Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Federal Department of Home Affairs FDHA
Federal Food Safety and Veterinary Office FSVO
Research Management

Animal health **Zoonotic diseases**

Rapid identification and epidemiological analysis of OIE-notifiable viral animal diseases

Matthias Licheri[1,2], Manon Wider[1], Jenna N. Kelly[3], Ronald Dijkman[1]
[1] Institute for Infectious Diseases, University of Bern, Bern, Switzerland, [2] Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland, [3] Institute of Virology and Immunology, Bern and Mittelhäusern, Switzerland

Key words
Whole genome sequencing, African swine fever virus, avian influenza A virus, SARS-CoV-2.

Aim of the study
The purpose of this project is to establish a novel analysis pipeline that is based on the state-of-the-art Oxford Nanopore sequencing technology in conjunction with a powerful bioinformatic-based data analytic and user-friendly visualization tool. The latter will aid in the rapid identification and epidemiological understanding of OIE-notifiable viral pathogens, such as avian influenza virus (AIV), and African Swine Fever (ASF), to improve the outbreak response time.

Material and methods
For the evaluation of nanopore sequencing protocols for influenza A virus (IAV) we evaluated different primers sets that are based on the 3' and 5' conserved terminal regions of the viral genomic segments that can be used to amplify the complete genome or only the hemagglutinin (HA), neuraminidase (NA), and matrix (M) genomic segments using viral RNA as template [1,2]. To estimate assay sensitivity, viral RNA was extracted from six 10-fold serial dilutions of cell culture supernatant containing the pandemic H1N1 influenza A virus (A/Hamburg/4/2009 H1N1) WT or $NS1_{R38A}$-mutant virus. The latter has two nucleotide mutations in the Non-Structural 1 (NS1) gene. The viral load in the six 10-fold serial dilutions was quantified with a universal diagnostic qRT-PCR assay targeting the Membrane (M) gene of influenza A viruses. Following nucleic acid extraction, viral genomic segments were amplified, and sequence as described previously by our group [3]. Downstream Data analysis was performed with a local instance of the web-based bioinformatics suite INSaFLU ("INSide the FLU") (https://insaflu.insa.pt/) [4].
For the establishment of a nanopore whole genome sequencing protocol for African Swine Fever (ASF) virus different viral DNA enrichment approaches were evaluated using nucleic acids extracted from sucrose-purified ASFV samples, as well as whole serum or EDTA-blood samples from SPF-pig infected with AFSV (isolate Arm08). The first approach was focused on depleting host chromosomal DNA using the NEBNext Microbiome DNA enrichment kit (NEB). The second approach was to amplify the whole ASF genome (170 – 194 kbp) using isothermal multiple displacement amplification (MDA) using a recombinant Phi29 DNA polymerase (Equiphi29™, Thermofisher) in combination with either random primers or with a custom set of 7 primers that were designed to selectively bind multiple times to the ASF genome. The last enrichment approach was based on evaluating the efficienty of isothermal MDA directly on clinical samples using either random primers, or the ASF specific primer set (7 primers). Following the different enrichment approaches, samples were prepared for sequencing using the Oxford Nanopore Technology (ONT) rapid barcoding sequencing kit (SQK-RBK004). The barcoded samples were pooled together and loaded on a MinION flowcell or Flongle flowcell (ONT, R9.4.1) mounted on a MinION MK1b device. Sequencing and data acquisition and real-time high-accuracy (HAC) basecalling and sample demultiplex was performed using MinION software (v21.10.4). Downstream Data analysis with the ASFV reference genome (Arm08) was performed in Geneious Prime (v2022, Biomatters).
Because human-animal spillover events of SARS-CoV-2 have been reported, although not included in the OIE list of Diseases, notifications of infections in animals still must be submitted to the OIE's World Animal Health Information System. Therefore, during the start of the SARS-CoV-2 pandemic it was decided to implement the ARTIC Nanopore sequencing pipeline for SARS-CoV-2 [5].

