Validation and Implementation of CLIA-Compliant Whole Genome Sequencing (WGS) in Public Health Laboratory

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Abstract

Background

Public health microbiology laboratories (PHL) are at the cusp of unprecedented improvements in pathogen identification, antibiotic resistance detection, and outbreak investigation by using whole genome sequencing (WGS). However, considerable challenges remain due to the lack of common standards.

Objectives

1) Establish the performance specifications of WGS applications used in PHL to conform with CLIA (Clinical Laboratory Improvements Act) guidelines for laboratory developed tests (LDT), 2) Develop quality assurance (QA) and quality control (QC) measures, 3) Establish reporting language for end users with or without WGS expertise, 4) Create a validation set of microorganisms to be used for future validations of WGS platforms and multi-laboratory comparisons and, 5) Create modular templates for the validation of different sequencing platforms.

Methods

MiSeq Sequencer and Illumina chemistry (Illumina, Inc.) were used to generate genomes for 34 bacterial isolates with genome sizes from 1.8 to 4.7 Mb and wide range of GC content (32.1%-66.1%). A customized CLCbio Genomics Workbench - shell script bioinformatics pipeline was used for the data analysis.

Results

We developed a validation panel comprising ten *Enterobacteriaceae* isolates, five gram-positive cocci, five gram-negative non-fermenting species, nine *Mycobacterium tuberculosis*, and five miscellaneous bacteria; the set represented typical workflow in the PHL. The accuracy of MiSeq platform for individual base calling was >99.9% with similar results shown for reproducibility/repeatability of genome-wide base calling. The accuracy of phylogenetic analysis was 100%. The specificity and sensitivity inferred from MLST and genotyping tests were 100%. A test report format was developed for the end users with and without WGS knowledge.

Conclusion

WGS was validated for routine use in PHL according to CLIA guidelines for LDTs. The validation panel, sequencing analytics, and raw sequences will be available for future multi-laboratory comparisons of WGS in PHL. Additionally, the WGS performance specifications and modular validation template are likely to be adaptable for the validation of other platforms and reagents kits.

Introduction

Clinical and public health microbiology laboratories are undergoing transformative changes with the adoption of whole genome sequencing (WGS) [1, 2]. For several years, leading laboratories have published proof-of-concept studies on WGS-enabled advances in the identification of pathogens, antibiotic resistance detection, and disease outbreak investigations [3-6]. The technologies also referred to as next generation sequencing (NGS) have yielded more detailed information about the microbial features than was possible using a combination of other laboratory approaches. Further developments of WGS platforms had allowed remarkable in-depth inquiry of pathogenic genomes for the discovery of genetic variants and genome rearrangements that could have been missed using other DNA methods [3, 7, 8]. The enhanced investigations of disease outbreaks have led to new understanding of transmission routes of infectious agents [9-11]. WGS-enabled metagenomics and microbiome discoveries have revealed a new appreciation for the role of microbes in health and disease [12-15]. The innovations are continuing at such an unprecedented pace that WGS is expected to become an alternative to culture-dependent approaches in the clinical and public microbiology laboratories [16-18].

Notwithstanding its promises, several challenges remain for the adoption of WGS in microbiology laboratories [19-22]. The accelerated obsolescence of the sequencing platforms presents several obstacles in bridging the gap between research and routine diagnostics including standardizations efforts [23]. The downstream bioinformatics pipelines are also unique challenges for the microbiology laboratory both in terms of infrastructure and skilled operators [24-27]. Overall, WGS ‘wet bench-dry bench' workflow represents an integrated process, which is not easily amenable to the traditional quality metrics used by the microbiology laboratories [27-29]. The capital investments and recurring costs of WGS for clinical laboratories although rapidly declining still remain relatively high to allow multi-laboratory comparisons for the standardization of the analytical parameters. Finally, the regulatory agencies have not yet proposed WGS standard guidelines for the clinical microbiology [30], and external proficiency testing programs are still in development for the clinical and public health microbiology laboratories [31, 32].

There are other notable recent developments towards standardization and validation of next generation sequencing in clinical laboratories. The US Centers for Disease Control and Prevention (CDC) sponsored the Next-generation Sequencing: Standardization of Clinical Testing (Nex-StoCT) workgroup to propose quality laboratory practices for the detection of DNA sequence variations associated with heritable human disorders [33, 34]. The workgroup developed principles and guidelines for test validation, quality control, proficiency testing, and reference materials. Although not focused on infectious diseases, these guidelines provide a valuable roadmap for the implementation of WGS in clinical microbiology and public health laboratories. The College of American Pathologists' (CAP) published eighteen requirements in an accreditation checklist for the next generation sequencing analytic (‘wet bench') and bioinformatics (‘dry bench') processes as part of its' molecular pathology checklist [30]. These ‘foundational' accreditation requirements were designed to be broadly applicable to the testing of inheritable disorders, molecular oncology, and infectious diseases. Along the same lines, the feasibility of *in silico* proficiency testing has been demonstrated for NGS [35]. Clinical and Laboratory Standards Institute (CLSI) has updated its’ “Nucleic acid sequencing methods in diagnostic laboratory medicine” guidelines with considerations specific to the application of next generation sequencing in microbiology [36]. Thus, a broad technical framework is now available to design WGS validation protocols that will be most relevant for the clinical and public health laboratories. Our aims for the current study were to establish performance metrics for the workflow typical in the microbiological public health laboratories, design modular templates for the validation of different platforms and chemistries, finalize user-friendly report format, and identify a set of bacterial pathogens that could be used for WGS validation and performance assessments.

Methods

Bacterial isolates and sequences

A set of 34 bacterial isolates representing typical workflow in the PHL, was used for validation and quality control of WGS. These included ten *Enterobacteriaceae* isolates, five gram-positive cocci bacterial pathogens, five gram-negative non-fermenting bacterial pathogens, nine *Mycobacterium tuberculosis* isolates and five miscellaneous bacterial pathogens (Table 1). This Whole Genome Shotgun project has been deposited at GenBank under the accession MTFS00000000-MTGZ00000000. The version described in this paper is version MTFS01000000-MTGZ01000000. Raw and assembled sequences are available for download (see Supplementary Table 1 for the accession numbers).

Reference whole genomes

The genome sequences of ATCC strains, isolates characterized by CDC, and other representative isolates were downloaded from NCBI database (http://www.ncbi.nlm.nih.gov/genome/) to be used as reference per the recommendations in the CLSI guidelines [36], (Table 1).

WGS wet bench workflow

The whole genome sequencing was performed on Illumina MiSeq sequencer (Figure 1). The Nextera XT library preparation procedure and 2x300 cycle MiSeq sequencing kits were used (Illumina Inc., San Diego, CA, USA). Illumina Nextera XT indexes were used for barcoding. Bacterial DNA was extracted using Wizard Genomic DNA Kit (Promega, Madison, WI, USA). The bacterial DNA concentrations were measured using Qubit fluorometric quantitation with Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA purity was estimated using NanoDrop 2000 UV-Vis Spectrophotometer (NanoDrop Products, Wilmington, DE, USA). The Mastercycler nexus was used for tagmentation incubation and PCR (Eppendorf North America, Hauppauge, NY, USA). The library concentration was measured using Qubit HS kit. DNA library size distribution was estimated using 2100 BioAnalyzer Instrument and High Sensitivity DNA analysis kit (Agilent Technologies, Santa Clara, CA, USA). Ampure beads were used for size selection. Manual normalization of libraries was performed. The PhiX Control V3 sequencing control was used in every sequencing run (Illumina, Inc. San Diego, CA, USA). Genomes were generated with the depth coverage in the range of 15.71x-216.4x (average 79.72x, median 71.55x).

