Final Report

Project title (Acronym)
The application of Next-Generation Sequencing technology for the detection and diagnosis of non-culturable organisms: viruses and viroids (NGSdetect)

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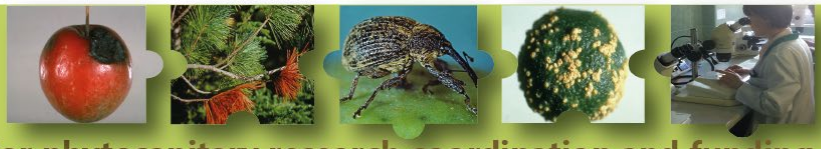
Contents



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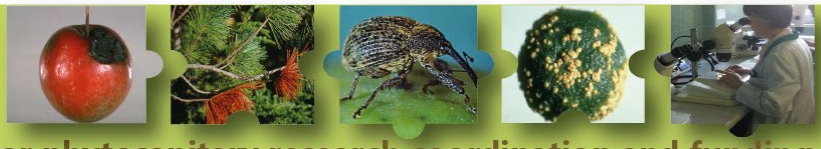

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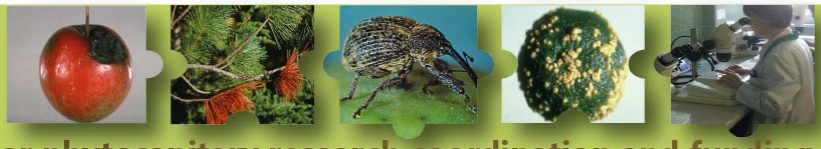

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2. Short project report

2.1. Short executive summary

High-throughput sequencing (HTS, formerly also known as next-generation sequencing or pyrosequecing) technologies have seen a tremendous evolution in terms of technical developments. These technologies are more and more used for plant virus discovery, metagenomic and ecological studies but are also increasingly being used in plant virus diagnostic settings, post-entry quarantine investigations or pre-export diagnostics. Conventional virus detection methods based on serology (i.e., enzyme-linked immunosorbent assays) or nucleic acids (polymerase chain reaction (PCR), real-time PCR), requires in depth knowledge for the target pathogen to develop antisera or primers limiting these methods to the detection of mostly known viruses.

The use of HTS for virus detection is not dependent on *a priori* knowledge about the viruses to be detected which is a major advance in diagnostic testing.

This project aimed to harmonise sample enrichment strategies and HTS workflows for plant virus and viroid detection within a diagnostic framework. Existing bioinformatics approaches were investigated during a proficiency test and a test performance study which demonstrated the power of HTS for diagnostics but also the limitations if diagnosticians are not experienced with the analyses of HTS data. HTS and newly developed sequencing technologies such as Oxford Nanopore Sequencing have a great potential for diagnostics but further work is required to set up guidelines for validation of HTS workflows.

2.2. Project aims

Over all, this project aimed to optimise the application of HTS in diagnostics, including sample preparation and enrichment of viral sequences, library preparation, comparison of different sequencing platforms, bioinformatic analysis and communication of results including provision of reference data for other Euphresco projects (VirusCollect II).

2.2.1. Development of sample preparation strategies

In order to reduce the sequencing of host matrix sequences, it is desired to enrich samples for viral sequences. The commonly used extraction methods for viral enrichment include double-stranded RNA (dsRNA) or small RNA (RNA) molecules that are enriched in virus -or viroid-sequences in infected plants. Total RNA or total RNA depleted of plant rRNAs can also be used, but does contain a higher percentage of host genomic sequences. Circular single-stranded DNA viruses (such as nanoviruses or begomoviruses) can also be enriched by rolling-circle amplification (RCA). This project aimed to share experiences with these extraction methods as well as optimisation of extraction methods from “difficult” hosts such as grapevine, fruit trees or pollen.

