ESCMID Postgraduate Education Course

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Meeting report

ESCMID postgraduate education course: regional capacity building for integration of next-generation sequencing in the clinical microlab

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1. Introduction

Next-Generation Sequencing (NGS) is increasingly being used in clinical and public health microbiology laboratories. The main applications of NGS include whole genome sequencing of cultured pathogens (Whole Genome Sequencing - WGS), study of microbial populations via 16S amplicon sequencing (Microbiomics), and culture-independent analysis of samples using shotgun metagenomics, which analyses all genomic content of the sample. With its multitude of benefits, NGS is becoming the gold standard in microbiology and its wide implementation, depends amongst other factors, on the training of personnel to facilitate the 'wet' and 'dry' components of NGS (see Ref. [1] for a review).

The workshop intended to introduce the emerging role of NGS in clinical and public health microbiology and support the building of national and regional capacity. Special focus was placed on available sequencing technologies, their implementation in the microbiology laboratory settings, the use of various bioinformatics tools for analysing NGS outputs and clinical interpretation. Capacity building involved relevant exemplars such as surveillance of infection, outbreak investigation and control, antimicrobial resistance and food and water microbiology. Moreover, the course aimed to create new networks between microbiologists, infectious disease specialists, bioinformaticians and other allied professions in order to facilitate the application of NGS. Several take-home messages appear in Table 1.

2. Participants and venue

The workshop was co-organised by several Study Groups of the European Society for Clinical Microbiology and Infectious Diseases (ESCMID), including the Genomic Molecular Diagnostics (ESGMD), Molecular Markers (ESGEM), Legionella Infections (ESGLI) and Forensic and Post-Mortem Microbiology (ESGFOR). This ESCMID event was also co-organised with the Israeli Society for Infectious Diseases as the local organiser. Prof. Jacob Moran-Gilad (ESGMD Chairperson) opened the workshop, noting that this was the first time an ESCMID workshop was organised in Israel. This was followed by welcome notes by the Chair of the Israeli Society for Infectious Diseases, Prof. Miriam Weinberger. The workshop involved more than 100 delegates from 18 countries, conveniently taking place at the Herzeliya, in the greater Tel Aviv area (Fig. 1).

3. NGS and new technologies in medical microbiology

Prof. Alexander Friedrich (University Medical Center Groningen, The Netherlands) opened the workshop presentations with an eye to the future, highlighting the anticipated challenges to clinical microbiology for the coming 10 years. An eldering society and requiring a more and more complex and invasive healthcare needs a focus on the prevention of healthcare-associated infections and prevention of the spread of antimicrobial resistance (AMR). This necessitates technological innovations for reliable diagnostic tests, allowing a more personalised microbiology with awareness not

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Table 1
Take-home messages and highlights from the ESGMD workshop.

Topic	Highlights
NGS and new technologies in medical microbiology	• Future focus will be on the prevention of healthcare-associated infections and prevention of the spread of AMR. • As an alternative to classical approaches, SNP analysis and whole genome and core genome MLST will become the standard. • There's a need for more people to be capable of NGS performance, analysis and interpretation.
Basics of NGS	• There is no one solution for these procedures that fits all analyses. • For QC, internal controls, changing barcode sequences between sequencing runs on the same machine, and genome size evaluation as a contamination marker, are important.
Advanced NGS	• Application of WGS for outbreak analysis and surveillance, particularly in the form of wg and cgMLST is anticipated to become the standard for bacterial typing. • Clinically relevant information can be inferred from bacterial WGS as a “one stop shop”. • Still remains the need to further translate analysis results for clinical experts, to assist in clinical decisions.
Metagenomics (mainly 16S amplicon sequencing)	• Microbiomics is changing our understanding of microbial–host interactions, with application in a very broad range of fields.
Interactive discussion	
Interpretation of NGS findings – the clinical point of view	• Debatable at what threshold one may consider phylogenomic results to be of clinical or epidemiological importance. • For NGS interpretation, one will always need epidemiological meta-data in order to rule in or out an infection control decision.
Quality issues in implementing new technology in the micro lab	• With the incorporation of NGS into the standard clinical microbiology and public health workflow, what is the gold standard? and is ISO accreditation possible and acceptable? Remain debatable questions.

only at the hospital level but also at the regional level. In so, Prof. Friedrich presented a number of outbreak studies that demonstrated the complexity and necessity of preventative measures [2]. Among the approaches mentioned were classical approaches such as *spa*-typing, single nucleotide polymorphism (SNP) analysis, and *in silico* pulsed-field gel electrophoresis (PFGE) but the standard will be the use of whole genome/core genome multilocus sequence typing (wg/cg MLST) [3]. Novel interdisciplinary approaches will be real time in vivo imaging of infections [4], and metagenome analysis tools [5], all of which highlighted the successful use of NGS for tailor-made diagnostics (also termed “theragnostics”, see Ref. [6]). Prof. Friedrich emphasised that there is a clear need for more people capable of NGS performance, analysis and interpretation, in order to provide more on-demand, real-time, patient-oriented interventions, while also aiding preventive measures on the local/hospital, regional, and the cross-border scales.