Bioinformatics pipeline

Paired-end reads were quality trimmed with the threshold of Q30, and then used for mapping to the reference and *de novo* assembly on CLCbio Genomic Workbench 8.0.2 (Qiagen, Aarhus, Denmark). The BAM files generated after mapping to the reference genome were taken through series of software suites to generate the phylogenetic tree. A customized shell script was created to automate the subsequent steps after mapping that included: 1) SNP calling in coding and non-coding genome areas using SAMtools mpileup (v.1.2; [37]); 2) Converting into VCF matrix using bcftools (v0.1.19; http://samtools.github.io/bcftools/); 3) Variants parsing using vcftools (v.0.1.12b; [38]) to include only high-quality SNPs (hqSNPs) with coverage ≥30x, minimum quality > 200; with InDels and the heterozygote calls excluded. 4) Converting SNP matrix into FASTA alignment file for the export back to the CLCbio GW 8.0.2 for the generation of the phylogenetic tree.

hqSNP-based genotyping - The Maximum Likelihood phylogenetic trees were generated based on high-quality single nucleotide polymorphisms (hqSNPs) under the Jukes-Cantor nucleotide substitution model; with bootstrapping.

16S rRNA gene-based identification - Genomes were annotated with prokka v1.1 tool [39] and species identification was performed by comparing 16S rRNA gene sequences against the Ribosomal Database Project (RDP) database [40].

In silico MLST - *In silico* multi-locus sequence typing (MLST) was performed using the Center for Genomic Epidemiology (CGE) online tool [41].

ABR genes detection was performed using the CGE ResFinder online resource [42]. ATCC reference strains designated for use as antibiotic susceptibility controls were analyzed for the presence of antibiotic resistance genes. Negative controls were chosen among strains which were described by the CLSI M100-S25 document [43] as susceptible, with no known antibiotic resistance genes. Positive controls were chosen among strains, which according to the CLSI M100-S25 resistance determinants.

Validation Plan

Thirty-four bacterial isolates were sequenced in triplicate. For between-run reproducibility assessments, all replicates were generated starting from fresh cultures except for *M. tuberculosis* where DNA samples were used. Between run replicates were processed on separate days by different operators. For within run replicates, one DNA extract was used, but independent library preparations were done, with final samples being included in one sequencing run.

Results

A number of CLIA-required quality parameters were adopted with some modification for validation on WGS (Table 2). The modular validation template and a summary of performed here WGS validation for 34 bacterial isolates are presented in figure 2.

Accuracy of WGS

The accuracy of WGS was divided into three components: platform accuracy, assay accuracy, and bioinformatics pipeline accuracy.

Platform accuracy - Platform accuracy was assessed as the accuracy of identification of individual base pairs in the bacterial genome. The accuracy of the platform was established by determining the proximity of agreement between base calling made by MiSeq sequencer (measured value) and NCBI reference sequence (the true value). We determined MiSeq Illumina platform accuracy by mapping generated reads to the corresponding reference sequence and identifying Single Nucleotide Polymorphisms (SNPs). Few validation samples differed from reference genome by several SNPs. However, 99% (324 out of 327) of those SNPs were reproducible among all five replicates we have sequenced for each sample. Since sequencing errors are random between different library preparations and it is unlikely that the same erroneous SNP will occur in all 5 replicates, we can conclude that those discrepancies were not caused by sequencing errors, but most likely were a result of accumulation of mutations in the reference strains or previous sequencing mistakes in the reference sequence. In both cases, whether we take into the account all SNPs detected between validation and reference sequence, or only those SNPs which don't appear in all of the replicates (true sequencing errors), we observed > 99.999% agreement of generated whole genome sequences with the reference sequences for each tested sample.

Assay accuracy - Assay accuracy was determined by an agreement of the assay result for the validation samples with the assay result for reference sequences of the same strains. Four applications of WGS were used to validate the accuracy of the assay: *in silico* Multilocus Sequence Typing (MLST) assay, 16S rRNA gene species identification (ID) assay, an assay for detection of antibiotic resistance (ABR) genes, and genotyping assay using high-quality Single Nucleotide Polymorphisms (hqSNPs).

The definition of the correct result for MLST corresponds to a correct identification of each of the MLST alleles in the validation sequence. For all validation samples each of the sequences of the seven housekeeping genes used in the typing scheme (or 6 genes- for *Aeromonas hydrophilia*) were identified correctly, resulting in 100% allele identification accuracy.

For ABR genes detection the comparison of validation sequences was performed against each entry in the ResFinder database, which at the moment of validation contained sequences of 1719 antibiotic resistance genes, resulting in a total of 1719 tests performed for each validation sample. In negative control samples, all 1719 tests gave negative results.

In positive controls, 1 out of 1719 tests gave a positive result, and the rest must remain negative, as expected. Thus, the accuracy of the assay for ABR genes detection was 100%.

For 16S rRNA ID assay, variations only in one gene were detected, so the species ID results as a whole (e.g. "*Escherichia coli*") was considered as a single test. The identity of 16S rRNA sequence extracted from validation sample showed 100% match with 16S rRNA sequence extracted from the reference sequence.

To assess the accuracy of the genotyping test, phylogenetic trees were built using reference sequences and validation sequences, and resulting trees were compared. For better comparison, we used at least five strains of the same species in the phylogenetic tree. The accuracy of the genotyping test was determined using two approaches: 1) Topological similarity between reference tree and validation tree using Compare2Trees software, and 2) Comparison of clustering pattern of validation tree and reference tree. The phylogenetic trees were generated for five bacterial isolates. All five validation trees had matching clustering patterns and 100% of topological similarity with corresponding reference trees (Supplementary Table 2).

Bioinformatics pipeline accuracy - Accuracy of the bioinformatics pipeline used for hqSNP genotyping was assessed by performing phylogenetic analysis on raw WGS reads of bacterial isolates from well-characterized outbreaks and comparing validation results to the previously published phylogenetic results (Table 3). Two studies, presenting a phylogenetic analysis of outbreaks, caused by the gram-positive pathogen in one study [44] and gram-negative in another study [45] (at least six isolates/study), were used for validation of the bioinformatics pipeline (Figure 3). The clustering of validation tree completely replicated clustering of Study 1 [44] tree (Figure 3A-C), e.g. isolates 4 and 5 were identical and clustered together according to the Study 1, and the same results were shown in validation tree, with isolates 4 and 5 sharing the same node. All conclusions in regards to the genetic relatedness of the isolates that can be drawn from Study 1 tree can also be made from analysis of validation tree 1. The group of related isolates from Study 1 was compared with epidemiologically unrelated isolates suggested by the same study (no tree available from publication). The phylogenetic analysis using the PHL bioinformatics pipeline showed that epidemiologically unrelated isolates did not cluster with the group of outbreak isolates and appeared to be genetically distant (Figure 3D). Thus, the resulting phylogenetic tree produced by our bioinformatics pipeline showed complete concordance with the epidemiological data.

From the Study 2 [45], we have selected nine isolates, which were representative of 4 independent outbreaks and two isolates were epidemiologically unrelated controls (Figure 3E-G). The clustering of validation tree was identical to the clustering of Study 2 tree. For example, isolates 6 and 7 were a part of the same outbreak, while isolate 8 is an epidemiologically unrelated control used in the study. By epidemiological data and Study 2 tree, the validation tree showed that isolates 6 and seven do cluster together, but not with isolate 8. All observations about the genetic relatedness of the isolates drawn from Study 2 tree could be replicated from the analysis of validation tree 2. In summary, based on analysis of simulated data from both studies accuracy of the pipeline for phylogenetic analysis was 100%.

WGS repeatability and reproducibility.