2.2.2. Construction of HTS libraries

Nucleic acids extracts cannot be sequenced directly; adapters in form of a so called “library” need to be ligated to the nucleic acid molecules. These adapters depend on the HTS platform used. Commercial kits are available but costly; also outsourcing this step to commercial sequencing suppliers can be quite costly. The aim of the project was to develop alternative library preparation protocols that are reliable, cost-effective and suitable for multiplexing of different samples in a sequencing run.

2.2.3. Comparison of sequencing platforms

As HTS technologies evolved rapidly over the last few years, the aim was to evaluate different sequencing platforms in terms of their error rate and of the number and length of reads.

2.2.4. Development of bioinformatic pipelines for data analysis

HTS generates a huge amount of sequence data that need to be analyzed with specialized software. Based on existing bioinformatic pipelines such as VirTool or VirusDetect, common workflows were developed to automate sequence data analysis.

2.2.5. Validation of complete workflows (from extraction to data analysis) and comparison with existing technologies

For the abovementioned objectives, developed protocols were validated through inter-laboratory comparisons and compared with regard to sensitivity to existing diagnostic methods (ELISA, PCR).

2.2.6. Inclusion of reference data in public databases

The data obtained through this project will have major implications on quarantine policies and certification schemes. HTS technologies will allow detecting novel viruses and viroids and variants of existing and well-characterized pathogens. It is therefore important to make the data available to public databases such as Q-bank and GenBank. Additionally, the data will help to optimise bioinformatic pipelines such as VirTool or VirusDetect.

2.3. Description of the main activities and results

2.3.1. Development of sample preparation strategies

At the first project meeting in 2016 in Braunschweig, participants shared their experience with the different protocols available for enrichment of viral sequences in nucleic acid preparations. The most popular enrichment methods included the extraction of double-stranded RNA (dsRNA), small RNAs (sRNAs) and ribo-depletion of total RNA extracts. If DNA viruses with a circular genome were suspected in a sample, rolling circle amplification was also applied. Well-working “in-house” protocols were shared amongst the participants so that these protocols could be adapted and verified in the different partner laboratories. A comparative study was carried out comparing different enrichment strategies on a range of different viruses (Gaafar and Ziebell, 2019a). The Canadian partner (CFIA) offered *Phaseolus vulgari*s seeds that were infected with two endornaviruses (Phaseolus vulgarus endovirus -1 and -2; PvEV-1 and PvEV-2). Germinated plant material could be used as positive controls for both extraction and sequencing quality.

2.3.2. Constructions of in-house libraries for HTS

Only two laboratories reported that they would be using in-house prepared libraries. The Peruvian partner (CIP) shared their protocol for library preparation on sRNAs for sRNA sequencing and the Hungarian partner (NEBIH) were interested in the development of libraries for IonTorrent sequencing. All other partners are using either commercial kits for library preparation or are outsourcing this step to commercial sequencing providers so that it was decided that the partners interested in in-house library preparation protocols would pursue this aim on a bilateral basis.

2.3.3. Comparison of sequencing platforms

Based on experience, it was concluded that the type of sequencing platform was not critical provided that the sequencing depth was sufficient to discover unknown viruses/viroids (see also Visser, M., R. Bester, J.T. Burger und H.J. Maree, 2016: Next-generation sequencing for virus detection: covering all the bases. Virology Journal 13, 85, DOI:10.1186/s12985-016-0539-x.). It was noted that the sequencing depth would depend on the scope of the HTS analyses: for virus discovery, in general, partial genomic sequences would be sufficient but for full genome recovery (e.g., for evolutionary studies), more sequencing depth would be required. It was therefore decided to investigate the factor "sequencing platforms" in WP 5 within the frame of a proficiency test and a test performance study. The development of new sequencing technologies such as Oxford Nanopore (ONT) was noted and tested by several laboratories. The use of ONT for plant virus diagnostics is investigated in detail in a separate Euphresco project (VIRFAST).

