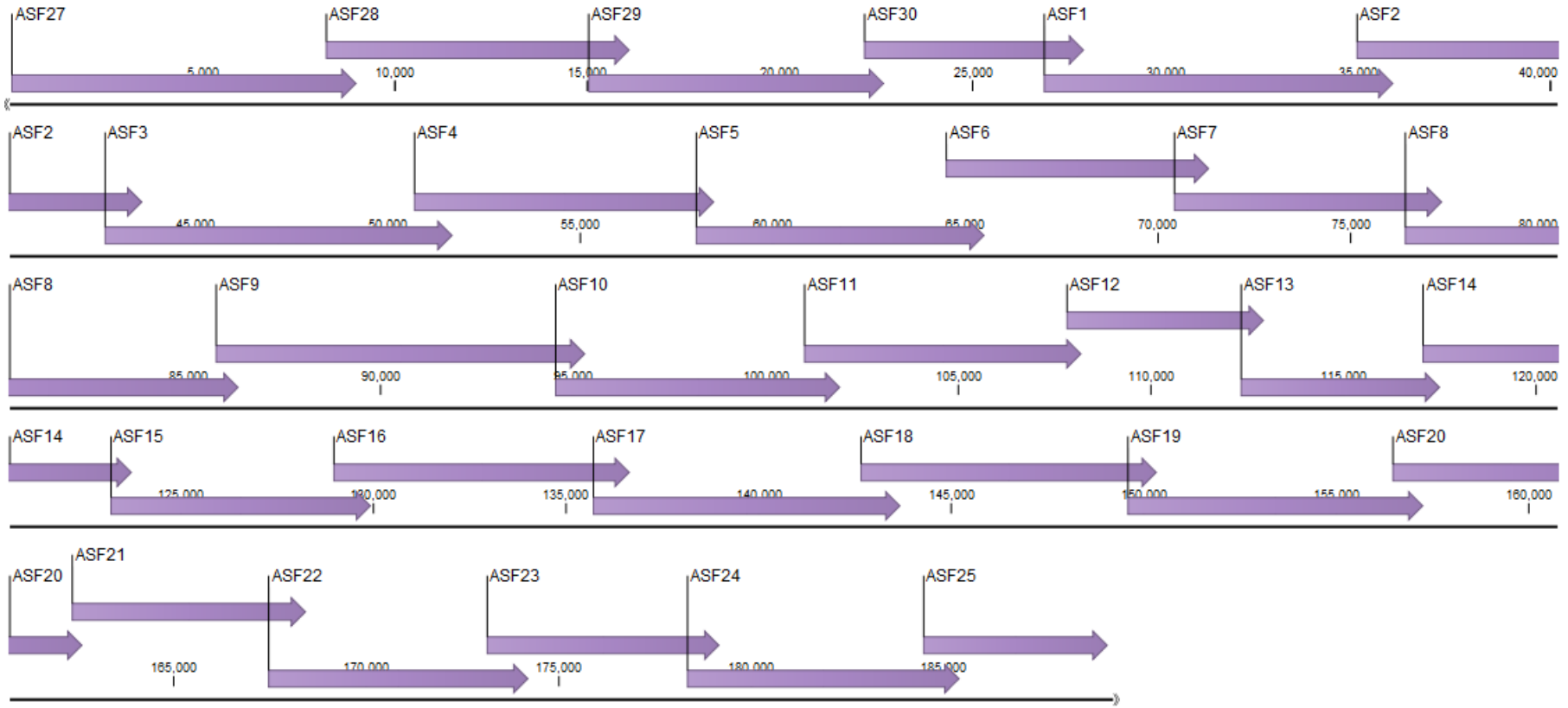


Warr et al, 2021

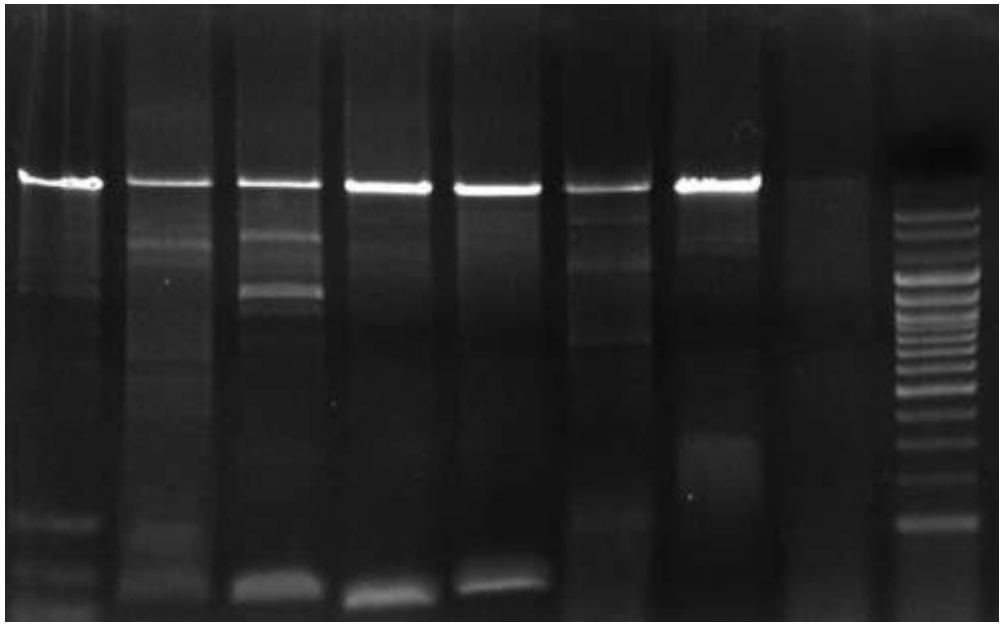


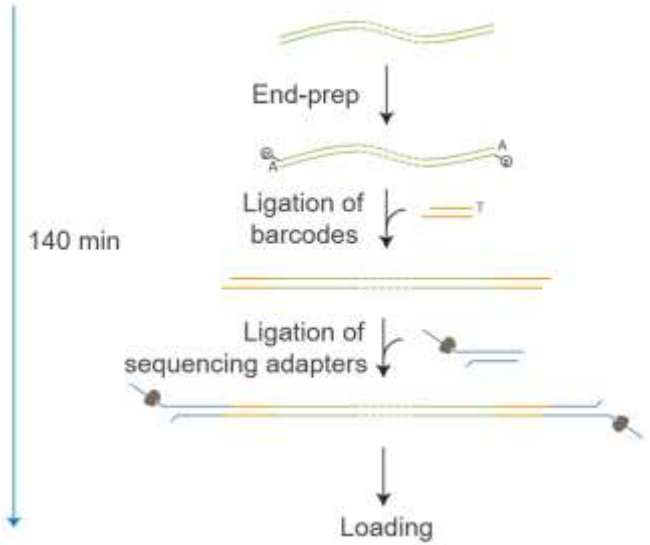
Li et al, 2024

Design new primer set for amplifying ASFV whole genome

