Multicenter validation of whole genome nanopore sequencing for AMR surveillance

Stephan Beisken, Thomas Weinmaier, Johannes Weinberger, Arne C. Materna
Ares Genetics GmbH, Karl-Farkas-Gasse 18, 1030 Vienna, Austria. Contact: services@ares-genetics.com

SEPTEMBER, 2021

Contents

1. Abstract

The purpose of this multicenter validation study was to assess the performance of Oxford Nanopore Technologies (ONT) sequencing of bacterial isolates to enable fast, cost effective, and easily deployable genomic antimicrobial surveillance. To this end we validated end-to-end workflows for ONT sequencing and AREScloud for bioinformatics analysis.

Despite higher single-molecule error rates typical for ONT, compared to other established sequencing platforms, the here presented workflows proved accurate and robust. Seven bacterial species were investigated for the effect of different extraction methods and sequencing labs on pathogen identification, antimicrobial resistance (AMR) marker detection, and predictive antimicrobial susceptibility testing (AST).

Pathogen identification was found to be 100% accurate and reproducible across extraction methods and labs. For AMR marker detection and predictive AST accuracies of up to 97% and a reproducibility across labs and protocols of 96% or higher were reached. Plasmid typing performed similarly well with an accuracy up to 90% and a reproducibility of consistently 100%.

Moreover, while we confirmed that ONT data are not yet sufficiently accurate for subtyping via core genome MLST, we expect that further advances of the nanopore sequencing platform will reduce error rates and enable this application.

Summarizing, we found that ONT sequencing together with AREScloud enable accurate and robust genomic antimicrobial surveillance.

2. Introduction

The role of next-generation sequencing for the surveillance, diagnosis and treatment of antimicrobial resistant pathogens

The burden of antimicrobial resistance (AMR) and its accelerating progression has been acknowledged worldwide by leading health institutes such as the WHO and the CDC[1–3]. Besides the need for new antibiotics, efforts are required in the field of AMR surveillance and diagnostics for effective infection prevention and informed selection of appropriate first and second line treatment options[4].

In recent years, next-generation sequencing (NGS) has emerged as an alternative to established culture-based and molecular tests for the detection, identification, typing and functional characterization of AMR pathogens[5]. The WHO, amongst other leading health organizations, has acknowledged the importance of whole genome sequencing (WGS) of bacterial isolates for surveillance and infection control. In a 2020 report on WGS, the WHO stated that the technology "provides a vast amount of information and the highest possible resolution for pathogen subtyping. The application of WGS for global surveillance can provide information on the early emergence and spread of AMR and further inform timely policy development on AMR control[1]."

Advantages and limitations of next-generation sequencing

The advantage of NGS over other molecular tests such as PCR resides in the technique's potential to integrate into a single assay i) the sensitive and precise detection of pathogens with ii) the comprehensive characterization of etiological or epidemiological traits, such as AMR markers (genes/variants) or plasmid replicon types, based on genomic information.

1. WHO GLASS whole-genome sequencing for surveillance of antimicrobial resistance. 2020.
2. Rochford, C. et al. Global governance of antimicrobial resistance. Lancet 391, 1976–1978 (2018).
3. WHO Global tuberculosis report 2018; World Health Organization, 2018.
4. Livermore, D. M. The need for new antibiotics. Clin Microbiol Infec 10, 1–9 (2004).
5. Guitor, A. K. et al. Capturing the Resistome: a Targeted Capture Method To Reveal Antibiotic Resistance Determinants in Metagenomes. Antimicrob Agents Ch 64, (2019).

While NGS is undergoing rapid adoption for the epidemiological analysis of bacterial isolates (for instance for the surveillance of foodborne pathogens[6]) several limitations impair the adoption of NGS for surveillance, particularly in low to mid income countries (LMICs). Arguably the most critical limitations include cost, turnaround time, and the ability to deploy NGS-based assays in the field or in low complexity lab environments.

Nanopore sequencing for antimicrobial surveillance

The emergence of nanopore sequencing, as commercialized by Oxford Nanopore Technologies (ONT), as an alternative to the de-facto standard short-read sequencing platforms has the potential to overcome the aforementioned bottlenecks that are impacting the adoption of NGS for AMR surveillance.

In contrast to the sequencing by synthesis approach employed by, for example, Illumina, ONT uses single molecule sequencing that also enables the possibility of real-time data analysis[7]. To interrogate a genomic sequence, individual DNA molecules are translocated in a highly parallelized fashion through biological pores across a barrier. A steady flow of ions through these pores creates a measurable current which fluctuates in nucleotide sequence-specific patterns during the DNA translocation process. These patterns in electrical current are then computationally converted into base calls corresponding to the sequence of the translocated DNA molecule.

