

The utility and perspectives of NGS-based methods in BSL-3 and BSL-4 laboratory – sequencing and analysis strategies

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Abstract

Modern diagnostics is in general based on molecular biology methods. Nowadays sequencing-based methods, especially whole genome sequencing, are becoming increasingly important. Implementation of such methods into routine diagnostic of highly dangerous pathogens, like *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, Ebola virus, MERS, Lassa virus etc. would be very helpful. The best diagnostic strategy would be the metagenomic sequencing directly from the clinical sample. Implementation of majority of currently available WGS platforms inside the BSL-3 or 4 laboratory is impractical because of the size of the equipment and time consuming wet lab part (e.g. library preparation). Nowadays there is a possibility to implement pocket size MinION - real time whole genome sequencer into BSL-3 and 4 laboratory for rapid and precise diagnostic purposes.

Key words: Whole genome sequencing, molecular diagnostics, BSL-3, BSL-4

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Modern molecular diagnostics

Diagnostics schemes of different diseases and pathogens changed a lot during last decades. At present, commonly available diagnostic tests are often based on molecular biology techniques, quite often without any preculture step. Such culture-free diagnostic is very valuable especially in the context of highly pathogenic bacteria like e.g. *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis* [1–5] or viruses like e.g. Ebola virus, MERS, SARS, Lassa virus etc. [6–9], because every culture step can be difficult and, what is even more important, dangerous. Most of the molecular diagnostics is based on PCR methods, both regular and real-time version. There are also other advantages of such diagnostic strategy. The result can be rapidly obtained when compared with the classical, culture based techniques (bacterial culture takes at least one day, whereas result of PCR reaction can be obtained within few hours). In most cases molecular methods have also much higher sensitivity and specificity. But even these methods seems not to be ideal and

sometimes their high sensitivity can result in occurrence of false positive results. This is because of the fact, that all PCR methods are based only on the specificity (but not identity) of only short oligonucleotide fragments (and more or less accurate distance between these primers). In this case contamination with high amount of different nucleic acids that can be present inside the probe (e.g. patient DNA/mRNA) may introduce additional noise to the test. Because of that, for example, during complex molecular diagnostics of suspected Ebola case directly from the clinical sample it is recommended to confirm every positive result with another molecular test focused on different genetic target or by sequencing [10].

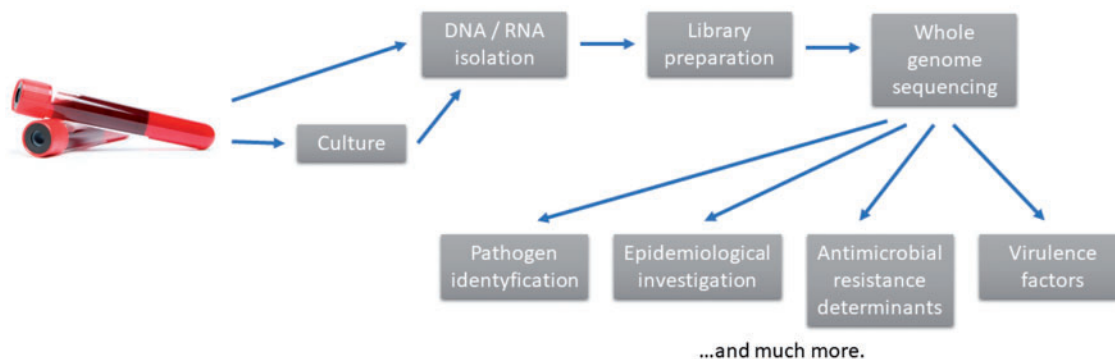
Advantages of introducing of whole genome sequencing into diagnostic shame

Modern diagnostics is changing day by day and methods based on whole genome sequencing analysis (WGS) are of increasing importance nowadays [11]. WGS sequencing techniques, like e.g. most prevalent Illumina, IonTorrent, PacBio, Nanopore and

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Table 1. Comparison of efficiency of most important WGS platforms.