Repeatability (precision within run) was established by sequencing the same samples multiple times under the same conditions and evaluating the concordance of the assay results and performance. Reproducibility (precision between runs) was assessed as the consistency of the assay results and performance characteristics for the same sample sequenced on different occasions. Thirty-four validation samples each were sequenced three times in the same sequencing run (for repeatability) and in 3 times in different runs (for reproducibility). Between run replicates were processed on different days, altering two operators, as recommended CLSI MM11A document [46]. For within run replicates, one DNA extract was used, but independent library preparations were done, with final samples being included in one sequencing run. Therefore, for each sample, the number of intra-assay replicates and inter-assay replicates were three each, and the total numbers of repeated results were five. All quality parameters [depth of coverage, uniformity of coverage, and accuracy of base calling (Q score)] remained relatively constant within and between runs.

Two methods of evaluating precision were used: evaluation of absolute inter- and intra-assay precision per replicate and evaluation of precision relative to the genome size. One out of 3 within-run replicates of isolate C50 *Pseudomonas aeruginosa* ATCC 27853 had a 1 SNP difference from other within-run replicates (see Supplementary Table 3). All validation samples except C50 yielded identical whole genome sequences for all three within-run replicates. The inter-assay precision was 99.02% as per replicate. Three validation samples had one of the between-run replicates each differing from other between-run replicates. Sample C47 *Staphylococcus epidermidis* ATCC 12228 had one between-run replicate with 2 SNPs difference from other replicates. Samples C49 *Streptococcus pneumoniae* ATCC 6305 and C55 *Escherichia coli* ATCC 25922 each had one of the between-run replicates differing from other replicated sequences by 1 SNP. Intra-assay precision per replicate was 97.05%. If precision per base pair is estimated (in relation to the covered genome size), both inter- and intra-assay precision were > 99.9999%.

We also estimated reproducibility and repeatability for MLST and 16S rRNA ID assays. For MLST total number of alleles analyzed for either within- or between-run replicates was 441. Each single allele in all validation samples was identified consistently among within- and between-run replicates. Within- and between-run replicates had repeatable/reproducible sequences of 16S rRNA gene and resulted in repeatable/reproducible species identification. Within and between run precisions of allele detection and species identification for corresponding assays were 100%.

WGS Sensitivity and Specificity

Analytical sensitivity and specificity of WGS were estimated for genotyping and MLST.

Genotyping sensitivity and specificity - to estimate analytical sensitivity and specificity of WGS-based genotyping, the hqSNPs phylogenetic trees generated from the validation sequences were compared to the trees generated from the reference sequences for the same strain. All generated validation trees repeated clustering and had 100% of topological similarity with corresponding reference trees, indicating absence false negative or false-positive results in the genotyping test. Both analytical sensitivity and analytical specificity of the hqSNP-based genotyping assay were 100%.

MLST sensitivity and specificity - As described above, using organism-specific MLST databases sequence type of validation sequences and their reference sequences was determined. For MLST number of the true positive results corresponds to the number of alleles correctly identified in the validation samples. For the true negative results, we performed a comparison of validation sequences against MLST databases for unmatched species, e.g. search of alleles for C1 *Escherichia coli* validation sample against MLST database for *Salmonella enterica*. In the latter case, the MLST assay is not supposed to be able to identify any alleles.   All alleles in positive validation samples were identified correctly. None of the alleles in negative controls were identified. Both analytical sensitivity and analytical specificity of *in silico* MLST test were 100%.

WGS reportable range

The following information about the sequenced genome was collected for the reportable range: genome-wide hq SNPs, housekeeping genes used in MLST schemes, 16S rRNA gene, and antibiotic resistance genes included into ResFinder database.

Reporting language was developed to assist interpretation of the results by an end user with or without specific WGS knowledge- the template and examples are provided in the Supplementary Document 1.

Quality assurance and quality control of WGS

The quality assurance (QA) and quality control (QC) measures were developed as the results of valuation to ensure high quality and consistency of further routine testing using MiSeq Illumina platform. QC must be performed during both pre-analytical (DNA isolation, library preparation), analytical (quality metrics of sequencing run) and post-analytical (data analysis) steps of the WGS. On the stage of data analysis, QC includes three steps: raw read QC, mapping quality QC (or/and *de novo* assembly QC), variant calling QC. PHL should use the WGS validation to establish the thresholds of quality parameters, which can be used in following routine testing to filter out poor quality samples and data and this way minimize the chance of false results. We suggest spiked-in positive and negative controls for routine testing as well as more comprehensive monthly positive and negative controls. Since traditional CLIA rules require the positive and negative control to pass through all the pre-analytical steps, including DNA isolation, laboratory may choose to follow this guidance and perform DNA isolation and sequencing of positive and negative control in each run, or alternatively, implement Individualized Quality Control Plan (IQCP) [as per 42CFR493.1250] and use more economical spiked-in control instead. Type and complexity of positive and negative controls should be determined by each laboratory individually based on specifics of their workflow (most probable source of contamination), type of microorganisms and assays which are most commonly used. Regular and monthly QC practices are summarized in Supplementary Figure 1. The complete QA&QC manual established for WGS applications used in microbiological PHL can be found in Supplementary Document 1.

Validation Summary

WGS assay was shown to have >99.9% accuracy, >99.9% reproducibility/repeatability, and 100% specificity and sensitivity, which meets CLIA requirements for laboratory-developed tests (LDTs).

Discussion

This study established the workflow and reference materials for the validation of WGS for routine use in PHL according to CLIA guidelines for LDTs. The validation panel, sequencing analytics, and raw sequences generated during this study could serve as a resource for the future multi-laboratory comparisons of WGS. Additionally, the WGS performance specifications and modular validation template developed in the study could be easily adapted for the validation of other platforms and reagents kits. These results strengthen the concept of unified laboratory standards for WGS enunciated by some professional organizations, including the Global Microbial Identifier (GMI) initiative [30, 31, 33, 47]. A few other groups have also highlighted the challenges and solutions for the implementation of WGS in clinical and public health microbiology laboratories [21, 48].

Using a combination of reference strains and corresponding publicly available genomes, we devised a framework of ‘best practices' for the quality management of the integrated ‘wet lab' and ‘dry lab' WGS workflow (‘pipeline'). The importance of reference materials for validation and QC of wet- and dry-lab WGS processes has been noted earlier [28, 31, 33] . Unlike in human genomics [49], there is no well-established source of reference materials for WGS validation in microbiological PHL. The main challenge of creating customized validation set is the lack of reference materials, in other words, strains that can be easily acquired by the PHLs and which have high-quality well-characterized reference genomes available. While using complete genomic sequences of ATCC strains from NCBI is an option, it is far from being perfect. The genome sequences available from public databases are generated by using different methods, chemistries, platforms, which may yield different error rates, therefore deposited sequences are not guaranteed to be free of such errors. With the perpetual development of new sequencing technologies and improvements in the quality of sequences, it is not unlikely that the genomes sequenced with old methods may appear less accurate than the validation sequences generated by the laboratory during validation. In addition to this, there is a possibility of mutations accumulation in the control strains, e.g. ATCC cultures, which are propagated by the different laboratories. In this sense, there is no gold standard available for use as a reference material for bacterial WGS validation. Nevertheless, NCBI, ENA, and similar public genome depositories remain to be the best resource for the genomic sequences of control strains, which can be used for validation. In future, it would be optimal to have a network/agency/bank which could distribute panels of thoroughly sequenced isolates, with curated and updated genomic sequences available online for WGS validation. In the absence of such resource, we developed a validation set of microorganisms, which can be used for future validations of WGS platforms. Bacterial genomes vary differently in size, GC content, abundance of repetitive regions, and other properties, which affect the WGS results. We created a validation set which reflects the diversity of the microorganisms with various genome sizes and GC-content, which are routinely sequenced by the PHL. Different species of gram-positive and gram-negative microorganisms and *M. tuberculosis* were included to account for the differences in DNA isolation procedures as well.

