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Key considerations for the implementation of high throughput sequencing based metagenomics (mNGS) in diagnostic clinical, food and veterinary labs : a no-nonsense pointer.

the Metastava consortium*

* Steven Van Borm, Frank Vandenbussche, Kris De Clercq, Andy Haegeman, Sigrid De Keersmaecker, Kevin Vanneste, Michael Peeters, Steven Van Gucht, Yannick Blanchard, Nicole Pavio, Sandra Martin-Latil, Fabrice Touzain, Isabelle Kempf, Lihong Liu, Mickhayil Hakhverdyan, Mikael Leijon, Alex Bossers, Wim van der Poel, Marcel Hulst, Bas Oude Munnink, Miranda de Graaf, Sander van Boheemen, Marion Koopmans, Martin Beer, Anne Pohlmann, Dirk Höper.

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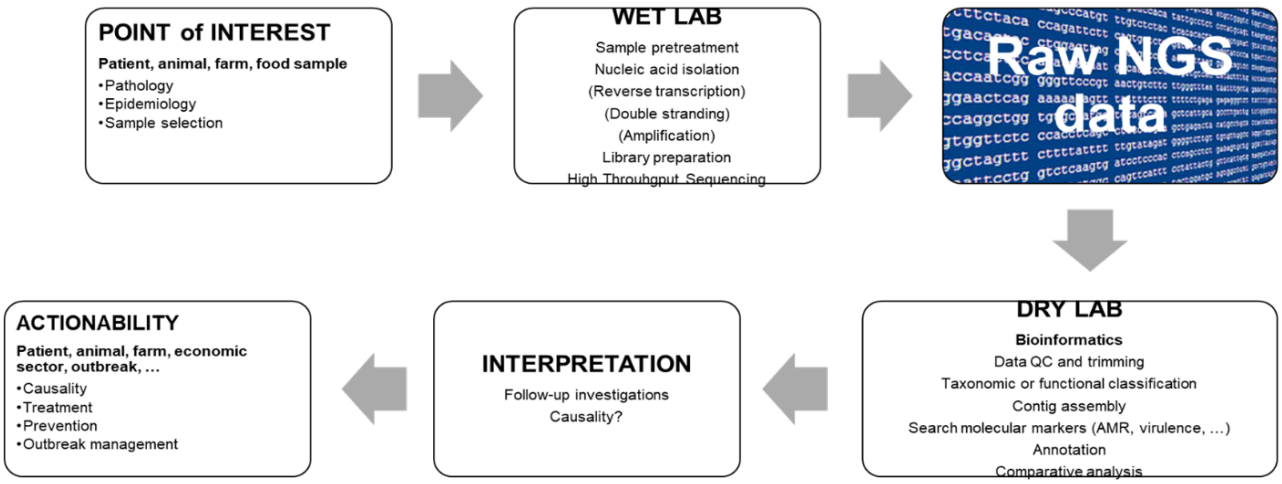
1. Introduction

Metagenomics, here defined as random access nucleic acid high throughput sequencing for unbiased identification and characterization of pathogens in a sample of interest, is a booming field in human, veterinary and food microbiology since the commercialization of high-throughput sequencing platforms. Its translation to the diagnostic microbiology laboratory for the hypothesis-free identification of pathogens – diagnostic metagenomics - is currently ongoing. It offers considerable added diagnostic value when targeted assay panels are inconclusive and when no isolate can be obtained from the sample for further characterization. We refer to metagenomic NGS (mNGS) as (diagnostic) metagenomics through high-throughput sequencing methodologies. A typical metagenomics workflow (figure 1) spans expertise fields including pathology, epidemiology, molecular biology, and bioinformatics, complicating an informed metagenomics experimental design.