Sequencing platform	Single read length	Amount of data per flow-cell	Run types
Sanger sequencing	800-1500	–	–
Roche 454	300–500	0, 4–0, 7 Mb	Single end
Illumina MiSeq	100–140	0, 3–15 Gb	Single and paired end
Illumina HiSeq	150-250	125–600 Gb	Single and paired end
IonTorrent	200-400	1, 5–2 Gb	Single end
PacBio	10 000–20 000	5–8 Gb	Single end
Nanopore - MinION	10 000–300 000	5–10 Gb	1D and 2D reads

**Figure 1.** WGS strategies and possible analyses.

many other give the possibility of rapid perform thousands of reads of DNA sequence and because of that are able to generate sequences more or less covering whole microbial genome (comparison of most prevalent sequencing platforms in table 1). There are some Institutes, where these methods are already implemented into routine diagnostic, and the WGS approaches often are replacing classical methods [12, 13]. The reason for this is that WGS analysis could give much more information than any other molecular and classical method. For example, in a single PCR it is possible to analyse only the occurrence of two short sequences homologous to used primers, within relatively short distance. By contrast, WGS based analysis could yield precise information about every sequence in the genome and allows to perform many accurate comparisons [14] (analytical strategies are shown in the figure 1). Such information, especially during an ongoing public health threat, where accurate and timely information is extremely important, whole genome analysis can provide near real-time insights into pathogen's origin, transmission dynamics, mechanisms of adaptation, resistance traits and evolution. That is because of the fact, that rapidly performed WGS analysis will show every changes in the pathogen genome from single point mutations to acquisition of new genes, plasmids etc. Of course it has to be taken into account that the accuracy of different sequencing techniques can vary and different techniques can give slightly different results, especially in the context of point mutations (for example because of the error rate). Additionally whole genome metagenomics analysis of clinical samples can identify many different pathogens in a single analysis. For example, Gire et al. [15] during their analysis of 35 samples from suspected Ebola virus disease (EVD) cases (but tested negative for Ebola virus) identified other known pathogens like Lassa virus, HIV-1, enterovirus A and malaria parasites. Metagenomics sequencing was useful also in the case of a novel filovirus that caused an outbreak in Uganda in 2007 [16]. The advantages of metagenomic

sequencing include also its relative speed, the ability to detect non-culturable pathogens and, as it was shown, detect of new pathogens (what is impossible using other techniques). There are also some problems with such metagenomic sequencing for diagnostic purposes. Because of the fact that this is still quite new methodology there are no consistent standards what constitutes identification of the pathogen in the sample, how many positive reads are necessary for identification of a true positive result. Because of that it is hard to estimate the efficiency of the method, but it looks that it should be even more sensitive than RT-PCR (which is 1-2 orders of magnitude more sensitive than classical PCR) and the efficiency can be different for different pathogens. The situation seems to be easier with the positive results – detection of even one pathogen specific read in a clinical sample, that are not found in control samples is likely to be interpreted as a positive result. However, for negative results important is the knowledge about limits of detection (LoD). There are many analysis showing that LoD level can vary between different sequencing platforms, variation in the extraction techniques or used sample preparation kit. All these variables can affect the composition of sequence reads [17, 18, 19]. For example Cheval et al. [20] compared Roche-454 pyrosequencing with Illumina in a detection of eleven different viruses with known concentrations in cerebrospinal fluid and plasma samples. In this study it was shown that Illumina gives higher output that resulted in more sensitive detection of the tested viruses per run. Also Frey et al. [21] were compared three WGS platforms: Roche-454 Titanium, Illumina MiSeq and Ion Torrent for metagenomics using among others Dengue virus and *Bacillus anthracis*. They found out that Roche-454 Titanium platform produced longer reads, whereas Illumina MiSeq platform produced consistently greater depth and breadth of coverage while the Ion Torrent has higher speed of sequencing.

To increase the amount of pathogen DNA (or RNA) in a clinical sample it is also possible to amplify it by the PCR (or RT-PCR)