Samples were validated based on four core elements also reflected in the assay report: 16S rRNA-based species identity, *in silico* MLST, hqSNP phylogenetic analysis, and the presence of AR determinants. Overall, we achieved high accuracy, precision, sensitivity and specificity for all test analytes ranging from 99-100%, which well exceeds 90% threshold for these performance parameters for LDT as per CLIA. These findings are in agreement with recent reports of 93%-100% accuracy in WGS identification, subtyping, and antimicrobial resistance genes detection in a number of pathogens [50-53].

The successful CLIA integration of the WGS would also obligate a laboratory to implement a continuous performance measurement plan via an internal or external proficiency testing (PT) program. Such PT programs are under active development with the Global Microbial Identifier (GMI) network, the Genetic Testing Reference Materials Coordination Program (Get-RM), the Genome in a Bottle (GIAB) Consortium, and the CDC PulseNet NextGen being the most prominent [31, 49]. More generic standards have been proposed by the College of American Pathologists' (CAP) molecular pathology checklist (MOL)[30]. The proposed quality standards include both live cultures as well as ‘sequence only' formats for a comprehensive assessment of the WGS pipeline. Our validation set of isolates is amenable to both internal and external quality assurance testing. In preliminary internal PT, we were able to successfully assess the entire workflow and personnel performance (details not shown).

Microbial WGS remains a dynamic technology, and therefore, any validated pipeline is unlikely to remain static. For this reason, implementation of modular validation template becomes crucial for the seamless and timely introduction of changes to the 'pipeline,' e.g. we had to carry-out several amendments to the protocol since its implementation in the laboratory. These included a new processing algorithm for highly-contagious pathogens and some adjustments to the data analysis algorithm. The changes were accomplished via minor modifications in the ‘pipeline' with corroborative testing using developed by us modular validation template. We also performed a two-sequencer comparison to allow for processing of increased volume of samples (see the protocol for the correlation study in Supplementary Document 1).

The WGS report format continues to pose challenges. Reporting language was designed to be able to convey the WGS-based assay results to the end user with or without the extensive knowledge of WGS to avoid erroneous interpretation of the results by the final user and provide actionable data. Disclaimers are particularly important to guide the potential use of the data in clinical settings, e.g. a disclaimer that detection of antibiotic resistance genes by WGS do not guarantee resistance of the strain *in vivo* and that phenotypic susceptibility test is required to confirm antimicrobial resistance.

The study possesses certain limitations. Firstly, only a limited number of WGS-based assays were included into the validation study based on the most common PHL applications. Other types of WGS assays/analytics would have to be validated in a similar manner to determine the performance specifications, which are required to generate accurate and reproducible results, e.g. a threshold for the base calling accuracy of the platform, or a depth of coverage of specific genes. Secondly, not all validation set samples had available NCBI database entries to provide comparison sets. Thirdly, the absence of any eukaryotic pathogens in the current validation is another shortcoming and therefore, additional validation studies would be needed to implement a pipeline for the pathogenic fungi and parasites.

As the clinical and public microbiology community implements high-quality WGS, it would be opportune to consider the available models for the delivery of these services [54]. Since their inception, most WGS activities have taken place in the reference facilities with rather large supporting infrastructure. Although inevitable in the early stages, the centralization of services presents several challenges on the turnaround time and access to the specific expertise on the local population structure of a given pathogen, which are crucial for the management of infectious diseases at the local and regional levels. WGS services could now be delivered locally, more easily with the affordable sequencers, standardized reagents, and well-defined quality metrics. The local delivery model would also be more responsive to the needs of the target client and enhance the adoption of WGS across the healthcare systems. Another alternative is a hybrid model with complimentary central and local services to balance the need for speed with the advanced expertise and resources [54]. Two prominent examples of the hybrid models in the United States are the Food and Drug Administration (FDA) GenomeTrakr network for the tracking of food-borne pathogens, and the CDC Advanced Molecular Detection (AMD) initiative for the improved surveillance of infectious diseases [55, 56]. The AMD and GenomeTrakr frameworks rely on a participatory model with enhanced analysis, curation and data storage at a central site. However, these resource-intensive networks focus on few selected pathogens at present. Notably, there still remain significant challenges for the implementation of the comprehensive WGS services at the local level [48, 57]. It is hoped that the quality framework proposed in the present study would advance the localization of comprehensive WGS services in clinical and public health laboratories.

In summary, the salient achievements of this study included: 1) establishment of the performance specifications for WGS in the application to public health microbiology in accordance with CLIA guidelines for the LDTs, 2) the development of quality assurance (QA) and quality control (QC) measurements for WGS, 3) formatting of laboratory reports for end users with or without WGS expertise, 4) a set of pathogenic bacteria for further validations of WGS and multi-laboratory comparisons and, 5) development of an integrated workflow for the ‘wet bench' and ‘dry bench' parts of WGS.

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FIGURE LEGENDS

Figure 1. **WGS wet and dry bench workflow**

Figure 2. **The summary of the WGS validation.**

Figure 3. **Bioinformatics pipeline validation with two groups of outbreak isolates. A.** "Study 1 tree", a phylogenetic tree of outbreak isolates, which was published in the study 1. The isolates from the study which were picked for validation have arrows pointing at them and numbers assigned for purposes of validation (1-7). **B.** A tree representing phylogenetic connections between chosen isolates from original study tree. **C.** "Validation tree 1", a phylogenetic tree generated using the PHL bioinformatics pipeline. The same isolates in the original tree and validation tree are marked with the same numbers. **D.** Comparison of the group of related isolates (1-7) from Study 1 with epidemiologically unrelated isolates from the same study using the PHL bioinformatics pipeline. **E.** "Study two tree", a phylogenetic tree combining epidemiologically related and nonrelated isolates published in the study 2. The isolates from the study two which were picked for validation marked with green node circles and had numbers 1-11 assigned for purposes of validation. **F.** A tree representing phylogenetic connections between chosen isolates from original study tree. **G.** "Validation tree 2", a phylogenetic tree generated using the PHL bioinformatics pipeline. The same isolates in the tree from Study 2 and the validation tree are marked with the same numbers.