The ONT real-time sequencing technology offers unique advantages, which make it an attractive alternative to de-facto standard sequencing platforms for pathogen surveillance. These advantages include longer sequencing read lengths of up to several thousand base pairs, lower equipment and per run cost, a reduction in turnaround time from days to 4-6 hours[8-10], high portability due to the lack of optics and the tight integration of detectors with the consumables, and less complex sample preparation amenable to clinical and field-based laboratories[11].

One downside of ONT sequencing, however, resides with the platform specific error rate of sequencing reads. While long sequencing reads are advantageous for the de-novo assembly of pathogen genomes, ONT reads have a lower nucleotide fidelity than standard platforms and might therefore require specialized bioinformatics to deliver comparable data quality.

In order to further the utility of the ONT platform, we here present the validation of an end-to-end workflow for fast, cost effective whole genome sequencing of bacterial isolates and data interpretation via automated cloud based bioinformatics.

3. Multicenter validation of ONT sequencing of bacterial isolates

Study design

A multicenter study approach was followed in order to establish and validate best practice protocols for ONT-based WGS of bacterial isolates.

To realize the advantages of affordable, centralized and scalable compute resources, we have evaluated the bioinformatics pipelines and databases as implemented on AREScloud. AREScloud is a web application combining Ares Genetics´ proprietary AMR reference database ARESdb with state-of-the-art bioinformatics tools for infectious disease testing. While the application has previously been validated for microbial identification & typing from short-read Illumina data[12], this study extended the assessment of the application's utility beyond short-read data to long-read ONT data.

The study objective was to assess the analytical performance for pathogen identification, microbial subtyping, plasmid detection, AMR marker detection, and predictive Antimicrobial Susceptibility Testing (pAST).

Figure 1 and Table 2 illustrate the study design and sample designations. Table 1 describes the reference species representing the ground truth for the study. The seven reference materials were previously used for the validation of Illumina based WGS workflows and have been extensively characterized[12].

Sample ID	Reference Species	Label	Reference ID	Database (Accession)
ID244	*Acinetobacter baumannii*	ACB	ATCC BAA-1605	ATCC Genome Portal (n.a.)
ID245	*Pseudomonas aeruginosa*	PAP	ATCC 27853	ATCC Genome Portal (n.a.)
ID246	*Klebsiella pneumoniae*	KPM	ATCC 700603	ATCC Genome Portal (n.a.)
ID247	*Staphylococcus aureus*	SAS	ATCC BAA-2312	ATCC Genome Portal (n.a.)
ID248	*Escherichia coli*	ECO	ATCC 35218	ATCC Genome Portal (n.a.)
ID249	*Enterococcus faecium*	EFM	ATCC 700221	RefSeq (GCF_001594345.1)
ID250	*Enterobacter cloacae*	ENC	NCTC 13464	RefSeq (GCF_900447465.1)

Table 1: Reference species serving as ground truth for the validation of the ONT isolate sequencing workflow.

6. Allard, M. W. et al. Practical Value of Food Pathogen Traceability through Building a Whole-Genome Sequencing Network and Database. J Clin Microbiol 54, 1975–1983 (2016).
7. Jain, M. et al. Improved data analysis for the MinION nanopore sequencer. Nat Methods 12, 351–356 (2015).
8. Charalampous, T. et al. Nanopore metagenomics enables rapid clinical diagnosis of bacterial lower respiratory infection. Nat Biotechnol 37, 783–792 (2019).
9. Schmidt, K. et al. Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing. J Antimicrob Chemoth 72, 104–114 (2016).
10. Greninger, A. L. et al. Rapid metagenomic identification of viral pathogens in clinical samples by real-time nanopore sequencing analysis. Genome Med 7, 99 (2015).
11. Quick, J. et al. Real-time, portable genome sequencing for Ebola surveillance. Nature 530, 228–232 (2016).
12. Lepuschitz, S. et al. Analytical Performance Validation of Next-Generation Sequencing Based Clinical Microbiology Assays Using a K-mer Analysis Workflow. Front Microbiol 11, 1883 (2020).