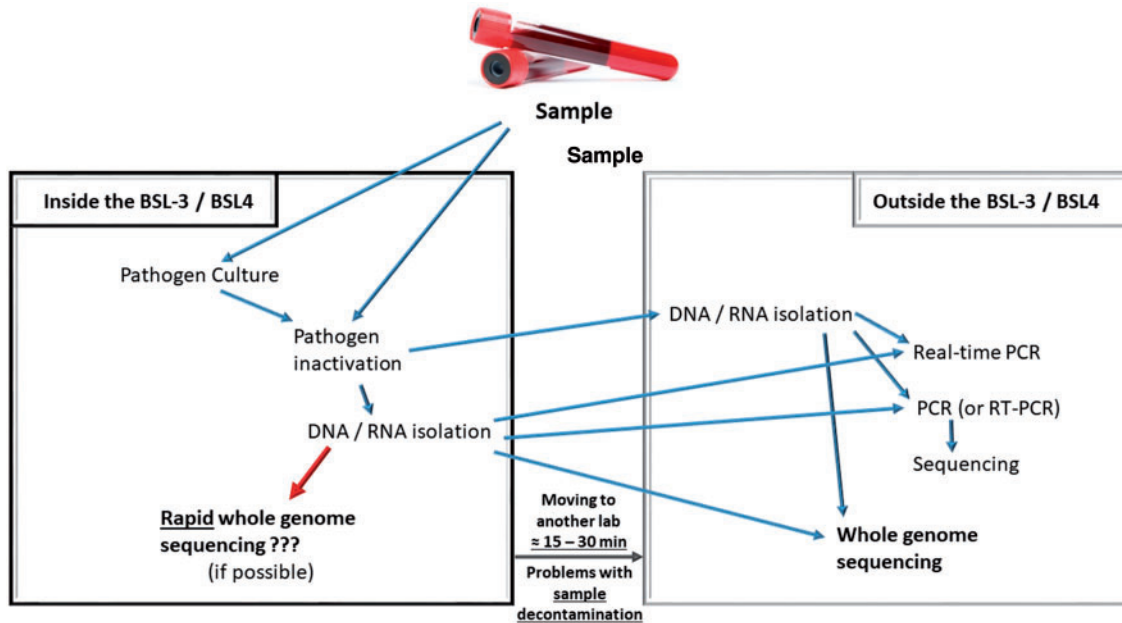


Figure 2. Compilation of different analytical steps performed inside and outside the BSL-3 or BSL-4 laboratory.

reaction (preferably multiplex PCR) before sequencing. Such multiplex PCR enrichment protocol was proposed by Quick et al. [22] for viruses like e.g. Zika detection. The selectivity of such pre-amplification step and described previously limitation of PCR/RT-PCR can add some additional bias into the analysis.

In this context, would it be possible, and reasonable to easily implement such analysis into BSL-3 or BSL-4 lab, for the purposes of diagnosis of most dangerous pathogens, both bacterial and viral.

WGS methods in the BSL-3 and 4 laboratories environment

BSL-3 and BSL-4 are very sophisticated and specialised environments, with multiple restrictions concerning manipulations of the examined material, safety procedures and usage of laboratory equipment (for review of all important rules and recommendations of designing, constructing and operating procedures in different classes laboratories see WHO "Laboratory biosafety manual" [23] and CDC "Biosafety in Microbiological and Biomedical Laboratories" [24]). This class of laboratories is designed for working with highly dangerous pathogens that pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease. Assignment of the pathogen to the appropriate safety class (from class 2 to 4) is defined in relevant local regulations [25]. There are also some differences in the pathogen classification due to differences in the type of research conducted. For example, diagnostics of potential Ebola virus disease cases should be performed in the BSL-3 class laboratory. But scientific researches of Ebola viruses should be rather performed in the BSL-4 class laboratory because of the need of work with the larger inoculum of the pathogen.

BSL-3 and 4 laboratories have highly limited access through few locks. Inside this kind of laboratory all manipulations of potential infectious materials within the laboratory must be in appropriate class II or III biosafety cabinets (BSC). All personnel that is working inside the laboratory have to wear special protective, multilayer clothes, masks, gloves and/or special suits. Despite of these difficulties every procedure has to be done

carefully, usually by two people (one person is working inside the BSC and another person is assisting). That makes working inside such laboratory very uncomfortable. Because of that it is nearly impossible to perform all the manipulations directly like in BSL-1 or BSL-2 laboratory. Additionally, every laboratory equipment must be routinely decontaminated, especially after every potential contamination or before removal from the laboratory. Because of these restrictions only necessary equipment should be stored inside the laboratory. This also limits the use of large, sophisticated (and because of that quite delicate) equipment like e.g. most NGS sequencers.