Supplementary Figure 1. **WGS quality control scheme.**

Figure 1. **WGS wet and dry bench workflow**

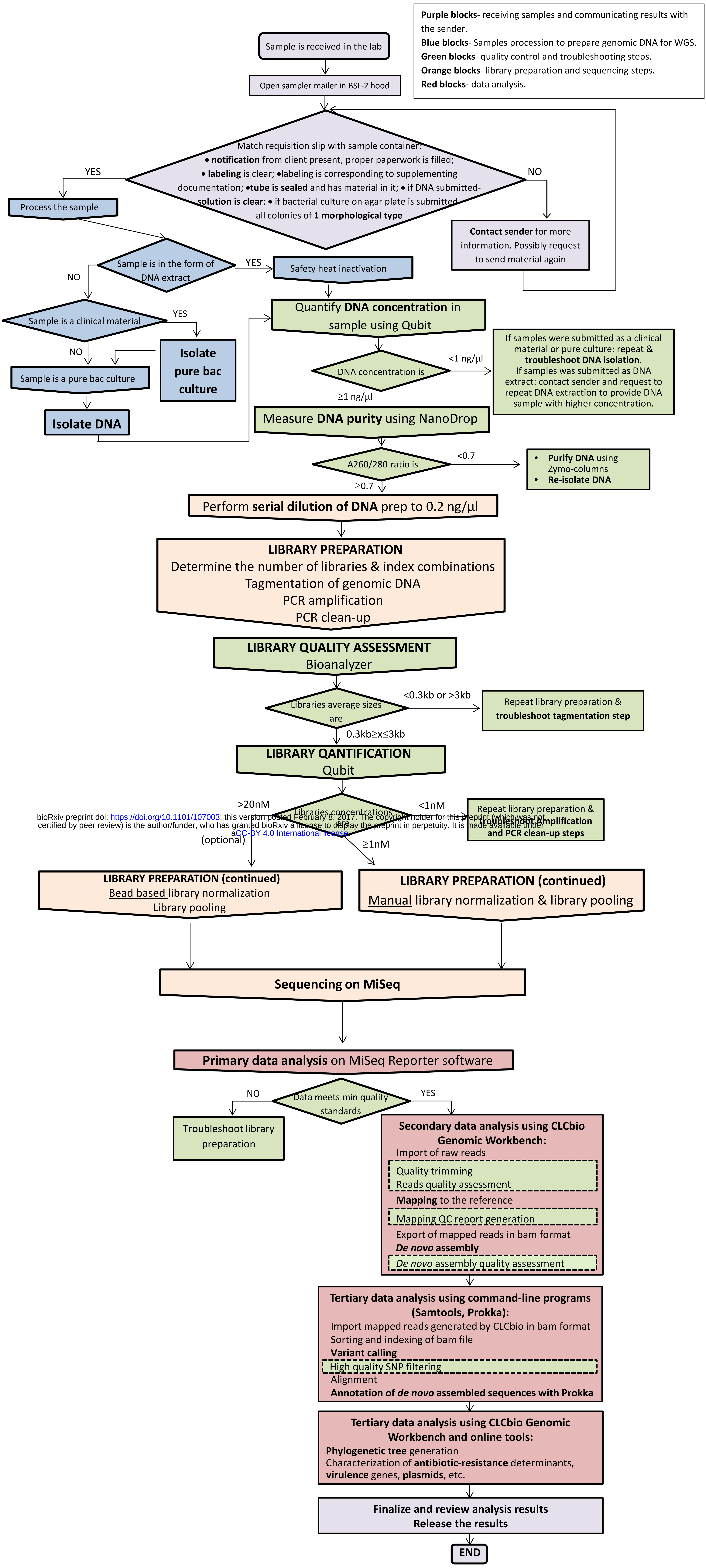
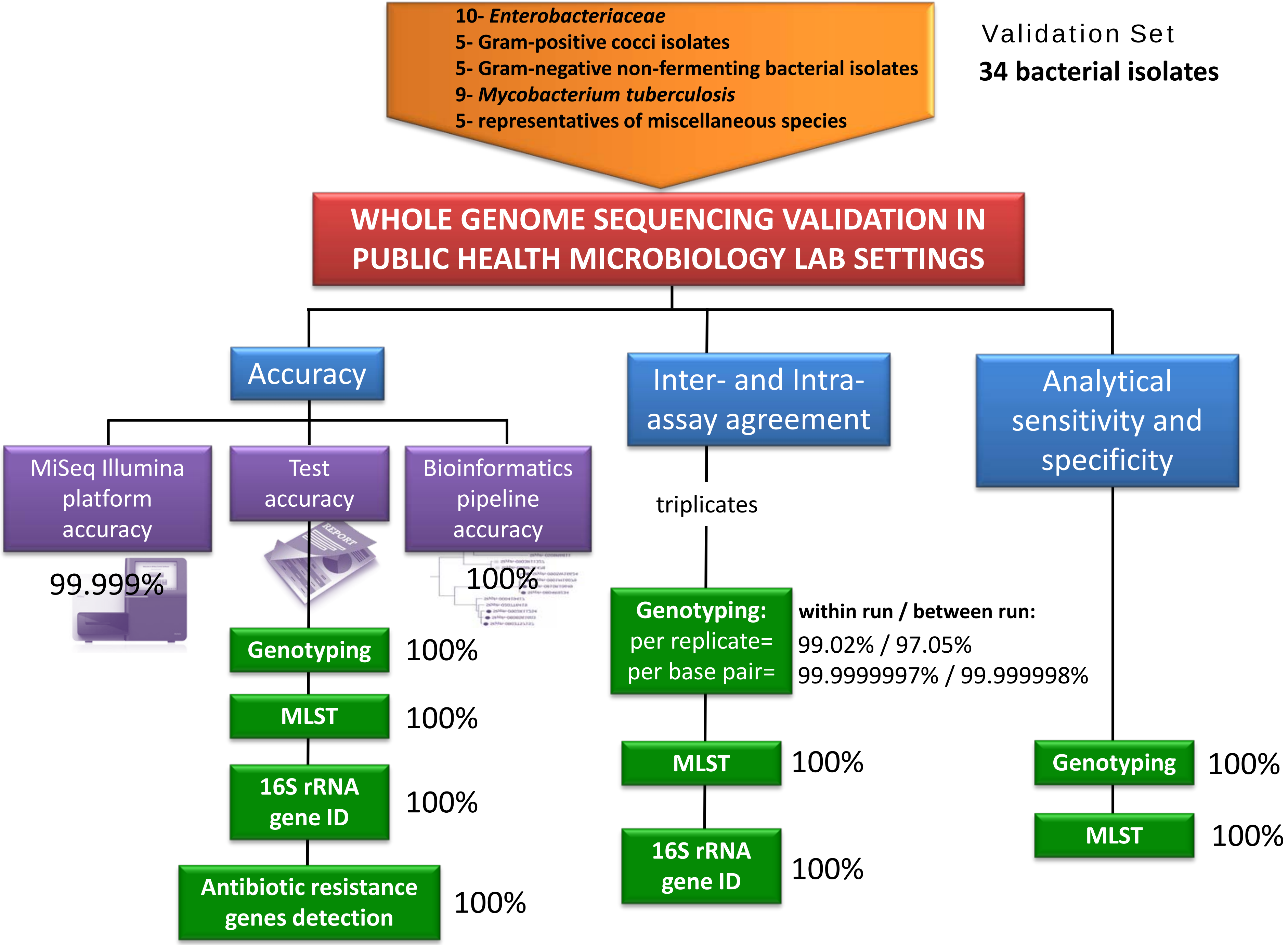