Figure 1. A typical mNGS workflow



METASTAVA (https://onehealthejp.eu/jrp-metastava/) is a small transnational research project were European partner labs from the public med/vet/food sector investigate key validation aspects of diagnostic metagenomics. These include analytical sensitivity, repeatability and robustness, implementation of quality controls, and standardization of methods for data generation and data analysis. A large number of high quality review papers have recently addressed the promises and current hurdles of diagnostic metagenomics, and several research groups and consortia have addressed aspects of method standardization and workflow validation. In the context of this rich research and development landscape, this document seeks to help labs envisaging a diagnostic use of metagenomic sequencing by pointing them to information resources we find particularly relevant. It is by no means intended as yet another inclusive review on diagnostic metagenomics, but rather as a pointer document to information we find particularly relevant to the field. In addition we provide some guidelines for the informed design of metagenomic experiments as we strongly believe an informed and critical (i.e. scientific) reflection about all workflow steps involved in diagnostic metagenomics is important.

2. Research initiatives addressing diagnostic metagenomics & guidelines/standards that may be relevant to the field.

Research on diagnostic metagenomics is plentiful. Great expert teams, projects and workgroups worldwide are working on the standardization of methods, validation and reporting guidelines and standards, etc. Even beyond work focusing on mNGS, some guidelines and standards from other clinical application fields of NGS are relevant e.g. when envisaging QC parameters for NGS experiments in general.

Initiative	Description	Links
METAGENOMICS SPECIFIC INITIATIVES		
H2020-COMPARE Research project	Large multidisciplinary research network on the standardization of strategies and methodologies of NGS, including mNGS and other sequence based pathogen characterization methods with a focus on delivering actionable results. Of particular interest is their library with protocols and SOPs	https://www.compare-europe.eu https://www.compare-europe.eu/library/protocols-and-sops
EFFORT against antimicrobial resistance Research project	Finished Project (2018) studying the complex ecology of antimicrobial resistance in animals, the food chain and the environment. It includes standardization of mNGS methodologies. Interesting position paper on mNGS based AMR detection in swine herds	http://www.effort-against-amr.eu/ http://www.effort-against-amr.eu/page/publications.php https://pubmed.ncbi.nlm.nih.gov/28115502 https://mmp.sfb.uit.no/ https://doi.org/10.1093/gigascience/gix047

Global Microbial Identifier Consortium	Global initiative focusing on developing curated whole genome databases for the identification of microorganisms to detect outbreaks and emerging pathogens. Includes a working group on laboratory and bioinformatic analytical approaches	https://www.globalmicrobialidentifier.org/ https://www.globalmicrobialidentifier.org/workgroups#work-group-3
STROBE-metagenomics reporting guidelines Consortium	Multidisciplinary, international expert group to establish reporting guidelines that address specimen processing, nucleic acid extraction, sequencing platforms, bioinformatics considerations, quality assurance, limits of detection, power and sample size, confirmatory testing, causality criteria, cost, and ethical issues	https://pubmed.ncbi.nlm.nih.gov/32768390/
Microbiome quality control consortium Consortium	Collaborative effort to comprehensively evaluate methods for measuring the human microbiome	https://www.mbqc.org/
NIST NGS Standards Governmental Organisation	NIST (National Institute of Standards and Technology) workgroup focusing on the use of metagenomics for viral adventitious agent detection in biologics	https://www.nist.gov/news-events/events/2019/09/workshop-standards-ngs-detection-viral-adventitious-agents-biologics-and
Advanced Virus Detection Technologies Interest Group (AVDTIG): Efforts for High Throughput Sequencing (HTS) for Virus Detection International Organisation	International association for biological standardization (IABS) workgroup on HTS for virus detection	https://www.ncbi.nlm.nih.gov/pubmed/27593693
FP7-International Human Microbiome Standards Project (IHMS) Research project	IHMS coordinated the development of standard operating procedures designed to optimize data quality and comparability in the human microbiome field	www.microbiome-standards.org
Elixir Marine Metagenomics community Research project	Elixir provides a range of tools including benchmark datasets for taxonomic classification that are broadly applicable also to diagnostic metagenomics	https://elixir-europe.org/communities/marine-metagenomics
CAMI (Critical Assessment of Metagenome Interpretation) Consortium	focuses on taxonomic classification by providing benchmark datasets. Includes some datasets for clinical pathogens.	https://data.cami-challenge.org/)