4. The basics of NGS

Dr. Eric Claas (Leiden University Medical Centre, The Netherlands) presented an excellent overview of the historical development of NGS technologies with their underlying chemistry, including first generation Sanger sequencing, second generation short reads Illumina sequencing, and third generation long reads pacific Biosciences sequencing. All of which were explained briefly and clearly with the use of diagrams and videos. Dr. Claas recommended further reading [7] and [8], while also taking advantage of publicly available animations.

With that background, Dr John Rossen (University Medical Center Groningen, The Netherlands) presented the workflow for nucleic acid extraction and library preparation for microbial NGS. Dr Rossen's laboratory houses two Illumina MiSeq sequencers allowing for five to six sequencing runs per week. For this throughput, a number of recommendations were highlighted, in order to optimise downstream efforts and reduce costs. For example, in the nucleic acid isolation procedures one needs to know beforehand what is needed to be isolated (DNA and/or RNA, virus/bacteria/parasites, moulds) and if depletion of human nucleic acids is desirable or required. Dr Rossen shared his experience with different manual and automated extraction products and emphasised that not one solution fits all.

One critical step for successful sequencing is the sample library preparation, which entails (a) measuring DNA/RNA concentration and quality, (b) a mechanical or enzymatic method for library preparation (e.g. Illumina's Nextera XT), (c) sample clustering, (d) library pooling and indexing quality control (QC), (e) library amplification, and finally, (f) assembly. Recently, Dr Rossen's laboratory obtained ISO15189 certification for validation of WGS (see Refs. [9] and [10] for their methodology and results), which involved reproducibility, repeatability and comparisons to established methods.

Further to those presentations, an interactive discussion on the topic of ‘How to setup wet NGS in hospital microbiology lab’ was undertaken with Prof Friedrich (moderator), Dr Claas, and Dr Rossen on the panel. Among the topics of discussion were the requirement for internal controls for QC in library preparation, platform dependence in contamination issues, ethical and legal issues involving analysis, the advantages and disadvantages of centralisation as opposed to decentralisation of NGS, and avenues for capacity building for NGS.

It was suggested that internal controls were important for all 16-23S amplicon sequencing and shotgun metagenomic samples and that changing barcode sequences between runs on the same sequencer and that larger than expected genome sizes due to presence of more than one microbial genome (as a marker of contamination) are important for quality control. The legal implication of answering unasked questions and the impact on payment was discussed, as was the issue of privacy with respect to possible identification of patients via their microbiome. Decentralisation of sequencing was said to be preferred due to the speed, flexibility and quality, but has cost implications, while decentralisation of the dry lab component may follow a different model. With respect to human resource it was suggested that wet lab technicians need training for simple bioinformatics analyses, while it was questioned whether it is preferred to hire bioinformaticians and train them in microbiology or hire microbiologists and train them in bioinformatics.

5. Advanced NGS

Leaving the wet lab and entering the dry lab, Dr Joao Andre Carriço (University of Lisbon, Portugal) presented a bioinformatics-oriented overview of the available molecular typing approaches. With the aim to discriminate strains within a species or subspecies, microbiologists have adopted three approaches, namely phenotypic (including growth, morphology, physiology, serotyping),