Two partners of the project (NIB and FERA) have conducted an in-depth comparison of the performance of sRNA and ribosomal RNA depleted RNA sequencing (Illumina platform) for the detection of an array of plant viruses with different genome types. The results of the comparisons showed similar performance of the two methods, however, sequencing of the ribosomal RNA depleted RNA was shown to be a better choice for discovery of new viruses present in plants in very low titer. The study was published as a scientific paper (Pecman *et al.*, 2017b).

2.3.4. Development of bioinformatic pipelines for data analysis

Many bioinformatics softwares have been developed for the analysis of HTS data (including commercially available softwares such as CLC Genomics Workbench, Sequencher, Geneious Prime as well as Galaxy-based software packages). However, often these programs require a substantial understanding of bioinformatics knowledge as many scripts have numerous parameters that can substantially influence the outcome of the analyses. Alternative approaches were developed within the plant virology community, that would take into account the specific requirements of plant virus sequencing data. VirusDetect was developed by the Peruvian partner (CIP) (ZHENG, Y., S. GAO, C. PADMANABHAN, R. LI, M. GALVEZ, D. GUTIERREZ, S. FUENTES, K.-S. LING, J. KREUZE UND Z. FEI, 2017: VirusDetect: An automated pipeline for efficient virus discovery using deep sequencing of small RNAs. Virology 500, 130–138, DOI:10.1016/j.virol.2016.10.017). This automated pipeline is designed to analyse deep sequencing data from sRNAs with the intention of virus discovery, but can only process siRNA reads. In contrast, VirTool, developed by the Canadian partner (CFIA), aims to provide an easy platform for the analysis of Illumina HTS data with the aim to discover both known and unknown virus sequences. As most partners of the NGSdetect consortium were using Illumina sequencing for their samples, it was decided to use both in house bioinformatic pipelines and VirTool in both a proficiency test for the detection of fruit tree and grapevine viruses as well in a test performance study using one sequencing provider.

2.3.5. Validation of complete workflows (from extraction to data analysis) and comparison with existing technologies

2.3.5.1. Proficiency test on the detection of fruit tree and grapevine viruses by HTS

The Canadian partner (CFIA) organized a proficiency test for interested participants. One or two sets of freeze-dried tree fruit and grapevine viruses, respectively, were provided. Each laboratory had to use their own extraction and enrichment methods as well as their own sequencing provider to process these samples. The initial bioinformatics analyses were also carried out by the participants themselves using their preferred in-house bioinformatics pipeline. The results and raw data were supplied to the Canadian partner (CFIA). All data supplied were re-analysed using VirTool. As a result, it was suggested to implement extraction/sequencing controls such as PvEV-1 to check the performance of RNA extraction and sequencing. This would also allow to check whether the minimum number of total reads/mapped reads was appropriate for the sample as it was noted that low read numbers would increase the number of false negative results whereas a high read number would increase the risk of false positive results. Therefore, it depends on the expertise of the diagnostician/bioinformatician to determine the correct results of the HTS analyses.
In general, it was difficult to compare the results of the individual laboratories due to the high number of deviations in terms of extraction methods, library constructs, sequence read lengths, read numbers and the different methods of analyses. A test performance study was therefore proposed.