GENERAL GUIDELINES and GUIDELINES FROM OTHER NGS APPLICATION FIELDS		
DECATHLON guidelines minimum performance parameters NGS Research project	Resulting from an EU project, guidelines for the development of cost efficient advanced DNA-based methods for specific traceability issues and high level on-site applications, mostly related to targeted sequencing in the field of GMO, food pathogens and customs issues, not metagenomics	https://cordis.europa.eu/project/rcn/111292/reporting/en
ISO/DIS 23418 'Microbiology of the food chain - Whole genome sequencing for typing and genomic characterization of foodborne bacteria - General requirements and guidance' International Organisation	ISO norm on whole genome sequencing of isolates in food microbiology. Includes a chapter on validation. Some quality assurance aspects are relevant for mNGS	https://www.iso.org/standard/75509.html
OIE standards HTS, bioinfo & computational genomics chapter 1,1,7 International Organisation	World Animal Health Organisation (OIE) guidelines on NGS use. Animal health focus	http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.07_HTS_BGC.pdf
FDA guidance for next generation sequencing-based testing: balancing regulation and innovation in precision medicine Governmental Organisation	Human health focus (clinical care)	https://www.nature.com/articles/s41525-018-0067-2
FDA: Infectious Disease Next Generation Sequencing Based Diagnostic Devices: Microbial Identification and Detection of Antimicrobial Resistance and Virulence Markers Governmental Organisation	Recommendations for studies to establish the analytical and clinical performance characteristics of Infectious Disease Next Generation Sequencing Based Diagnostic Devices for Microbial Identification and Detection of Antimicrobial Resistance and Virulence Markers	https://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM500441.pdf
FDA: Considerations for Design, Development, and Analytical Validation of Next Generation Sequencing (NGS) – Based In Vitro Diagnostics (IVDs) Intended to Aid in the Diagnosis of Suspected Germline Diseases Governmental Organisation	Focus on human genomics (hereditary disease), Guidance for Stakeholders and Food and Drug Administration Staff	https://www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm509838.pdf
Association for Molecular Pathology and the College of American Pathologists. Standards and Guidelines for Validating Next-Generation Sequencing Bioinformatics Pipelines Consortium	Focus on human genomics (hereditary disease)	https://jmd.amjpathol.org/article/S1525-1578(17)30373-2/fulltext

EuroGenTest. Guidelines for diagnostic next-generation sequencing. Focus genetic disorders Research project	Guidelines for the evaluation and validation of next-generation sequencing (NGS) applications for the diagnosis of genetic disorders. Focus on human genomics (hereditary disease)	http://www.eurogentest.org/index.php?id=214&tx_ttnews%5Btt_news%5D=179&cHash=449e71664700b0bd827e4574db021cf6 https://www.nature.com/articles/ejhg2015226
Minimum Information about any (x) Sequence (MIxS) Consortium	Developed by the Genomic Standards Consortium (GSC). A unified standard for describing sequence data and to provide a single point of entry for the scientific community to access and learn about GSC checklists. MIxS currently consists of three separate checklists; MIGS for genomes, MIMS for metagenomes, and MIMARKS for marker genes.	https://gensc.org/mixs/ https://www.nature.com/articles/nbt.1823
Minimum Information About an Uncultivated Virus Genome (MIUViG) Consortium	Extension of MIxS standard for reporting sequences of uncultivated virus genomes. MIUViG standards developed within the Genomic Standards Consortium framework and include virus origin, genome quality, genome annotation, taxonomic classification, biogeographic distribution and in silico host prediction, to improve the reporting of uncultivated virus genomes in public databases.	https://www.ncbi.nlm.nih.gov/pubmed/30556814
Euformatics 2017. Worldwide overview guidelines, genetics Company	Interesting pointer compiled by clinical bioinformatics and data management company Euformatics to worldwide NGS standards and guidelines (mostly in genetics)	https://euformatics.com/evolving-standards-in-clinical-ngs/?cn-reloaded=1
JRC working group "Benchmarking of NGS bioinformatics pipelines for AMR " Consortium		https://f1000research.com/articles/7-459/v2