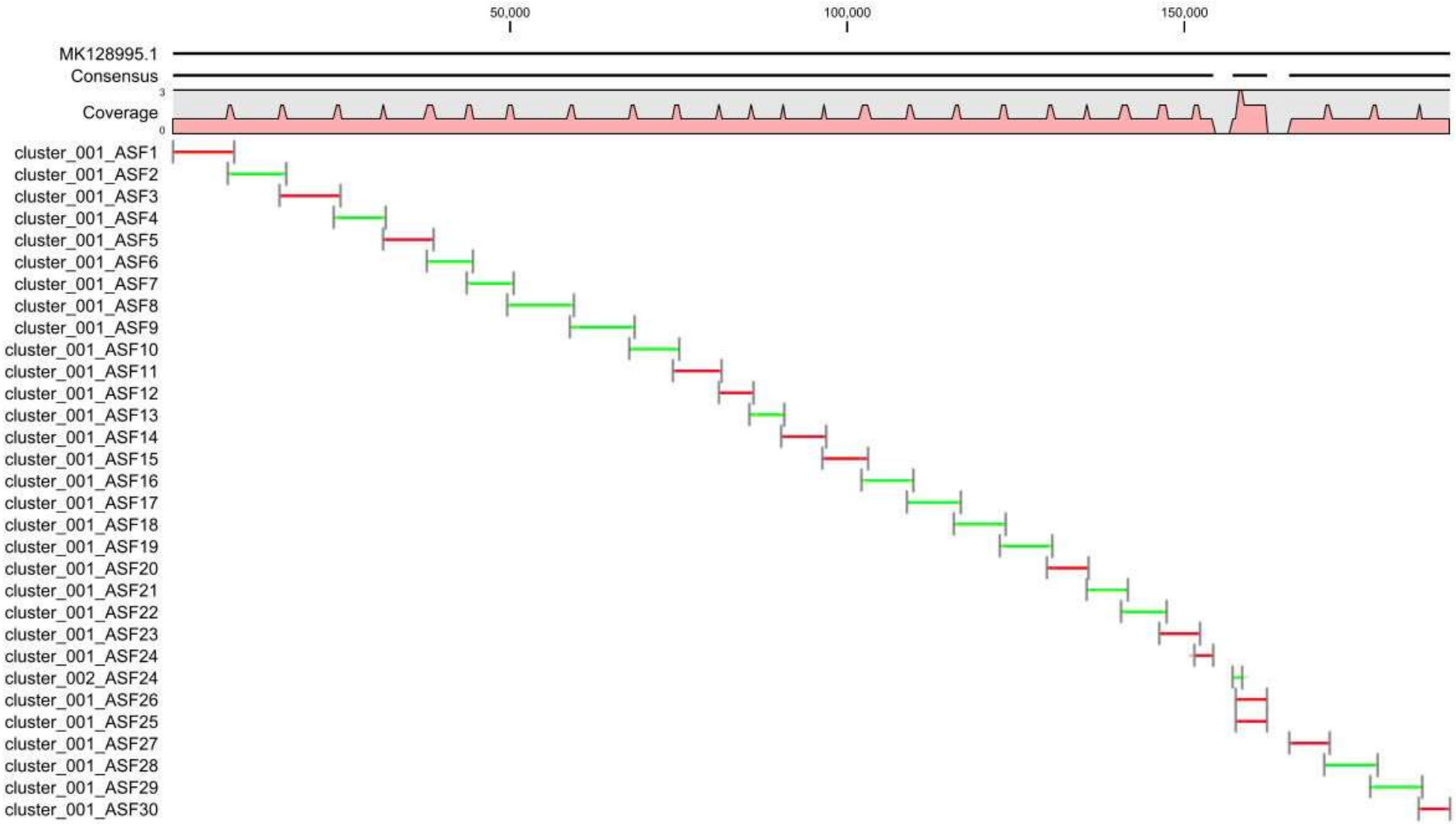


Suscess amplification of ASFV whole genome and Nanopore sequencing

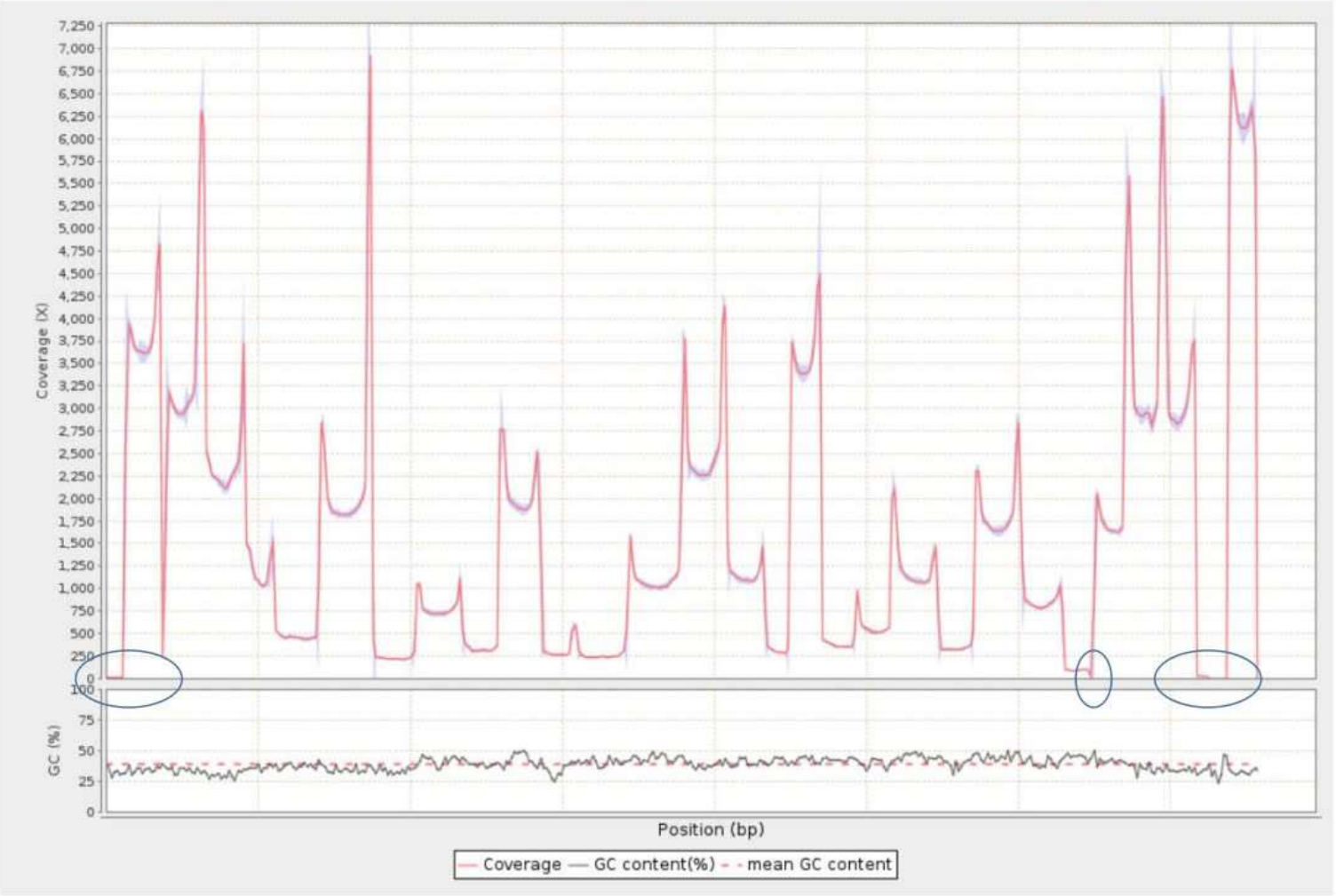




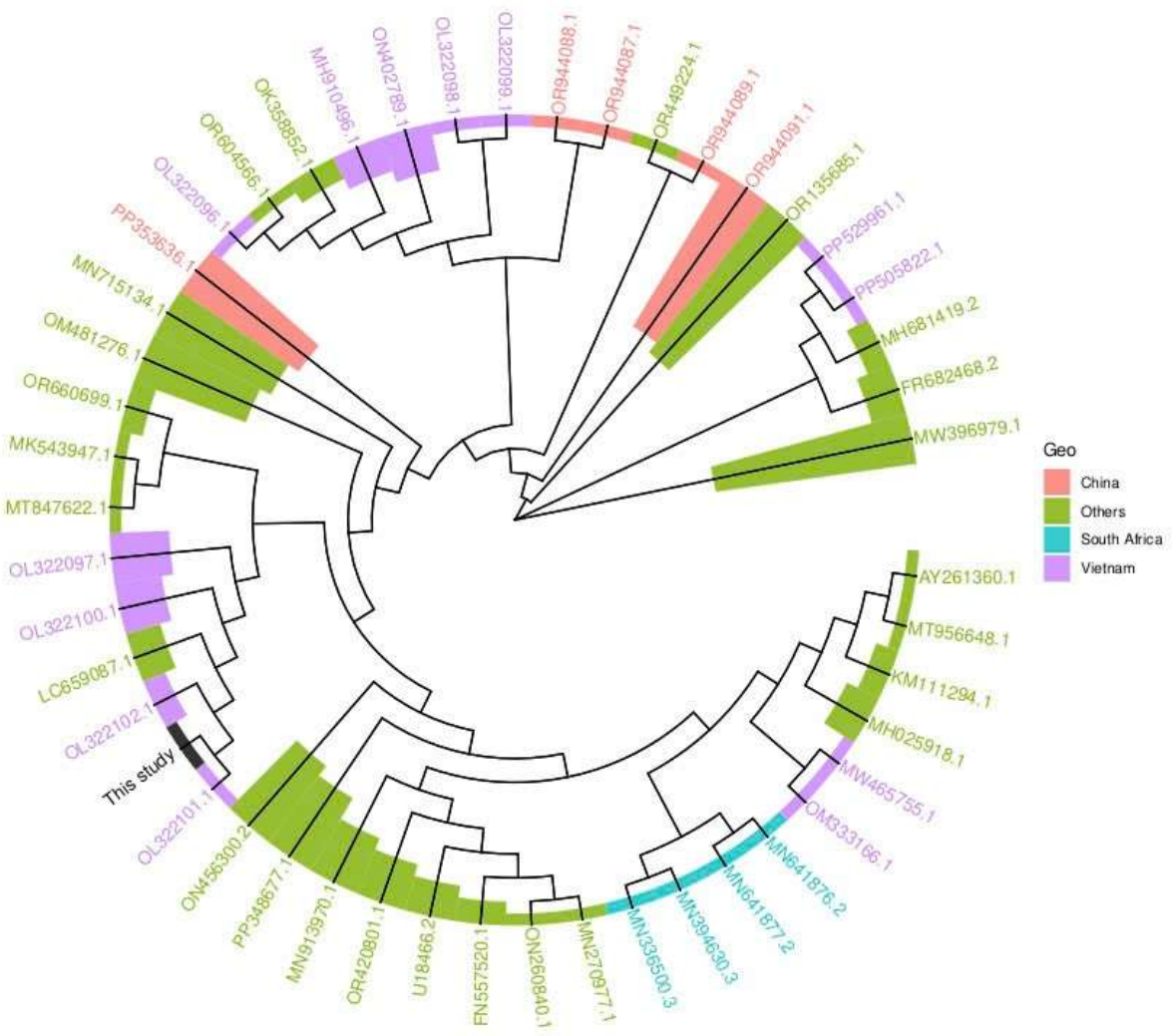
Assembly of ASFV whole genome



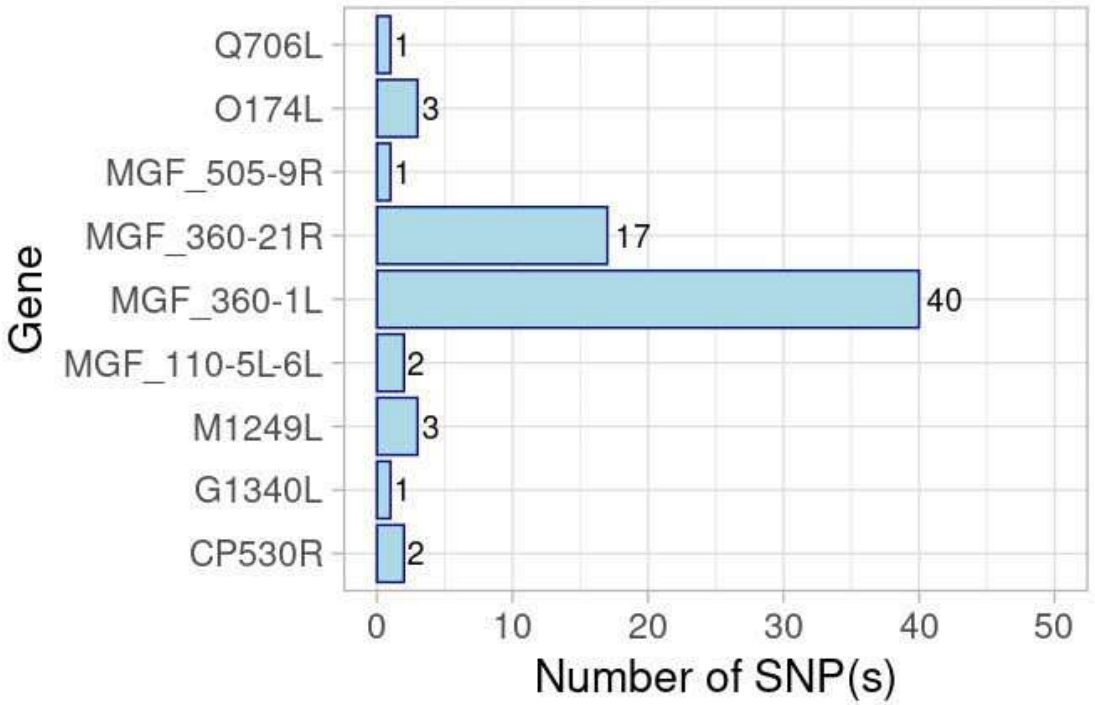
Assembly of ASFV whole genome: Coverage



Phylogenetic analysis



Analysis of mutations on individual genes

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CONCLUSIONS and FUTURE WORKS

- *Enrichment of ASFV DNA by long PCR and following by Nanopore long read sequencing facilitate the study of ASFV on genomic level*
  - *Eliminate host DNA in final result*
  - *Effective on low viral titer samples*
  - *High accuracy*
  - *Comprehensive and almost complete genome (except telomeric repeat regions)*
  - *No need to polish by short read sequencing*
- *Validate the mutations by Sanger sequencing*
- *Build the deeper analysis pipeline for functional study*

Thank you ^^