2.3.5.2. Test performance study on the detection of plant viruses by HTS

Two pools of freeze-dried plant material samples containing a range of different plant viruses unknown to the participants were prepared by the German partner (JKI). The samples were sent to participants together with the chemicals needed for total RNA extraction (provided by the Dutch partner NVWA) and ribo-depletion (provided by the German partner JKI). The participants were asked to send their ribo-depleted total RNA extracts for Illumina sequencing to one designated HTS provider. To test the proficiency of the participants for HTS data analysis, they were asked to pre-analyse their HTS data with their own bioinformatics pipelines. After submission of the results, they shared their raw data with the German partner (JKI) who used VirTool to re-analyse all data with one bioinformatic pipeline. Of the 17 invited laboratories, 9 participated in the test performance study but only 8 laboratories submitted the results. Despite having provided the exact same amount of plant material and chemicals for RNA extraction/ribodepletion, the quantity of RNA showed high variation ranging from 3.75 ng/µl to 30 ng/µl depending on the laboratory. Although 2x5 million reads were requested for pool 1, the laboratories received between 13 million and 54 million reads. However, 4 out of 8 participants correctly identified all viruses in pool 1; 3 out of 8 participants reported 100 % accurate result for pool 2. Only one laboratory reported on false positive results (avian coronavirus). Most deviation was observed with alfalfa-associated nucleorhabdovirus which sequence at the time had not been placed on GenBank and was therefore missed by some participants. This could not be linked to any particular bioinformatic pipeline as these participants all used different programs for the analysis. VirTool showed a great advantage over all other bioinformatics pipelines as all viruses in the pools were detected by the algorithms used in Virtool (PathoscopeBowtie and NuVs) in all datasets. The discovery of unknown viruses/virus sequence is therefore not trivial and might need certain adjustments by the bioinformaticians. This highlights that bioinformatic expertise is required when performing HTS sequence analysis to identify the viruses present.
In addition, the French partner (INRA) did a direct comparison of HTS analyses and classical diagnostics (e.g., based on ELISA, RT-PCR or molecular hybridization). Interestingly, several

false positive results were obtained by HTS, despite a background subtraction. The most likely explanation was the presence of high titer viruses that led to sample cross-contamination.

2.3.6. Inclusion of reference data in public databases

During the course of this project, many new viruses and virus variants were published. As custom for most journal, the sequencing data were placed in public database such as GenBank. Sequences of quarantine viruses were also provided to EPPO Q-Bank.

2.4. Conclusions and recommendations to policy makers

HTS is a powerful technology that enables the simultaneous detection of plant viruses and viroids in a given sample without a priori knowledge on the pathogen(s). HTS can be used on samples where “traditional” methods such as serological or molecular biology methods failed to identify the cause of a disease. However, the implementation and execution of HTS is not trivial due to various constraints:

- In comparison to other technologies, HTS is still relatively expensive. For diagnostic laboratories, it is often not cost-efficient to buy and run a sequencer themselves unless there is a high sample number with regular throughput. Commercial sequence providers offer HTS and although prices have dropped considerably the last few years it is still too high for routine diagnostics and should be reserved for critical samples. Also, the end-users have no or only little influence on the turnaround time of sequencing result; in one extreme case, the contract had to be withdrawn from a commercial sequencing provider as the samples had not been processed 3 months after arriving in the sequencing facility – for critical cases, this turnaround time is not acceptable.
  There has been a constant development of new HTS technologies such as the Oxford Nanopore (ONT) sequencing. In comparison to other sequencing machines, ONT equipment is small and can run on laboratory computers; in comparison, ONT can be cheaper than other sequencing platforms for in-house sequencing. The sequencing error rate is still an issue but has been reduced over the last few years so that ONT technology is seen as having significant potential for plant virus diagnostics, although its performance at the sequencing depth it provides still remains to be evaluated in detail.
- The outcome of the HTS analyses depends on good sample preparation. Various methods exist to enrich viral sequences and, depending on the scope of the analyses, different enrichment methods may be chosen (Gaafar and Ziebell, 2020a). However, as the sample preparation is crucial for good sequencing results, the best method needs to be empirically validated in each laboratory and adjusted to the particular matrix under analysis. Currently, no one-fits-all approach is available as each enrichment method has its own advantages and disadvantages that could possibly be compensated by good bioinformatics analyses. The use of PvEV-1 is an excellent way of controlling the extraction phase as well as whether a sufficient sequencing depth has been achieved thus avoiding false negative results. This was highlighted by the proficiency test were some laboratories had difficulties in correctly identifying the viruses present in a sample in the case of an insufficient sequencing depth.
- Another major obstacle in implementing HTS lays in the bioinformatics analyses of HTS data. Commercial and freely available software suites have many different parameter settings, each of which can influence the analysis outcome tremendously. There is currently no solution that will fit all purposes and therefore significant bioinformatics expertise is required to analyse and correctly interpret HTS data. One potential solution may be the use of Virtool that was developed by the Canadian partner (CFIA). Virtool is freely available and has curated reference database thus minimizing the risk of wrongly annotated reference sequences (e.g., the GenBank entry Citrus exocortis Yucatan viroid represents in fact a mitochondrial sequence). Virtool can be installed on