Results and significance

To allow rapid identification of the genetic composition avian influenza viruses, we first evaluated the sensitivity of two different primer pairs, which either lead to the amplification of the complete genome or only several segments required for IAV subtyping, respectively. This indicated that the specific amplification the HA, NA, and M genes is approximately 100-fold more sensitive compared to the whole genome sequencing approach (lower detection limit Ct 30 versus 25). These results in combination with a user-friendly web-based server allows rapid genetic subtyping of avian influenza A viruses in clinical samples with relatively low viral RNA loads, such as those obtained from waterfowl or poultry during an avian influenza virus outbreak.

A major hurdle of sequencing the extremely large genome of ASF virus from clinical samples is the presence of contaminating host chromosomal DNA that negatively impacts the downstream processing of samples for sequencing. To this end we evaluated different enrichment methods using sucrose-purified ASF virussamples, as well as whole serum or EDTA-blood samples from ASF virus-infected SPF-pigs, to either remove and/or minimise the influence of host chromosomal DNA on the downstream sequencing procedure. This revealed that only host DNA depletion leads yields a genome coverage of 0.2 – 1.1%, whereas a subsequent isothermal multiple displacement amplification increased the overall ASF genome coverage to almost 80.0%, independent from using random or selective primers. Of note, the best results were obtained when nucleic acids are extracted from EDTA-blood samples, as regardless of the approach the genome coverage was always below 10% when serum samples of ASF-infected pigs were used. Interestingly, direct isothermal MDA with selective primers also yielded into an overall genome coverage of almost 80.0%, suggesting that the laborious and expensive host DNA depletion procedure is not required for genotyping ASF virus. Demonstrate that our selective whole genome amplification method, using a set of only 7 primers, allows for rapid enrichment of viral ASF viral DNA from clinical material in a relative short time frame that can be used to further optimized the whole genome sequencing of ASF virus. This paves the way for the development of a novel method to rapidly analyse and characterize ASF virus from infected pigs and wild boars.

Early during the SARS-CoV-2 pandemic we successfully implemented ARTIC Nanopore sequencing pipeline for SARS-CoV-2 and could demonstrating that no obvious signs of nucleotide transitions (adaptation) is required for SARS-CoV-2 to productively infect monkey and cat epithelial airway cell cultures [6]. This method has now been implemented for the SARS-CoV-2 research projects conducted at the IVI, which can also be implemented as a viral diagnostics tool to determine which viral variants are present in SARS-CoV-2 positive animals.

Thus, the current project led to the development of various Nanopore sequencing protocols that can aid in the rapid identification and epidemiological understanding of OIE-notifiable viral pathogens, such as avian influenza virus (AIV), and African Swine Fever (ASF).

Publications, posters and presentations

Gultom, M.; Licheri, M.; Laloli, L.; Wider, M.; Strässle, M.; Steiner, S.; Kratzel, A.; Thao, T.; Stalder, H.; Portmann, J.; Holwerda, H.; V'kovski, P.; Ebert, N.; Stokar-Regenscheit, N.; Gurtner, C.; Zanolari, P.; Posthaus, H.; Schuller, S.; Vicente-Santos, A.; Moreira-Soto, A.; Corrales-Aguilar, E.; Ruggli, N.; Tekes, G.; von Messling, V.; Sawatsky, B.; Thiel, V.; and Dijkman, R.; et al 2021, Susceptibility of well-differentiated airway epithelial cell cultures from domestic and wildlife animals to SARS-CoV-2. Emerg Infect Dis. 2021;27(7):1811-1820.

Licheri, M.; Mehinagic, K.; Wider, M.; Ruggli, N.; Dijkman, R.; et al 2021 Establishment of a selective whole genome amplification protocol for large complex DNA viruses. Meeting of Master- and Doctoral Students in Virology, Münchenwiler 28th - 29th October 2021

***Project** 1.20.02*

***Project duration** April 2020 – December 2021*