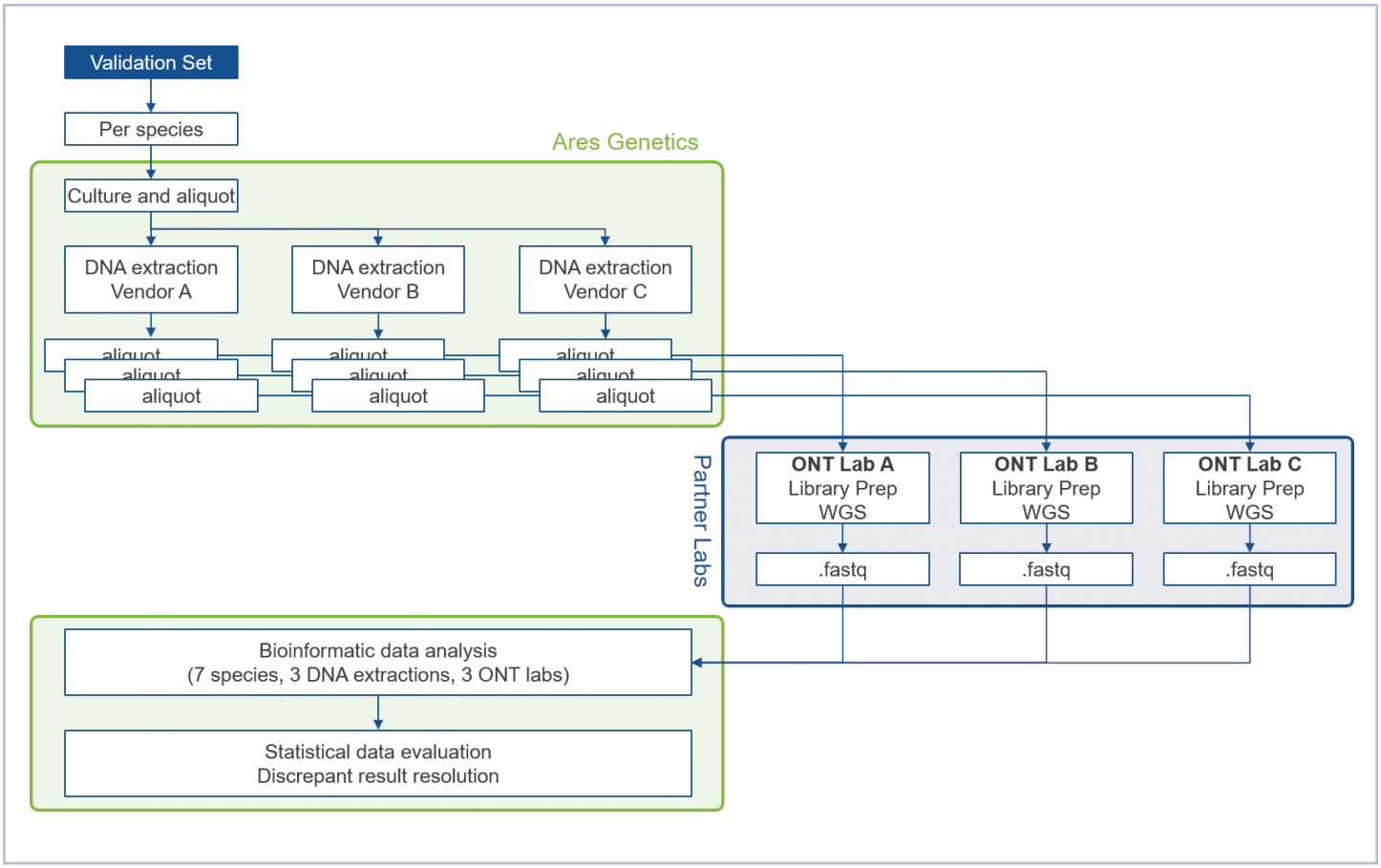

Figure 1: Validation of the ONT isolate sequencing workflow. The study aim was to establish and validate best practices for isolate whole genome sequencing based on ONT. Study endpoints included pathogen identity, subtyping (cgMLST), plasmid detection, AMR marker detection, and predictive Antimicrobial Susceptibility Testing (pAST).

Reference species, internal ID: **ID244..245..246..247..248..249..250**			
Extraction Kit / Partner Lab	**Clinical Partner Lab (A)**	**Commercial Lab (B)**	**Commercial Lab (C)**
DNeasy PowerSoil (PS)	[internal ID]-A-PS	[internal ID]-B-PS	[internal ID]-C-PS
QIAsymphony DSP DNA (BC)	[internal ID]-A-BC	[internal ID]-B-BC	[internal ID]-C-BC
MagAttract HMW DNA (HW)	[internal ID]-A-HW	[internal ID]-B-HW	[internal ID]-C-HW

Table 2: Sample designations. Reference materials were extracted using three different kits and split into aliquots. One aliquot per extraction kit and species were sent to each of the participating partner labs for QC, ONT library preparation and sequencing.