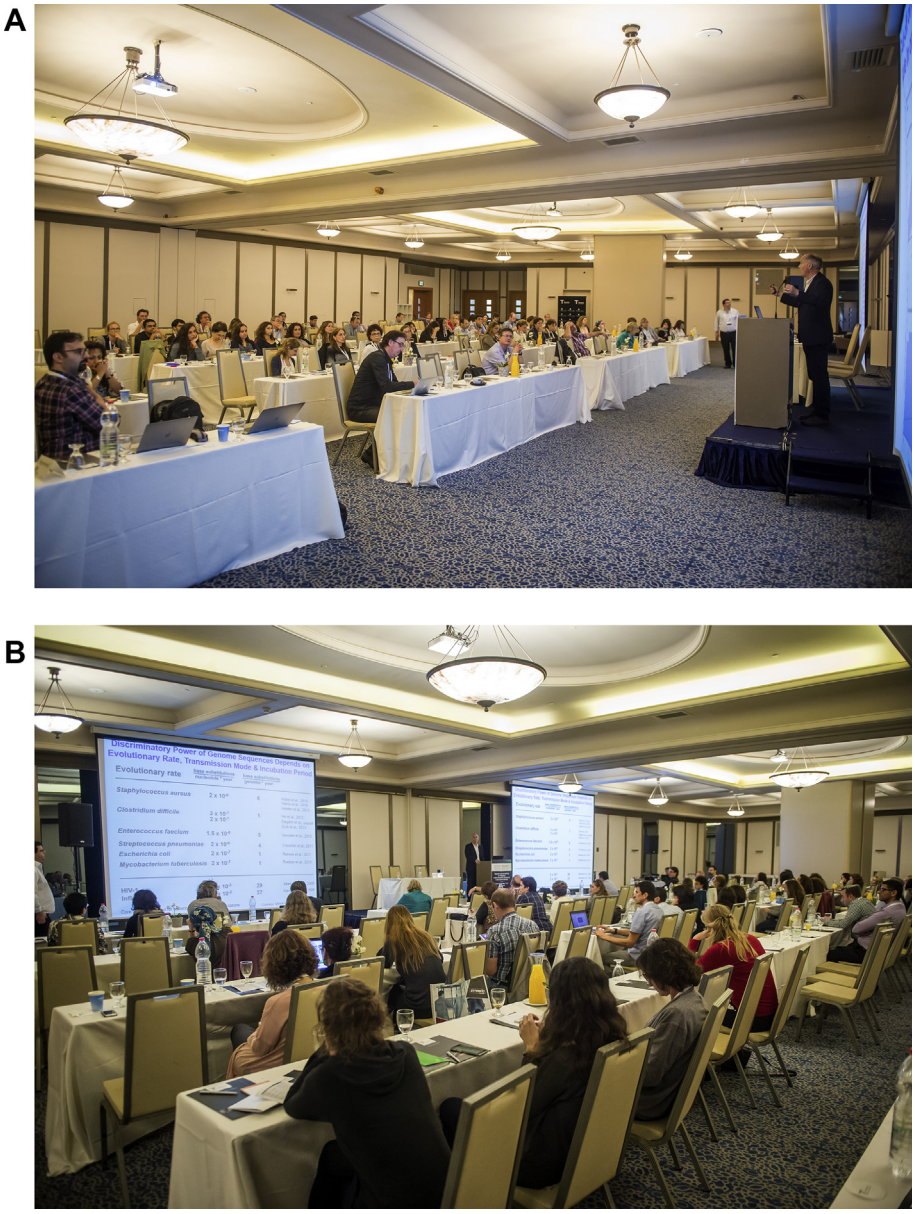

Fig. 1. ESCMID Capacity-building workshop on next-generation sequencing. Lectures (A) and interactives discussions (B) spanned a wide range of issues concerning the implementation of sequencing technology in clinical microlabs.

genotypic at the molecular level (chromosomal, extrachromosomal), and genotypic at the sequence level. The latter was the main focus of Dr Carriço's presentation, with informative descriptions of methods including PFGE (formerly considered the 'gold standard'), multilocus variable number of tandem repeats analysis (MLVA), MLST, *spa* typing (for *Staphylococcus aureus*). Dr Carriço also highlighted each method's advantages and disadvantages, while also noting some comparisons between the methods (see Refs. [11] and [12]). Dr Carriço also highlighted the gradual shift to NGS for molecular typing, such as the US PulseNet (https://www.cdc.gov/pulsenet/next-generation.html) initiative to utilise WGS.

Continuing the dry lab focus, Prof. Jacob Moran-Gilad (Ben-Gurion University, Israel) presented some real case examples of how clinically relevant information is inferred from bacterial WGS as a "one stop shop". Prof. Moran-Gilad described NGS as a disruptive technology that can be implemented into the traditional workflow of the clinical laboratory, either via WGS sequencing, microbiome studies, metagenomics of microbiological samples, and cross-cutting issues. Prof. Moran-Gilad highlighted how in a *S. aureus* case study using an in-house bioinformatics pipeline the dry lab could accurately analyse the resistome and virulome to identify mechanisms of AMR, while also provide molecular typing results (cgMLST, *spa*, wgMLST, and SNPs). Another case study was presented, together with Dr. Yair Motro from the same group, involving *A. baumannii*. Prof. Moran-Gilad emphasised that there still remained the need to further translate such data and results for the clinical experts, and that there is still a long way to go towards achieving a clinical decision. Though the incorporation of NGS for clinical relevance is continually progressing, for example predicting MICs from WGS [13], and the establishment of proficiency testing for NGS [14].