Purple blocks- receiving samples and communicating results with the sender.
Blue blocks- Samples procession to prepare genomic DNA for WGS.
Green blocks- quality control and troubleshooting steps.
Orange blocks- library preparation and sequencing steps.
Red blocks- data analysis.
Sample is received in the lab
Open sampler mailer in BSL-2 hood
Match requisition slip with sample container: • notification from client present, proper paperwork is filled; • labeling is clear; •labeling is corresponding to supplementing documentation; •tube is sealed and has material in it; • if DNA submitted-solution is clear; • if bacterial culture on agar plate is submitted all colonies of 1 morphological type
YES
NO
Process the sample
Contact sender for more information. Possibly request to send material again
Sample is in the form of DNA extract
YES
NO
Safety heat inactivation
Sample is a clinical material
YES
NO
Isolate pure bac culture
Sample is a pure bac culture
Isolate DNA
Quantify DNA concentration in sample using Qubit
DNA concentration is
<1 ng/µl
≥1 ng/µl
If samples were submitted as a clinical material or pure culture: repeat & troubleshoot DNA isolation. If samples was submitted as DNA extract: contact sender and request to repeat DNA extraction to provide DNA sample with higher concentration.
Measure DNA purity using NanoDrop
A260/280 ratio is
<0.7
≥0.7
• Purify DNA using Zymo-columns
• Re-isolate DNA
Perform serial dilution of DNA prep to 0.2 ng/µl
LIBRARY PREPARATION
Determine the number of libraries & index combinations
Tagmentation of genomic DNA
PCR amplification
PCR clean-up
LIBRARY QUALITY ASSESSMENT
Bioanalyzer
Libraries average sizes are
<0.3kb or >3kb
Repeat library preparation & troubleshoot tagmentation step
0.3kb≥x≤3kb
LIBRARY QANTIFICATION
Qubit
Libraries concentrations are
>20nM
(optional)
<1nM
≥1nM
Repeat library preparation & troubleshoot Amplification and PCR clean-up steps
LIBRARY PREPARATION (continued)
Bead based library normalization
Library pooling
LIBRARY PREPARATION (continued)
Manual library normalization & library pooling
Sequencing on MiSeq
Primary data analysis on MiSeq Reporter software
Data meets min quality standards
NO
YES
Troubleshoot library preparation
Secondary data analysis using CLCbio Genomic Workbench:
Import of raw reads
Quality trimming
Reads quality assessment
Mapping to the reference
Mapping QC report generation
Export of mapped reads in bam format
De novo assembly
De novo assembly quality assessment
Tertiary data analysis using command-line programs (Samtools, Prokka):
Import mapped reads generated by CLCbio in bam format
Sorting and indexing of bam file
Variant calling
High quality SNP filtering
Alignment
Annotation of de novo assembled sequences with Prokka
Tertiary data analysis using CLCbio Genomic Workbench and online tools:
Phylogenetic tree generation
Characterization of antibiotic-resistance determinants, virulence genes, plasmids, etc.
Finalize and review analysis results
Release the results
END

Figure 2. **The summary of the WGS validation.**



10- *Enterobacteriaceae*
5- Gram-positive cocci isolates
5- Gram-negative non-fermenting bacterial isolates
9- *Mycobacterium tuberculosis*
5- representatives of miscellaneous species

Validation Set
34 bacterial isolates

WHOLE GENOME SEQUENCING VALIDATION IN PUBLIC HEALTH MICROBIOLOGY LAB SETTINGS

Accuracy

MiSeq Illumina platform accuracy
99.999%

Test accuracy

Bioinformatics pipeline accuracy
100%

Genotyping 100%
MLST 100%
16S rRNA gene ID 100%
Antibiotic resistance genes detection 100%

Inter- and Intra-assay agreement

triplicates

Genotyping:
per replicate=
per base pair=

within run / between run:
99.02% / 97.05%
99.9999997% / 99.999998%

MLST 100%
16S rRNA gene ID 100%

Analytical sensitivity and specificity

Genotyping 100%
MLST 100%

Figure 3. **Bioinformatics pipeline validation with two groups of outbreak isolates.**

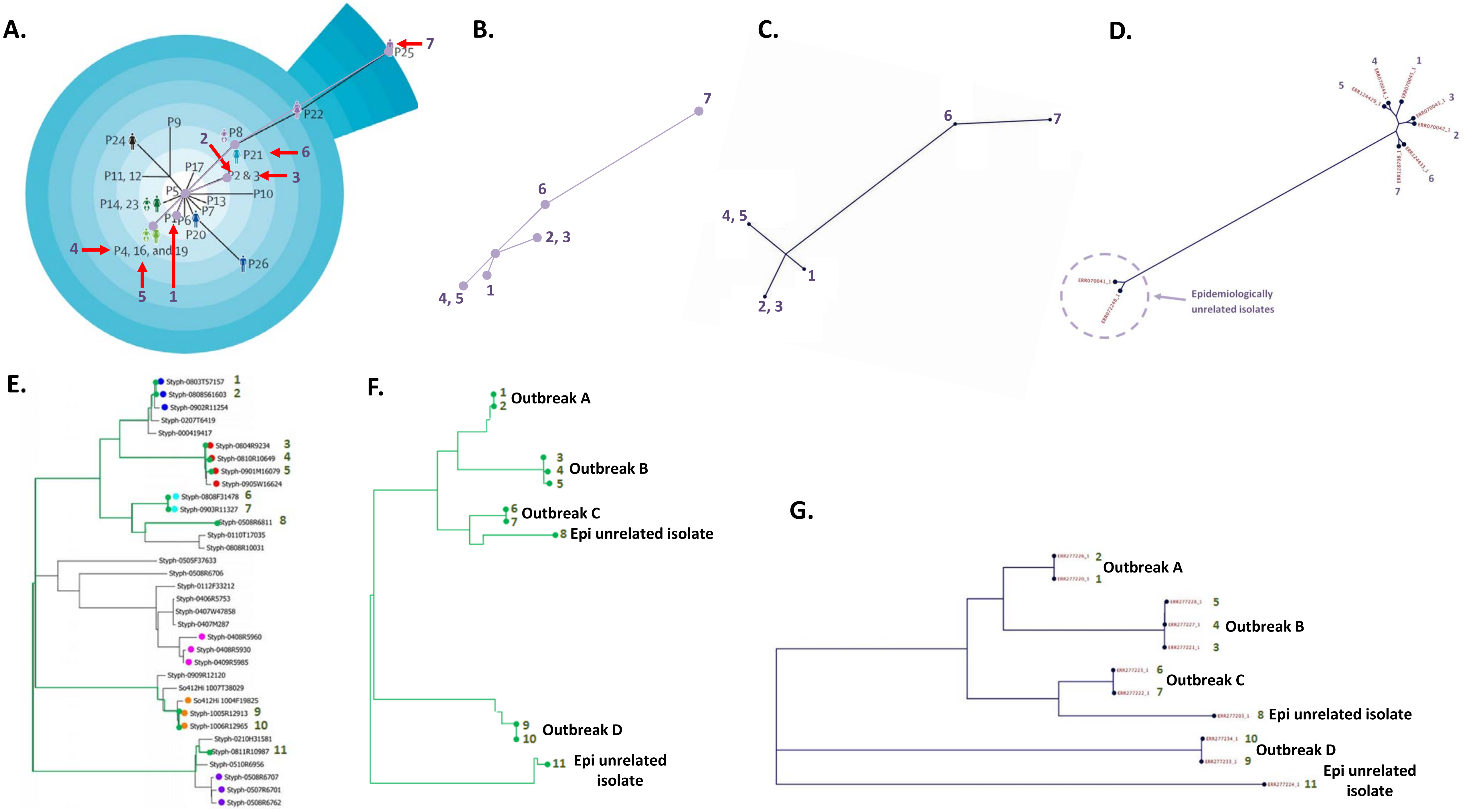


Table 1. List of strains used for validation and corresponding reference materials

Reference materials- NCBI strains

MDL ID	Species	Reference	
		NCBI Strain	NCBI Acc#
C1	*Escherichia coli* O157:H7 CDC EDL 933	O157:H7 CDC EDL 933	NZ_CP008957.1
C3	*Escherichia coli* ATCC 8739	ATCC 8739	NC_010468.1
C55	*Escherichia coli* ATCC 25922	ATCC 25922	NZ_CP009072.1
C4	*Enterobacter cloacae* ATCC 13047	ATCC 13047	NC_014121
C6	*Salmonella enterica* ser Typhimurium ATCC 14028	14028S	NC_016856
C5	*Staphylococcus aureus* ATCC 25923	ATCC 25923	NZ_CP009361
C46	*Enterococcus faecalis* ATCC 29212	ATCC 29212	NZ_CP008816
C47	*Staphylococcus epidermidis* ATCC 12228	ATCC 12228	NC_004461
C48	*Staphylococcus saprophyticus* ATCC 15305	ATCC 15305	NC_007350
C49	*Streptococcus pneumoniae* ATCC 6305	ATCC 700669	FM211187
C50	*Pseudomonas aeruginosa* ATCC 27853	FRD1	NZ_CP010555
C51	*Stenotrophomonas maltophilia* ATCC 13637	ATCC 13637	NZ_CP008838
C52	*Legionella pneumophila* SG-12 ATCC 43290	ATCC 43290	NC_016811
C53	*Moraxella catarrhalis* 87A-3084	ATCC 25240	NZ_CP008804
C54	*Acinetobacter baumannii* ATCC 17945	AB07	NZ_CP006963
C103	*Bacteroides fragilis* ATCC 25285	638R	NC_016776
C104	*Haemophilus influenzae* ATCC 10211	KR494	NC_022356
C2	*Aeromonas hydrophilia* ATCC 7966	ATCC 7966	NC_008570
C105	*Corynebacterium jeikeium* ATCC 43734	ATCC 43734	GG700813:GG700833
C106	*Neisseria gonorrhoeae* ATCC 49226	MS11	NC_022240
C56	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3
C57	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3
C58	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3
C59	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3
C61	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3
C65	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3
C67	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3
C68	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3
C69	*Mycobacterium tuberculosis*	H37Rv	NC_000962.3