Sample preparation and ONT sequencing

Each reference species was cultured and subjected to DNA extraction using three different kits, including the QIAGEN DNeasy PowerSoil Pro kit (PS), the QIAGEN MagAttract HMW DNA kit (HW), and the QIAsymphony DSP DNA Mini Kit (BC), which is run on the QIAsymphony system for automated liquid handling. Each extract was split into three aliquots, one of each per reference species was sent to the participating partner labs, resulting in 3 x 7 samples per lab. The partners were comprised of one clinical microbiology lab (A) and two commercial sequencing labs (B, C). Starting with 400 ng of genomic DNA per aliquot, each carried out sample QC, library preparation using the ONT Rapid Barcoding kit, WGS on the ONT platform using ONT R10.3 flow cells and the Guppy 4.3+ software for base calling.

The resulting FASTQ files were returned to Ares Genetics for subsequent bioinformatics analysis on ARESCloud and for the statistical evaluation and resolution of discrepant results.

Statistical interpretation

Reproducibility

Definitions and calculations for accuracy and repeatability/reproducibility as described by Kozyreva et al. (2017)[13] were adapted for pathogen identification, subtyping via cgMLST, AMR marker detection, predictive AST and plasmid typing.

Analytical sensitivity, limit of detection

The analytical performance of applied assays (taxonomic identification, AMR marker detection, and predictive AST) was assessed by downsampling of all validation samples. Required basepair counts for sequencing depths of 40×, 30×, 20×, and 10× ONT sequencing were determined based on the size of the reference genome for each species. The Limit of Detection (LOD) was defined as sensitivity ≥90%. As ground truth served hereby analysis results obtained using the respective public reference genomes.

Diagnostic sensitivity and specificity

Diagnostic sensitivity and specificity were assessed for predictive AST. For interpretation, results are defined as likelihoods and had to be classified either as true positives (TP), false positives (FP), true negatives (TN), or false negatives (FN) in comparison to the phenotypic broth micro-dilution (BMD) test results for the extensively characterized reference strains, which was done in concordance to recommendations by Kozyreva et al. (2017)[13]. Sensitivity for predictive AST was determined based on the ratio of numbers of compounds against which resistance was predicted for the validation data set divided by the number of compounds against which resistance was confirmed in phenotypic BMD tests. Specificity for predictive AST was determined based on the ratio of true negative resistance predictions as identified by BMD testing divided by the total number of negative cases.

4. Results

The ONT sequencing data from each participating partner lab were processed using the web-based ARESCloud application. The user friendly application automates the bioinformatics analysis of WGS data from Illumina and ONT sequencing platforms and conducts sequence QC, de-novo genome assembly, assembly QC, pathogen identification, typing using MLST and cgMLST schemes as applicable, AMR profiling, plasmid typing, and predictive AST.

Sequencing quality and genome assembly

The quality of de-novo genome assemblies was compared across partner labs and extraction methods (see Table 3). Notably, regardless of partner lab and extraction method, base calling quality (measured on the PHRED scale) of ONT data was with an average PHRED score of 11.43 below Illumina data, confirming the higher per base error probability.

Samples processed with the QIAGEN DNeasy PowerSoil Pro kit (PS) consistently yielded shorter ONT read lengths and a lower N50 contig length compared to the QIAGEN MagAttract HMW DNA kit (HW), and QIAsymphony DSP DNA kit (BC), likely due to the more aggressive cell wall disruption and DNA shearing in case of the PS kit. However, the PS kit proved more robust: no sample drop outs occurred and compared to HW/BC the resulting yields for ONT reads and bases were higher (29.2%/33.4% more reads, 0.1%/8.4% more bases). In contrast, three replicates had to be excluded due to low yield for the HW kit and one replicate for the BC kit, across all partner labs.

Partner Lab	Extraction	Reads	Bases	N50	PHRED	Median Length
A	PS	2115034	7768310140	6087	11.43	2524
B	PS	2548646	8881712547	5843	11.05	2319
C	PS	1193160	8012554326	5495	11.97	2319
A	HW	1578984	8334985587	10689	11.15	2949
B	HW	741671	4549672945*	12110	10.20	3571
C	HW	1224862	8086766586	12803	11.92	3933
A	BC	905367	5222377454*	10605	11.36	3487
B	BC	1599503	8685278690	10329	11.98	3149
C	BC	1397733	8689445038	11604	11.83	3769

Table 3: Sequencing quality and de-novo assembly statistics. *Sequencing run time was 72 hours, except for partner lab A-BC and B-HW with ~50 hours, which resulted in a lower base yield.