Linux-based servers and access is via a web-based interface, making the handling user-friendly. The software also records all analysis steps, including software version, thus providing all information required under e.g. accredited conditions. As our test performance study demonstrated, Virtool successfully detected known and unknown viruses from the sample pools where many participants failed to identify for example "unknown" viruses that were not present in public databases yet. We hope that a long-term solution for further development of Virtool and the curation of the database can be secured as it will be an asset to any diagnostic laboratory that is using HTS for virus/viroid diagnostics. Unfortunately, Virtool does not yet process sRNA data or sequencing data derived from ONT but hopefully these can also be incorporated in the future. It is recommended that regular training courses on bioinformatics analyses and interpretation of data are offered to diagnosticians; training courses within the COST Action "DIVAS" were very popular and highly successful to train many people with different bioinformatic backgrounds in the correct analysis of HTS data.

- As for any diagnostic technique, there is always the risk of false positive or false negative results. Therefore, any HTS workflow needs to have a level of validation when implemented in the laboratory. It is also important to implement quality control measures when reporting HTS-based diagnostic data, in particular if reporting new viruses. Confirmation tests need to be carried out and the results should be checked to evaluate whether they make sense at all (e. g., the detection of cytomegalo- or bunyaviruses in plant samples has been reported but most likely represents cross-contaminations from the wet-lab or from the sequencing provider, Lan *et al*., 2019).

2.5. Benefits from trans-national cooperation

This Euphresco project was highly successful in bringing together many partners with very different experiences in HTS. There were partners with little or no experience as well as proficient experts that had developed very advanced bioinformatic softwares for the analysis of HTS data for the presence of viral sequences. The consortium was very open and cooperative and many laboratory protocols were exchanged. As extraction and sequencing control, bean seeds containing endornaviruses were distributed freely by the Canadian partner (CFIA). The developers of Virtool and VirusDetect gave introductory lectures on the use of their respective bioinformatic tools so that consortium members could use these platforms for the analysis of their data.

Within this project, it was also possible to carry out one proficiency test and one test performance study. The data gained through these two tests provides valuable information for further HTS projects and will help to form guidelines for the validation and verification of HTS workflows and the setting of minimum quality criteria.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

Proficiency testing for plant virus detection using ribosomal RNA depletion and high-throughput sequencing (in preparation).

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

Gaafar, Y. Z. A., Sieg-Müller, A., Lüddecke, P., Hartrick, J., Seide, Y., Müller, J., *et al*. (2019e). First report of natural infection of beetroot with *Beet soil-borne virus*. *New Dis. Rep.* 40, 5. doi: 10.5197/j.2044-0588.2019.040.005

Gaafar, Y. Z. A., Sieg-Müller, A., Lüddecke, P., Herz, K., Hartrick, J., Maaß, C., *et al*. (2019f). First report of *Turnip crinkle virus* infecting garlic mustard (*Alliaria petiolata*) in Germany. *New Dis. Rep.* 39, 9. doi: 10.5197/j.2044-0588.2019.039.009

Gaafar, Y. Z. A., and Ziebell, H. (2019a). Complete genome sequence of a highly divergent carrot torradovirus 1 strain from *Apium graveolens*. *Arch Virol* 164, 1943–1947. doi: 10.1007/s00705-019-04272-3

Gaafar, Y. Z. A., and Ziebell, H. (2019b). Two divergent isolates of turnip yellows virus from pea and rapeseed and first report of turnip yellows virus-associated RNA in Germany. *Microbiol Resour Announc* 8. doi: 10.1128/MRA.00214-19