Reference materials- strains sequenced at CDC

MDL ID	Species	Reference raw reads generated by CDC		Reference used for mapping	
		CDC Strain	Accession #	NCBI Strain	NCBI Acc#
C72	*Escherichia coli* O121:H19	2014C-3857	SRR1610033	2011C-3493	NC_018658
C73	*Salmonella enterica* ser Enteritidis	CDC_2010K-1543	SRR518749	P125109	NC_011294.1
C74	*Salmonella enterica* ser Infantis	2014K-0434	SRR1616809	1326/28	NZ_LN649235
C75	*Salmonella enterica* ser Adelaide	2014K-0941	SRR1686419	P125109	NC_011294.1
C76	*Salmonella enterica* ser Worthington	2012K-1219	SRR1614868	P125109	NC_011294.1
C77*	*Salmonella enterica* ser Saintpaul	2014K-0875	SRR1640105	14028S	NC_016856

Footnotes: Green color designates cases when the genome of the strain sequenced by the PHL is available from the NCBI database. Yellow color designates cases when the genome of the strain sequenced by the PHL is NOT available from the NCBI database and an alternative reference genome was used for mapping.

*P.S.: Sample C77 was sequenced by PHL only for genotyping test accuracy validation. No replicates were done.

Table 2. Performance characteristics, definitions, and formulas used in validation. Summary of the validation for different assays.

Definition of the performance characteristic for WGS applications				Formula used for calculation	Assay used for validation of the parameter					
					hqSNP based genotyping		MLST	16S	Antibiotic resistance genes detection	Assay-specific definitions
Accuracy	The degree of agreement between the nucleic acid sequences derived from the assay (measured value) and a reference sequence (the true value).	accuracy of platform	Accuracy of base calling against reference sequence. The accuracy of the platform was established by determining the proximity of agreement between base calling made by MiSeq sequencer (measured value) and NCBI reference sequence (the true value).	$\% \text{ agreement with reference} = \frac{(\text{Covered genome length})-(\text{Total \# of SNP differing fromreference})}{\text{Covered genome length}}$ x 100%	99.999378%					Accuracy of the platform
		accuracy of assay	Test accuracy is determined by agreement of the test result of validation samples sequenced by PHL with the test result for reference sequences of the same strains.	$\text{Accuracy} = \frac{\text{\# of correct results}}{\text{total \# of results}} \text{x } 100\%$	congruence of phylogenetic trees built using reference sequences and validation sequences		Detection and correct identification of each of the MLST alleles	ID of 16S rRNA sequence of the validation sample matches the ID of 16S rRNA sequence of the reference sequence	Presence of ABR genes characteristic for reference strain, absence of any other ABR genes	Definition of correct results
					Individual sample clustering		Allele	16S rRNA ID result	Antibiotic resistance gene	Single test unit
					100%		100%	100%	100%	Accuracy of the assay
		accuracy of bioinformatics pipeline	Clustering suggested by previous investigators must match clustering achieved by the analysis using PHL validation bioinformatics pipeline.	$\% \text{ agreement} = \frac{\text{\# of outbreak isolates clustered correctly in validation tree}}{\text{Total \# of outbreak isolates clustered together in the study tree}} \text{x } 100\%$	100%		-	-	-	Accuracy of the bioinformatics pipeline
Precision	The degree to which repeated sequence analyses give the same result repeatability (within-run precision) and reproducibility (between-run precision).	Repeatability (precision within run)	was established by sequencing the same samples multiple times under the same conditions and evaluating the concordance of the test results and performance.	$\text{Inter} - \text{assay precision (Repeatability)} = \frac{\text{\# within}-\text{run replicates in agreement}}{\text{Total \# of tests performed for within}-\text{run replicates}}$ x 100%	inter- assay precision of single nucleotide variant detection.					Definition of correct results
					SNP (precision per replicate)	SNP (precision per base pair)	Allele	16S rRNA ID	-	Single test unit
					99.02%	99.9999997%	100%	100%	-	Repeatability
		Reproducibility (precision between runs)	was assessed as the consistency of the test results and performance characteristics for the same sample sequenced under different conditions, such as between different runs and different sample preparations.	$\text{Intra} - \text{assay precision (Reproducibility)} = \frac{\text{\# between}-\text{run replicates in agreement}}{\text{Total \# of tests performed for between}-\text{run replicates}}$ x 100%	intra- assay precision of single nucleotide variant detection.					Definition of correct results
					SNP (precision per replicate)	SNP (precision per base pair)	Allele	16S rRNA ID	-	Single test unit
					97.05%	99.999998%	100%	100%	-	Reproducibility

Continued

			Assay used for validation of the parameter				
Definition of the performance characteristic for WGS applications		**Formula used for calculation**	**hqSNP based genotyping**	**MLST**	**16S**	**Antibiotic resistance genes detection**	**Assay-specific definitions**
Analytical sensitivity (Limit of detection)	The likelihood that a WGS assay will detect a sequence variation when present within the analyzed genomic region (this value reflects a false negative rate of the test).	$\text{Analytical sensitivity} = \frac{\text{TP}}{\text{TP + FN}}$ x 100%	Clustering of related samples (#of validation samples with clustering results matching reference)	Number of correctly identified alleles	-	-	Definition of true positive results
			Number of validation samples which clustered together with samples, genetically distant according to the reference tree	Number of unidentified or misidentified alleles validation samples	-	-	Definition of false negative results
			Individual sample clustering	Allele	-	-	Single test unit
			100%	100%	-	-	Analytical sensitivity
Analytical specificity	The probability that a WGS assay will not detect sequence variation(s) when none are present within the analyzed genomic region (this value reflects a test's false positive rate).	$\text{Analytical specificity} = \frac{\text{TN}}{\text{TN + FP}}$ x 100%	No clustering between unrelated samples (#of validation samples with clustering results matching reference)	Number of unidentified alleles in negative control samples	-	-	Definition of true negative results
			Number of validation samples which failed to clustered together with samples, genetically similar according to the reference tree	Number of identified alleles in negative control samples	-	-	Definition of false positive results
			Individual sample clustering	Allele	-	-	Single test unit
			100%	100%	-	-	Analytical specificity
Reportable range	The region of the genome in which sequence of an acceptable quality can be derived by the laboratory test.	N/A	Genome-wide hq SNPs	Housekeeping genes used in corresponding MLST schemes	16S rRNA gene	Antibiotic resistance genes in included ResFinder database	