Due to all these limitations, inside the BSL-3 or 4 class laboratory only initial pathogen inactivation or nucleic acids isolation is usually carried out [26, 27, 28]. All further analyses can be conducted on inactivated nucleic acid samples in standard molecular laboratory, under normal operation procedures and standards. Such a course of action should be applied also during preparation of NGS analyses. So nowadays implementation of most of such techniques to diagnostic schemes of class 3 and 4 pathogens could be similar to those already adopted for other pathogens. Of course taking the sample out of the BSL-3 or BSL-4 laboratory can be dangerous. First of all not all potential pathogens present in the sample can be completely inactivated (because of e.g. occurrence of hardly damage spores or because of simply laboratory mistakes). Additionally, decontamination of the sample should be performed as precisely as possible, but on the other hand this process can damage the sample. Another problem with taking the sample outside is the time. Because of the multiple protective clothes and locks it can take additional 15-30 minutes. That is why it sometimes it would be better to have the possibility to perform as much analysis as possible inside the BSL-3 or BSL-4 laboratory. Short compilation of different steps performed inside and outside the BSL-3 or BSL-4 laboratory is shown in the figure 2.

There are also some disadvantages of implementation of WGS analysis for routine diagnostics of class 3 and 4 pathogens. First of all such diagnostics usually should be as fast as possible. The time needed for whole protocol (isolation of good quality DNA, constructing the library and sequencing itself) can be

estimate for at least 2-3 days [11]. Analysis of RNA viruses, like Ebola or Lassa viruses would last even longer because of the need of cDNA synthesis [15, 29]. Another weakness, but more debatable one, of this kind of analysis is its unit cost. First of all the cost of the sequencing platform and every other necessary equipment is still high. Additionally, cost of single analysis can be much lower than 100 Euro, but it usually requires a large number of analysis for maximum flow cell filling. Diagnostics of most dangerous pathogens is not required quite often, what it is good from the public health reasons, but unfortunately can substantially rise the unit price. In a larger scientific centres, with WGS techniques implemented for routine analysis, it should not be a problem to sequence already prepared probe on one flow cell with other samples to lower the sequencing costs.

Nanopore sequencing technology in the BSL-3 and 4 laboratory

Different scheme of work organization in BSL-3 and 4 laboratory, in the context of WGS analysis, can be implemented with Oxford Nanopore Technologies sequencing technology, especially with the MinION sequencer. This sequencing platform presents completely different approach to sequencing technology. In fact all other technologies are based on some kind of DNA modification and/or synthesis [30]. Whereas the mechanism of Nanopore sequencing is based on translocation of single nucleic acid strand through a special pore protein located in an electrically resistant polymer membrane [31]. The current is changed as the different bases pass through the pore in different combinations (for more precise information see www.nanoporetech.com). As a result, Nanopore DNA sequencing can be aligned to the simple reading of sequence of nucleotides in the nucleic acid strand.

This sequencing strategy gives multiple advantages. First of all it can generate extremely long reads (usually about 10-20 kb, but it is also possible to generate much longer reads, even about 300 kb). Also preparing of sequencing library can be quite easy and fast, because it can be limited only to ligation of specific adaptors. Usually two types of adaptors are used at the same time – standard double strand and hairpin shape (especially for 2D reads). The adaptors provide the mechanism of enrichment of the DNA fragments on the membrane within the flow cell, increasing the efficiency of the sequencing. Standard library preparation would take about 2-4 hours but there are many protocols where it can be reduced to about 20-30 min [32, 33]. But using equipment called Voltrax[®] it can be theoretically shortened to about 10 minutes [34]. That makes it possible to easily prepare the library everywhere, also inside the class 2 or 3 biosafety cabinet inside the BSL-3 or 4 laboratory (see Figure 3).

Another advantages of Nanopore technology is the possibility to create extremely small sequencers. MinION measures $9.5 \times 3.2 \times 1.6$ cm, draws its power from a laptop or tablet via a Universal Serial Bus connection, with a total mass of less than 100 grams so it is smaller than a smartphone. For working it have to be plug into a PC or laptop by the USB cable. That made it also possible to take it into International Space Station [35, 36] or in the Antarctic Dry Valleys [37] so it is also possible to implement it also in the BSL-3 and 4 laboratory environment and prepare all the sequencing steps inside the BSC. It would be also possible to use MinION in the field laboratory e.g. in Africa, that is in areas directly exposed to possible outbreak of e.g. Ebola virus [38, 39]. Moreover, the overall price of the sequencing chamber is relatively low, thus can be considered disposable in BSL3 or BSL4 applications. The only thing that would get contaminated and that would have to be get out from the BSC is the



Figure 3. Analysis with MinION inside the class 2 BSC.

flow cell. One flow cell should suffice for few more sequencing runs. Between them the flow cell can be easily wash with two simply reagents. When the flow cell will worn out it can be moved of the sequencer and throw away after sterilisation.