3. Absolutely fabulous review papers on mNGS

Excellent recent reviews point out limitations and unique strengths of the mNGS approach. Although being by design less cost-effective and sensitive than targeted approaches, the untargeted and hypothesis- and culture-free mNGS approach provides groundbreaking added value with major advantages for pathogen discovery, characterization of multiple infections, and diagnosis beyond pre-defined testing panels.

We're all very enthusiastic about novel technology and the potential it has to move the boundaries of our research and eventually diagnostic reach. For sure, mNGS is currently already a highly informative complementary tool to the targeted assay panels available to the diagnostic lab. Application fields will

grow. We stress the ongoing debate and the fact that diagnosticians and researchers considering using metagenomics should critically envisage the scope of the application intended as it has consequences for experimental design and bioinformatics data analysis workflow (sampling, lab, analysis; the correct interpretation of findings; and the correct follow up actions on metagenomics results). As carefully laid out by Greniger 2018 (https://pubmed.ncbi.nlm.nih.gov/29898605) for metagenomics in a clinical lab/healthcare setting, we must have the courage to consider these exciting tools against a wider set of arguments including healthcare economics and actionability of results. Another recent review (https://pubmed-ncbi-nlm-nih-gov.vdicp.health.fgov.be/33141913/) stated that although metagenomics is increasingly reported in diverse clinical studies, the proportion of patient cases with positive clinical impact of mNGS testing remains low to date. The road beyond the current great added value to targeted assays towards first line diagnostic assays is wide open, but signposted with hard work to be done on the hurdles listed in panel 1.

Panel 1: Major hurdles to the implementation of mNGS based tests as first-line diagnostic assays as outlined by must read reviews on diagnostic/clinical mNGS.

- Technical and methodological aspect (error, bias, analytical properties, etc.)
- Cost currently several orders of magnitude higher than for targeted assays (although serially executing a panel of targeted assays is also labor and cost demanding).
- Regulatory aspects and cost of diagnostic validation.
- Legal and ethical considerations
- Actionability of results (i.e. how do they lead to improved patient care or disease management?), including turnaround time and the economy of the healthcare or veterinary system.

We found the below listed review papers of particular interest to reflect the ongoing debate and the critical areas that should be focused on in the reflection towards future (first line?) mNGS based diagnostic assays.

Relevant review papers	link	Title
Andersen & Hoorfar 2019	https://pubmed.ncbi.nlm.nih.gov/29300319/	Surveillance of Foodborne Pathogens: Towards Diagnostic Metagenomics of Fecal Samples
Andersen et al 2017	https://pubmed.ncbi.nlm.nih.gov/28595575	Towards Diagnostic Metagenomics of Campylobacter in Fecal Samples
Carleton et al 2019	https://pubmed.ncbi.nlm.nih.gov/31170005/	Metagenomic Approaches for Public Health Surveillance of Foodborne Infections: Opportunities and Challenges
Chiang and Dekker, 2020	https://pubmed.ncbi.nlm.nih.gov/31538184/	From the Pipeline to the Bedside: Advances and Challenges in Clinical Metagenomics
Chiu and Miller 2019	https://pubmed.ncbi.nlm.nih.gov/30918369	Clinical Metagenomics
Filkins et al 2020	https://pubmed.ncbi.nlm.nih.gov/33141913/	Navigating Clinical Utilization of Direct-from-Specimen Metagenomic Pathogen Detection: Clinical Applications, Limitations, and Testing Recommendations
Forbes et al 2017	https://doi.org/10.3389/fmicb.2017.01069	Metagenomics: The Next Culture-Independent Game Changer