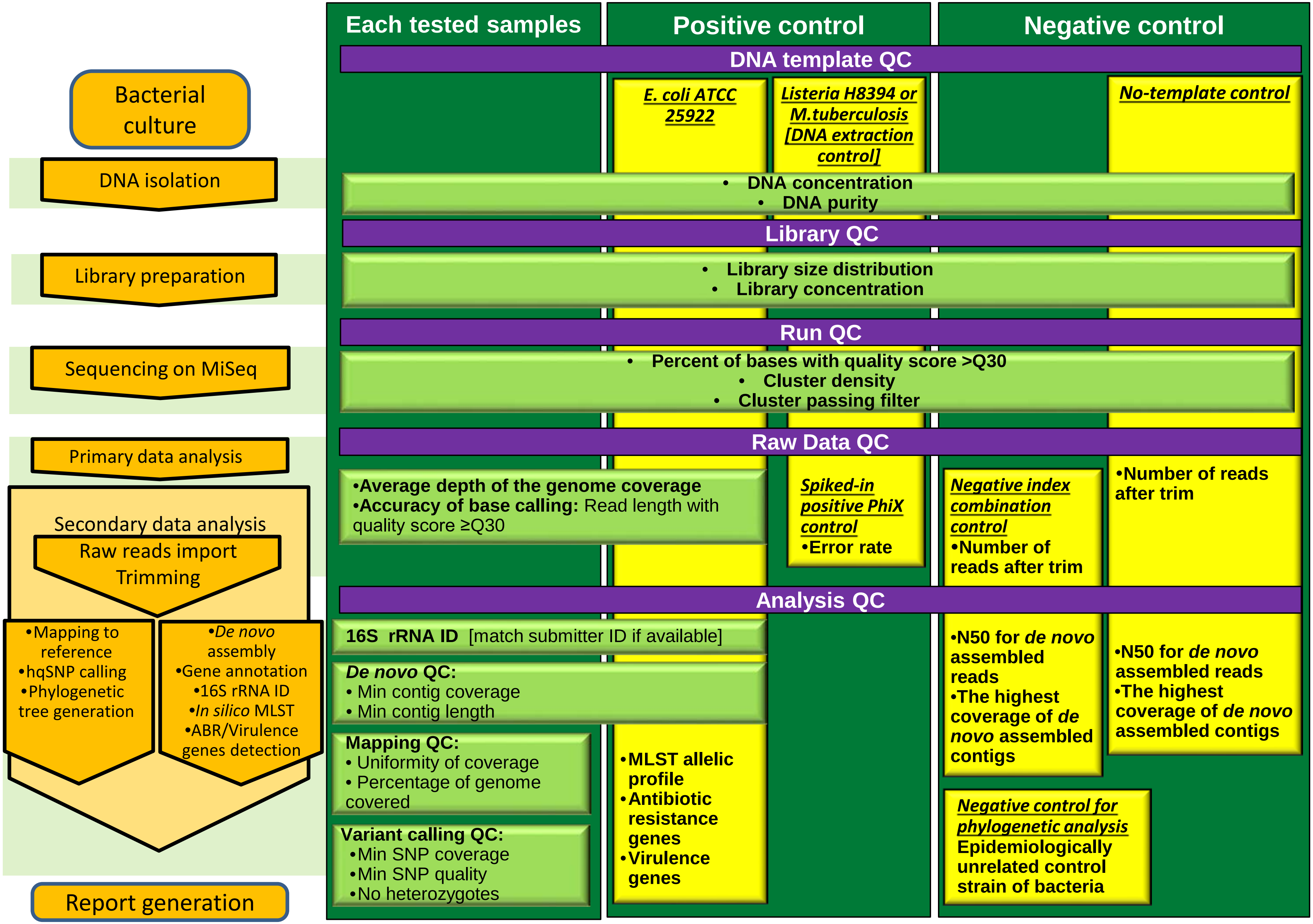
Footnotes: See details in Supplementary Document 1.

Abbreviations: TP- True positive results, TN- True negative results, FP- False positive, FN- False negative

Table 3. Summary of the studies used for validation of bioinformatics pipeline

Study	**Study 1.** SR Harris et al. Lancet Infect Dis 2013; 13: 130–36 [44]	**Study 2.** P Leekitcharoenphon et al. PLoS ONE 2014; 9(2): e87991 [45]
Microorganism	Methicillin-resistant *Staphylococcus aureus*	*Salmonella enterica* serovar Typhimurium
Source of isolates	Human	Human
Number of isolates analyzed	7 outbreak isolates (1 outbreak cluster) + 2 epidemiologically unrelated isolates	9 outbreak isolates (4 outbreak clusters) + 2 epidemiologically unrelated isolates
Type of outbreak	Hospital-associated outbreak	Foodborne outbreaks
ID of the samples in the study which were used for validation	P1, P2, P3, P4, P16, P21, P25, Identified by Infectious Control Investigation non-outbreak ST1, MRSA identified by searching microbiology database non-outbreak ST772	0803T57157, 0808S61603, 0808F31478, 0903R11327, 0811R10987, 0804R9234, 0810R10649, 0901M16079, 0110T17035, 1005R12913, 1006R12965
Accession ## of corresponding samples	ERR070045, ERR070042, ERR070043, ERR070044, ERR124429, ERR124433, ERR128708, ERR070041, ERR072248	ERR277220, ERR277226, ERR277223, ERR277222, ERR277224, ERR277221, ERR277227, ERR277228, ERR277203, ERR277233, ERR277234
# of clusters in study tree	1	4
# of clusters in validation tree	1	4
# of outbreak isolates in each cluster in the study tree	Cluster 1= 7	Cluster 1= 2, Cluster 2= 3, Cluster 3= 2, Cluster 4= 2
# of outbreak isolates in each cluster in validation tree	Cluster 1= 7	Cluster 1= 2, Cluster 2= 3, Cluster 3= 2, Cluster 4= 2
# of epidemiologically unrelated isolates in the set	2	2
# of epidemiologically unrelated isolates clustered with outbreak isolates	0	0
% agreement= (# of outbreak isolates clustered correctly in validation tree)x100%/ (Total # of outbreak isolates clustered together in the study tree)	(7x100/7) = 100%	(9x100/9) = 100%

WGS QUALITY CONTROL SCHEME



Bacterial culture

DNA isolation

Library preparation

Sequencing on MiSeq

Primary data analysis

Secondary data analysis

Raw reads import
Trimming

- Mapping to reference
- hqSNP calling
- Phylogenetic tree generation

- *De novo* assembly
- Gene annotation
- 16S rRNA ID
- *In silico* MLST
- ABR/Virulence genes detection

Report generation

Each tested samples | **Positive control** | **Negative control**

DNA template QC

E. coli ATCC 25922

Listeria H8394 or M.tuberculosis [DNA extraction control]

No-template control

- **DNA concentration**
- **DNA purity**

Library QC

- **Library size distribution**
- **Library concentration**

Run QC

- **Percent of bases with quality score >Q30**
- **Cluster density**
- **Cluster passing filter**

Raw Data QC

- **Average depth of the genome coverage**
- **Accuracy of base calling:** Read length with quality score ≥Q30

Spiked-in positive PhiX control
- **Error rate**

Negative index combination control
- **Number of reads after trim**

- **Number of reads after trim**

Analysis QC

16S rRNA ID [match submitter ID if available]

***De novo* QC:**
- Min contig coverage
- Min contig length

Mapping QC:
- Uniformity of coverage
- Percentage of genome covered

Variant calling QC:
- Min SNP coverage
- Min SNP quality
- No heterozygotes

- **MLST allelic profile**
- **Antibiotic resistance genes**
- **Virulence genes**

- **N50 for *de novo* assembled reads**
- **The highest coverage of *de novo* assembled contigs**

- **N50 for *de novo* assembled reads**
- **The highest coverage of *de novo* assembled contigs**

Negative control for phylogenetic analysis
Epidemiologically unrelated control strain of bacteria