Gaafar, Y. Z. A., and Ziebell, H. (2020a). Comparative study on three viral enrichment approaches based on RNA extraction for plant virus/viroid detection using high-throughput sequencing. *PLOS One* 15, e0237951. doi: 10.1371/journal.pone.0237951

Gaafar, Y. Z. A., and Ziebell, H. (2020b). Complete genome sequence of a soybean dwarf virus isolate from white clover in Germany. *Microbiol Resour Announc* 9. doi: 10.1128/MRA.00637-20

Gentit, P., Marais, A., Brans, Y., Renvoisé, J. P., Remenant, B., Saison, A., *et al*. (2019). *Comparaison directe de la performance des techniques classiques avec séquencage à haut débit (HTS) pour la détection des virus des arbres fruitiers: 17émes Recontres de Virologie Végétale. Aussois, France*.

Krizbai, L., Kriston, E., Kreuze, J., and Melika, G. (2017a). *Identification of Nectarine stem pitting-associated virus (NSPaV) by Next Generation Sequencing (NS) in Hungary. COST and Euphresco Meeting, Bari, Italy.*

Krizbai, L., Kriston, E., Kreuze, J., and Melika, G. (2017b). Identification of Nectarine stem pitting-associated virus infecting Prunus persica in Hungary. *New Dis. Rep.* 35, 18. doi: 10.5197/j.2044-0588.2017.035.018

Lan, Y., Yan, Z., Guo, Y., Duan, T., Li, C., Gao, P., *et al*. (2019). RETRACTED ARTICLE: Albinism and mosaicism in Apocynum venetum associated with viral infections in China. *Arch Virol* 164, 333. doi: 10.1007/s00705-018-4059-4

Marais, A., Faure, C., Bergey, B., and Candresse, T. (2018). “Viral double-stranded RNAs (dsRNAs) from plants: Alternative nucleic acid substrates for high-throughput sequencing,” in *Viral Metagenomics: Methods and Protocols*, eds. V. Pantaleo, and M. Chiumenti (New York, NY: Springer New York), 45–53.

Pecman, A. (2016). *Exploring NGS borders in plant virology: diagnostic, regulation, characterization & taxonomy: COST-DIVAS, Meeting 2; Thessaloniki, Greece*.
Pecman, A., Kutnjak, D., Gutiérrez-Aguirre, I., Adams, I., Fox, A., Boonham, N., *et al*. (2017a). *Next generation sequencing for detecion and discovery of plant viruses and viroids: comparison of two approaches: 7. Kongres Slovenskega Mikrobiološkega Drušva, Bled, Slovenia*.
Pecman, A., Kutnjak, D., Gutiérrez-Aguirre, I., Adams, I., Fox, A., Boonham, N., *et al*. (2017b). Next generation sequencing for detection and discovery of plant viruses and viroids: Comparison of two approaches. *Front Microbiol* 8, 537. doi: 10.3389/fmicb.2017.01998
Pecman, A., Kutnjak, D., Mehle, N., Tuŝek-Žnidarič, M., Fox, A., *et al*. (2018). *Comparison of NGS-aided plant virus discovery techniques. Final Meeting of COST-DIVAS Action, Liége, Belgium*.
Remenant, B., Mille, J., and Gentit, P. (2019). *Molecular characterization of a new world bipartite Begomovirus infecting green beans in French Caribean Island: 17ème Rencontres de Virologie Végétale, Aussois, France. Poster contribution.*
Svanella-Dumas, L., Marais, A., Depasse, F., Faure, C., Lefebre, M., Brans, Y., *et al*. (2019). First report of Citrus virus A (CiVA) infecting pear (*Pyrus communis*) in France. *Plant Dis* 103, 2703. doi: 10.1094/PDIS-01-19-0028-PDN

4. Open Euphresco data

Sequence data were made available to Q-Bank and Genbank.