Goldberg et al 2016 American Academy of Microbiology (AAM)	https://mbio.asm.org/content/6/6/e01888-15	Making the Leap from Research Laboratory to Clinic: Challenges and Opportunities for Next-Generation Sequencing in Infectious Disease Diagnostics
Greninger 2018a	https://pubmed.ncbi.nlm.nih.gov/29898605	The Challenge of Diagnostic Metagenomics
Greninger 2018b	https://pubmed.ncbi.nlm.nih.gov/29055712	A Decade of RNA Virus Metagenomics Is (Not) Enough
Gu et al 2019	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6345613/	Clinical Metagenomic Next-Generation Sequencing for Pathogen Detection
Höper et al 2016	https://pubmed.ncbi.nlm.nih.gov/27217170	Metagenomic Approaches to Identifying Infectious Agents
Höper et al 2017	https://pubmed.ncbi.nlm.nih.gov/29029726	Loeffler 4.0: Diagnostic Metagenomics
Kozyreva et al 2017	https://pubmed.ncbi.nlm.nih.gov/28592550	Validation and Implementation of Clinical Laboratory Improvements Act-Compliant Whole-Genome Sequencing in the Public Health Microbiology Laboratory
Miao et al 2018	https://pubmed.ncbi.nlm.nih.gov/30423048	Microbiological Diagnostic Performance of Metagenomic Next-generation Sequencing When Applied to Clinical Practice
Miller et al 2019	https://pubmed.ncbi.nlm.nih.gov/30992304	Laboratory Validation of a Clinical Metagenomic Sequencing Assay for Pathogen Detection in Cerebrospinal Fluid
Nooij et al 2018	https://www.frontiersin.org/articles/10.3389/fmicb.2018.00749/full	Overview of Virus Metagenomic Classification Methods and Their Biological Applications
Olson et al 2019	https://pubmed.ncbi.nlm.nih.gov/28968737/	assembly through the lens of validation: recent advances in assessing and improving the quality of genomes assembled from metagenomes
Oulas et al 2015	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4426941/	Metagenomics: Tools and Insights for Analyzing Next-Generation Sequencing Data Derived from Biodiversity Studies
Quince et al 2017	https://pubmed.ncbi.nlm.nih.gov/28898207/	Shotgun metagenomics, from sampling to analysis
Rossen, Friedrich and Moran-Gilad 2017	https://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(17)30630-4/fulltext	Practical issues in implementing whole-genome-sequencing in routine diagnostic microbiology
Roux et al 2017	https://peerj.com/articles/3817/	Benchmarking viromics: an in silico evaluation of metagenome-enabled estimates of viral community composition and diversity
Ruppé & Schrenzel 2019	https://pubmed.ncbi.nlm.nih.gov/30836173	Messages From the Third International Conference on Clinical Metagenomics (ICCMg3)

Schlaberg et al 2017	https://pubmed.ncbi.nlm.nih.gov/28169558	Validation of Metagenomic Next-Generation Sequencing Tests for Universal Pathogen Detection
Sekse et al 2017	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5655695/	High Throughput Sequencing for Detection of Foodborne Pathogens
Van Borm et al 2016	https://pubmed.ncbi.nlm.nih.gov/27217169	Next-generation Sequencing Workflows in Veterinary Infection Biology: Towards Validation and Quality Assurance
Kwok et al 2020	https://www.mdpi.com/1999-4915/12/1/107/	Systematic review on viral metagenomics in farm animals
Ten Hoopen P et al 2017	https://pubmed.ncbi.nlm.nih.gov/28637310/	The metagenomic data life-cycle: standards and best practices
Lambert D et al 2019	https://doi.org/10.5740/jaoacint.16-0269.	Baseline Practices for the Application of Genomic Data Supporting Regulatory Food Safety