The final presentation of the first day was delivered by Prof. Dag Harmsen (University of Münster, Germany), with further in-depth

discussion on the topic of bacterial typing applications using NGS. With the cost of NGS in clinical microbiology becoming rapidly cheaper [though it was highlighted that the costs using the popular Illumina MiSeq have remained fairly stable], application of WGS for outbreak analysis and surveillance is ever more viable (for a good summary of the available tools for WGS outbreak analysis, Prof Harmsen referred to [15]). Two main parts of WGS application were described, namely the assembly of WGS and the types of analysis widely performed in the context of surveillance and outbreak analysis. Two methods were described for assembly of WGS, reference-assisted and *de novo* assembly, with advantages and disadvantages highlighted (see Ref. [16] for recommendations). Of note, the issue of WGS associated data storage requirements was raised, with Prof Harmsen suggesting uploading data to public repositories as a cost-effective solution. Methods for analysis of WGS data presented by Prof Harmsen included multiple genome alignment, k-mer without alignment, average nucleotide identity (ANI) with alignment, genome-wide mapping and SNP calling, and gene-by-gene analysis, each of which had its principle described and a real-case application was highlighted. Of particular emphasis was the anticipation that wgMLST and cgMLST (see Ref. [17] for an excellent review) will become the standard for bacterial typing, with Prof Harmsen referring to a number of real case WGS analyses that were successfully applied for infection control, even at real-time, on resistant bacteria [see Refs. [9,18–20]].

6. Case presentations

The focus of the second day of presentations was on applied NGS case studies in a range of microbiological fields. Dr Colin Mackenzie (Heinrich Heine University, Germany) opened the session with description of a WGS-based investigation of a GIM-1 carrying multi-resistant *Pseudomonas aeruginosa* outbreak, which highlighted many challenges (see Ref. [21]). Dr John Rossen presented the identification of a plasmid-mediated colistin resistance in *Enterobacteriaecae* in patients and food using WGS [22]. In addition, the power of WGS for battling outbreaks was presented for a recent vancomycin-resistant *Enterococcus faecium* outbreak in a hospital setting that appeared in the end to be two different outbreaks. The importance of sharing genomic data before publication within healthcare networks was emphasized as well as the potential of a unique-marker real-time PCR based screening method for surveillance purposes [23]. Prof. Jacob Moran-Gilad also presented a recent Israeli Shiga-toxin producing *Escherichia coli* (STEC) outbreak (see Refs. [24] and [25] for background) investigated using WGS of outbreak isolates, that helped to disprove any link between this and a previous outbreak, demonstrating the application of NGS in One Health. The investigation also involved microbiome analysis for follow up of STEC shedding.

7. Metagenomics (mainly 16S amplicon sequencing)

Prof Paul Savelkoul (University of Maastricht, The Netherlands) was the first presenter for the main topic of the second day, namely microbiomics. Prof Savelkoul clearly and concisely introduced this rapidly advancing field (see Ref. [26] for an excellent review), highlighting how these advances are changing our understanding of the microbial–host interactions (for example see Ref. [27]). Prof. Savelkoul proceeded in describing the general microbiome workflow, emphasizing the points of difference when compared to other NGS workflows, while also highlighting recent applied microbiome-based research findings (see for example [28] where the gut microbiome was analysed with association to therapeutics, and antibiotics usage [29]; also see Ref. [30] where the potential use of the gut microbiome as a marker for inflammatory bowel disease was investigated). The use of mapping the gut microbiome to understand the efficacy of faecal microbiota transplantation was also presented [31] demonstrating how rapidly and broadly the field of microbiome metagenomics is advancing and influencing.