Important feature of Nanopore technology is that sequencing data are streamed into the computer in a real time and can be analysed in the same way [40]. The standard run can be conducted for 48 hours, and in this time is should generate around 5-10 Gb of DNA sequence data. But for the rapid diagnostic it would be better to perform real time analysis of obtained data looking for these sequences that would be homologous to known pathogens. Calculating that using newest “R9” chemistries and flow cell it is possible to sequence at a speed of around 250 bases per second per nanopore (MinION flow cell has 512 nanopores on the membrane surface) it will give around 76 million bases completely read after first 10 minutes. In such big amount of data it should be possible to find sequences matching to pathogens of interest.

In most sequencing technologies more problematic would be the diagnosis and sequencing of the RNA viruses (like e.g. viruses Ebola, Marburg or Lassa) because of the need of cDNA synthesis. Simple “reading” of non-modified nucleic acid on Nanopore sequencers makes it also possible to sequence and analyse the RNA directly, without cDNA synthesis step [41].

Completely different issue is the analysis of WGS data, and this is also a problem with the data produced by Oxford Nanopore sequencers. There are some bioinformatics tools, focused on WGS data and available on-line (like, e.g. Galaxy Tools or CGE tools)[42, 43], but they are usually unable to analyse raw files from Nanopore (files in the fast5 that use the HDF format instead of standard fastq file. Fastq files are inside the fast5 files and could be further extracted). Another analytical strategy assumes use of bioinformatics software like e.g. CLC Genomics Workbench, Lasergene package or BioNumeris, but none of these programmes analyse files in fast5 format (maybe it will be possible in the future). The greatest analytical possibilities are available by using Unix system tools and the command line, but using of these bioinformatics algorithms requires advanced bioinformatics skills. In this situation there is a possibility to use e.g. Canu genome assembler. There is also a possibility to use available web-based services for assembly the Nanopore reads, usually based on Canu assembler, like e.g. NanOnline (<http://minion.cent.uw.edu.pl/>) [44, 45]. But for rapid diagnostic of highly dangerous pathogens most useful would be the algorithm to perform real time comparison of the data obtained during the sequencing process to the database of known sequences, especially those that are pathogen specific. However

bioinformatics algorithms and scripts are developing very dynamically and detailed description of these topic exceeds the framework of this publication.

Conclusions

At present, molecular biology techniques, especially those referring to DNA sequencing, are developed rapidly. Because of this fact it is really hard to foresee the future of diagnostics. Classical PCR reaction, developed in the year 1983 is still in widespread use and, despite many changes and modifications, it is based on the same methodological foundations [46]. It seems obvious, also for many international experts [11, 47], that the main direction in a near future diagnostic will be associated with any whole genome sequencing methods, but it is really hard to foresee on which technology, because of the speed of technology development. Sanger sequencing method, developed in the year 1977 is also still in use [48], but nowadays it is widely supplanted by newer, Next Generation Sequencing methods. However, currently the life span of these modern methods is much shorter. For example pyrosequencing based sequencing technology was considered modern a few years ago [49]. Now it is widely replaced by more popular Illumina technology [50]. But even Illumina can also be easily supplanted by newer techniques, for example those that are able to produce much longer reads. And that is why more and more laboratories are implementing e.g. more modern PacBio technology [51]. That is also the problem with prediction, whether Nanopore sequencing technology [52], that can be considered as a real revolution in the field of nucleic acids sequencing, will constitute a basic diagnostic technique in the future. Maybe the existing problems with the throughput (maybe solved by introducing of bigger equipment like PromethION), quite high error rate (but lower in each new generation of the flow cell and chemistry) or lack of easily accessible and “microbiologist friendly” software will make the technology soon replaced by another one. Nevertheless, nowadays such WGS analysis looks like the best choice for rapid diagnostics of highly dangerous pathogens. Further development of these techniques would make it possible, to implement them into BSL-3 and 4 laboratory, what will reduce some analytical steps, will solve the problems with decontamination of the sample and will make it possible to receive the results much faster than it is now.

Key Points

- Whole genome sequencing methods are becoming increasingly important in the routine diagnostics.
- WGS platforms can be used for the purposes of diagnostics of high dangerous bacteria and viruses, especially for the metagenomics analysis.
- Most of the WGS platforms shouldn't be implemented inside the BSL-3 or 4 laboratory.
- Oxford Nanopore MiniON device is a sequencer that can be easily implement into the BSL-3 or 4 laboratory.

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