4. Guidelines for informed mNGS experimental design.

As with all diagnostic assays, a scientifically correct use of mNGS requires informed and critical reflection on the suitability of methodologies for all steps involved in the diagnostic workflow starting with the selection of suitable samples and methods down to the correct interpretation of results in view of known limitations of the methodology. Taking inspiration from the excellent research efforts and review papers above, we believe that this critical reflection should investigate the following fields:

SCOPE:

Each metagenomics approach should start with asking the following questions. What do we want to learn from our patient (whether human or animal) or sample? What taxonomic resolution are we aiming at (Genus? Species? Strain? Detection of AMR or virulence markers? Even specific single nucleotide polymorphisms?)? What actionable result do we envisage? What samples are suitable (e.g. anticipated target concentration)? What timeline is critical for a result to still be actionable? Can targeted methods or multiplex panels reach the same result? These reflections should lead to an **informed decision on fit-for-purpose metagenomics project design**.

METHODS FOLLOW SCOPE:

Are there any specific sample matrix requirements? Do we need to target viruses, bacteria or all microorganisms? What samples do we include and do we include a paired control group? Select fit-for-purpose methods for sample pretreatment, nucleic acid extraction, reverse transcription/double stranding, library preparation, and sequencing (what platform suits the question? What amount of sequencing data is needed?... e.g. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0160334). What raw data filtration and data analysis methods are suitable? With regards to data analysis: what databases (=balanced decision between curation status of sequence data and representation of genetic variation of target organisms) and bioinformatics methods (=tradeoff between more exhaustive methods that perform more analytical steps but may take days to week to run versus greedy approaches that are less performant but can be completed in a much quicker timeframe) are suitable? How to consider known biases such as the effect of low complexity sequence information (e.g. polyA or polyC stretches in viral genomes) and how these may affect sensitivity and specificity of the read classification or

assembly approach? How to integrate regulatory/ethics/legal considerations (e.g. requiring the removal of reads mapping to the human genome) in the approach?

QUALITY ASSURANCE: Validation is a prerequisite of a diagnostic assay. We refer to the above sections for relevant information and review papers. Currently, analytical and diagnostic validation data of mNGS methods is scarce. However, it is important to collect some information at least on analytical sensitivity and specificity. Currently, follow-up investigations of positive mNGS findings are needed (see under Interpretation), while the interpretation of negative mNGS findings is often difficult in lack of suitable validation data. Some examples of clinical mNGS validation approaches can be found in https://pubmed.ncbi.nlm.nih.gov/28169558 .

In a wider frame, quality assurance of metagenomic assay results not only includes analytical validation, but also the wider quality control (QC) environment including proper management of procedures, staff and equipment, adequate use of internal and/or external controls, proper documentation of key performance indicators, and reproducibility (including interlaboratory reproducibility testing). Specific metagenomics quality assurance guidelines and metrics do not always exist, but inspiration for intermediate outputs in your workflow (e.g. raw data quality from sequencer, library DNA QC, assembly quality) can of course be found in other NGS applications fields more focused on targeted sequencing.

We believe **monitoring** the following **metrics** can be useful for the quality assurance of mNGS findings:

1. Sampling selection and metadata: should be adequate, traceable, include paired controls where possible, etc. Repeated sampling or increased sample numbers may in some instances increase the sensitivity of the investigation. Sample metadata should include as much detail as possible (but considering legal/ethical constringency) including a clear description of sample type, date, geographical information, host type (species, breed, age, sex), relevant pathological information, additional epidemiological information, etc.
2. Selection and documentation of a suitable 'wet lab' workflow (= for generating the mNGS raw data), with tracing of wetlab QC parameters like:
    a. Documentation of any pretreatment of the sample
    b. Nucleic acid extraction methodology and QC (quantity, purity)
    c. Documentation of measures to avoid false positive and false negatives. These include positive and negative process controls, external spike-in controls (e.g. https://pubmed.ncbi.nlm.nih.gov/30373528 , https://pubmed.ncbi.nlm.nih.gov/32574649/) and their sequencing metrics. Of note, normal ranges (e.g. normalised read counts of a spike-in control) should be evaluated during the method implementation and validation.
    d. Sequencing effort per sample library QC and quantification (including size distribution)
    e. Sequencing platform output metrics, again balanced against predefined acceptability criteria.
        i. Read length & number of reads produced per sample (for raw vs trimmed datasets)
        ii. Cluster density (for Illumina sequencing)
        iii. GC content (although expected to be variable with a hypothesis free method like mNGS)
        iv. Sequencing chemistry spike-in controls like PhiX (Illumina)

f. Proper control of personnel training, QC of critical equipment, and protocol documentation.
g. Monitoring of the reproducibility of the assay, including within-lab reproducibility (e.g. evaluating repeated outputs of process controls) and interlaboratory reproducibility. The later can be assessed using interlaboratory proficiency testing which can include both wet lab (e.g. https://pubmed.ncbi.nlm.nih.gov/28932815/) and dry lab (e.g. https://pubmed.ncbi.nlm.nih.gov/31167846) aspects.

3. Selection and documentation of suitable Bioinformatics ('dry lab') workflows with documentation of QC metrics
    a. Dataset QC metrics
    b. Traceability and suitability of databases used
    c. Traceability of bioinformatics methods (tools, versions options and parameters)
    d. Trimming and filtering metrics
    e. Classification metrics and assembly metrics
    f. Proper control of personnel training, QC of IT infrastructure, and protocol documentation (workflow diagrams, end user documentation, technical documentation for developers).
    g. (Semi-)automation of (modular) workflows to reduce operator-introduced errors.
    h. Adherence to general 'best practises' for software development (code versioning, code review, basic testing…)
    i. FAIR (Findable - Accessible - Interoperable –Reusable) data management. Too many datasets from metagenomic studies are simply inaccessible as e.g. shown in a recent systematic review on viral metagenomics in farm animals (https://www.mdpi.com/1999-4915/12/1/107/).

INTERPRETATION:

Interpretation of metagenomic findings should take into account the known and potential biases and errors associated with the wet lab (enrichment, NA isolation, amplification, library prep, sequencing) and dry lab (algorithm, database) workflows.

The significance of metagenomics findings (candidate sequence hits from bioinformatic pipelines) should be evaluated against:

1. Suitability of sampling scheme
2. Suitability of workflow (both wet lab and dry lab). e.g. critical evaluation of results in view of known mNGS biases such as index jumping, cross-contamination, database limitations, bias introduced by low-complexity sequence regions, etc.
3. Suitability of QC measures
4. Validity of process control (and external control) results
5. Classification and assembly support. Intuitively we know a large number of mNGS reads identifying a certain taxon is of more significance than a single

hit. In general the following considerations should be taken into account to reflect on significance.

a. Number of reads. Normalised read numbers (corrected for the sequencing effort per sample) should be used when comparing samples. Especially low read numbers should be critically investigated against wet lab (index jump, contamination, …) and dry lab (database bias, low complexity regions, …) biases and errors.
b. Contig length when *de novo* assembling hit sequences.
c. Genome coverage of the taxon hit. High read counts in combination with Lander-Waterman complying genome coverage are more supportive.
d. Identity of hit/query (nucleotide or amino acid level similarity, query coverage, etc.)
e. Functional ORF prediction in the contig.
f. Nucleotide composition analysis can support a host-matched evolutionary history of the pathogen providing suitable reference data (e.g. https://pubmed-ncbi-nlm-nih-gov.vdicp.health.fgov.be/28335731/ ; https://pubmed-ncbi-nlm-nih-gov.vdicp.health.fgov.be/29442227/). A host-matched evolutionary history is suggestive of replication in that host, as opposed to detection of a non-replicating passenger (e.g. plant virus in a fecal sample may suggest a food origin).