Following Prof. Savelkoul's informative presentation, a number of microbiome/metagenomics applications in different microbiology fields were presented. Dr. Maria Luisa Ricci (Istituto Superiore di Sanita, Italy) presented applied microbiome-based studies for the detection and surveillance of *Legionella* in urban water systems, providing an excellent background and overview of the issues faced (see Ref. [32]), while highlighting recent findings (for example see Ref. [33]) and sharing experiences for a current ESCMID-funded research project on water microbiome. Dr Eddie Cytryn (Volcani Center, Israel) presented applications of both microbiomics and metagenomics in environmental surveillance of resistance, specifically source tracking resistance associated mobile gene cassettes along the sludge-effluent-soil-crop continuum (see Ref. [34]). Dr Amparo Fernandez Rodriguez (National Forensic Institute, Spain) presented the application of microbiomics in forensic microbiology, for example, post-mortem investigations (including possible cause of death) amongst others (including crime scene investigations). From their preliminary studies, they successfully identified 6 out of the 7 pathogens identified through standard procedures in infectious cause of death. Also, Dr Fernandez Rodriguez presented more unpublished results of a rape suspect identified by microbiome analysis, where traditional methods failed. (see for more examples [35]), all of which demonstrated great promise despite the legal and QC challenges that still lay ahead for forensic microbiology application.

8. Interactive discussion

8.1. Interpretation of NGS findings – the clinical point of view

Following the applications of microbiomics/metagenomics presentations, an interactive discussion moderated by Prof Harmsen was undertaken on the topic of interpretation of NGS findings from a clinical perspective. The underlying question that Prof Harmsen drew to attention was “how close is close enough?”, meaning at what cut off value can one consider in phylogenomic results to be of clinical or epidemiological importance? The discussion highlighted some rules of thumb (such as WGS is best for ruling out majority of isolates and cut-off values should never be absolutely applied and concluded that one will always need epidemiological meta-data in order to rule in or out an infection control decision. Cut off confounders were also discussed, with Prof Harmsen providing a number of case examples (see Refs. [36,37]).

8.2. Quality issues in implementing new technology in the micro lab

Another interactive discussion, moderated by Dr. Claas, considered the quality issues in implementing new technology in the microbiological lab. With WGS based bacterial typing, metagenomic detection and analysis of microbiota becoming more a part of the routine workflows in the microbiological lab, the question was if ISO accreditation is currently possible or acceptable, looking at public health and clinical microbiology? particularly since there are not much guidelines available for microbiological procedures, while also much of the NGS services are outsourced, and most importantly, what is considered to be a gold standard for NGS?

9. Bioinformatics

On the third and final day, Dr Carriço was given the opportunity to present the bioinformatics component of NGS (see Ref. [38] for

an encompassing review). In his first presentation, Dr Carriço focused on describing and highlighting the application of available databases and data processing work flows in NGS related work. The second presentation highlighted the applicability of graphical representation of WGS analysis, introducing the popular methods and software commonly implemented in the NGS analysis workflows and pipelines. His presentations involved demonstrations of different tools, notably software emerging from his research groups such as PHYLOViZ (http://www.phyloviz.net/).

10. I have sequenced a bacterial genome – what is next?

In the end of all the presentations, time was given to participants to ask questions in relation to NGS analysis. Questions included "what are the specifications of a good local computer for NGS analysis?", "what to do if phenotype doesn't match genotype?", data security methods, Dr Carriço was also asked "what is your favourite toolbox of software pipelines?". The topics were actively discussed with engagement of many of the faculty members.

11. Considerations in publication of NGS-based studies

Finally, Dr Ines Steffens (European Centre for Disease Prevention and Control) presented an Editor-in-chief's perspective on the considerations in publication of NGS-based studies. Dr Steffens discussed the importance of using a variety of metrics to measure the impact of a journal, and Eurosurveillance's interest in vision and proof of concept papers. To increase the chances of successfully publishing, Dr Steffens recommended that before submission, one selects an appropriate journal, with a fitting scope, good reputation, and an impact that will enhance the published work (should not only consider impact factors, but also dissemination and target audiences), while upon submission, one should make sure that all formal requirements are met, and should try to draw the editor's attention (using an informative title, highlight importance of findings in the abstract, and using the covering letter to the editor to add value and argue the case).

12. Conclusion

This capacity-building workshop was a very successful event in a series of courses and workshops organised by ESGMD under ESCMID. During 2018 and 2019, ESGMD plans to continue delivering a range of capacity-building activities to underpin the integration of new technologies in clinical and public health microbiology. Such notable courses include a practical hands-on course the will take place in Lausanne, Switzerland in September 2018 and a workshop dedicated to hands on shotgun metagenomics that will take place in Groningen, The Netherlands, in October 2018. Course participants expressed the need for capacity-building events that involve practical bioinformatics. One such course took place in October 2017 in Freiburg Germany, co-organised by ESGEM and ESGMD and the Study Group plan to such hands on computational workshops for 2019.

Conflict of interest

Authors declare no conflict of interest.

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