6. If available, analytical validation data for relevant target/sample matrix combinations for the exact workflow (wet & drylab) should be taken into account to interpret the predictive value of the findings.

FOLLOW-UP:

Potential follow-up investigations are outlined in this section. Some investigations are only relevant for new pathogen discovery or R&D and cannot be realised in a diagnostic context due to time constraints. However, these strategies can drastically increase the support for a hypothesised causative relation between initial nucleic acid signature findings and pathologies.

1. Confirm the presence of the sequence signature in original samples and extended affected population through additional sampling
    a. Quantitative (RT)PCR or digital PCR (dPCR) can be developed to confirm and quantify the sequence signature in the original sample
    b. Re-sampling the affected population, PCR screening can investigate a correlation between the prevalence of the signature and epidemiological/pathological information.
2. Pathogen confirmation
    a. Detect with Antibodies (requires pre-knowledge of pathogen)
    b. Electron microscopy (requires high pathogen load)
    c. Co-localisation with pathology (in situ EM /microscopy. Antibody or probe based technologies exist)

3. Strategies to confirm the presence of live pathogens (as opposed to inert genome sequence) include
    a. Virus isolation and infectivity assays. However, a workable isolation *in vitro* or *in vivo* system is rarely available in pathogen discovery.
    b. Stranded RNAseq or Novel synthesised RNAseq can confirm the presence of Nucleic acid types correlated with replicating virus/pathogen (e.g. https://pubmed-ncbi-nlm-nih-gov.vdicp.health.fgov.be/32660862/ ; https://pubmed-ncbi-nlm-nih-gov.vdicp.health.fgov.be/31167947/ ; https://pubmed-ncbi-nlm-nih-gov.vdicp.health.fgov.be/30992161/; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6553555/)
    c. Targeted (semi-) quantitative assays (qPCR, dPCR, NGS) can be used to measure growth in host cells (target quantity comparison between time points)
    d. And finally, the isolated pathogen (when possible) can be used to biologically characterise the virus, and attempt to fulfil Koch's postulates (but see https://cmr.asm.org/content/cmr/9/1/18.full.pdf ; https://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(16)30048-9/fulltext).

5. Conclusions.

Metagenomic sequencing is a booming field, both due to the continuing evolution of sequencing technologies, thereby enabling new approaches, and due to an impressive (collaborative) ongoing research effort in various application fields. No doubt that mNGS has gained an important role in diagnostic labs in areas where targeted assays fail (pathogen discovery, unexpected pathogens not reflected in routine test panels, etc.). However, certain aspects of diagnostic assay validation are currently less accessible for mNGS approaches due to the costs associated as well as the hypothesis-free nature of the method (e.g. how to define the "analyte" or test target in a completely random assay? What reference material to use for validation if your test aims at detecting…everything?). For the specific context of zoonotic and foodborne pathogens (including hepatitis E virus, Norovirus, zoonotic *Poxviridae*, STEC and ABR genes), the Metastava project provided exploratory analytical validation data, data on methodology reproducibility, exogenous spike-in control materials for metagenomics, and data generation and analysis methods. Building from this experience, we argue for a rational design of metagenomic studies, incorporating smart QC metrics and QA approaches to minimize technical uncertainties, as well as an informed and critical interpretation of mNGS results. We hope to have provided the aspirant mNGS diagnostician with food for critical reflection, reference to existing guidelines and ongoing research efforts, and common sense guidelines for an informed mNGS experimental design from diagnostic or research question to interpretation of the results. We strongly believe that any mNGS study should start with a bunch of critical questions to pair scope and method, and use common sense to keep track of all uncertainties during the process to be able to give a scientific interpretation of the outcome. That's what diagnostic assay development is all about, right?