13. Kozyreva, V. K. et al. Validation and Implementation of Clinical Laboratory Improvements Act-Compliant Whole-Genome Sequencing in the Public Health Microbiology Laboratory. J Clin Microbiol 55, 2502–2520 (2017).

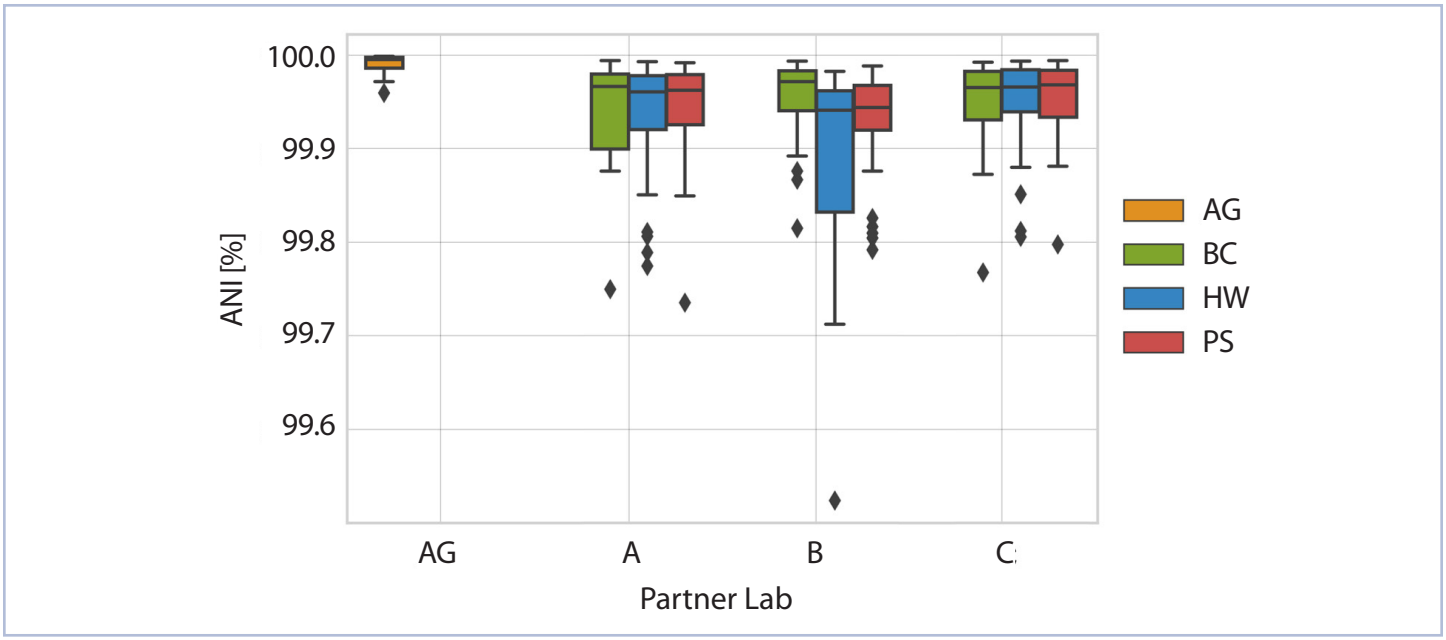

Figure 2: After de-novo assembly the average nucleotide identity (ANI) of the ONT- and Illumina NGS-derived assemblies to the public reference genomes was assessed across all reference species, participating partner labs and used DNA extraction methods. ANI values represent % identity. AG refers to the Illumina NGS-derived reference genomes previously published by Ares Genetics. While significant differences in read characteristics and base quality/error rates between ONT and Illumina data persist, both NGS data types produced high quality assemblies leading to high values for ANI.

Despite a lower base quality of ONT compared to Illumina, the quality of the resulting de-novo genome assemblies was consistently high (see Figure 2). The choice of partner lab or extraction method did not significantly affect the average nucleotide identity (ANI) of the ONT assemblies with the public reference genomes, in contrast to the choice of sequencing platform (as confirmed by Kolmogorov-Smirnov test, $p < 0.05$). Notably, the ANI significantly correlated with sequencing depth (40x-10x) across vendors and extraction methods (data not shown, Spearman rank-order correlation coefficient, $r = 0.6$, $p < 0.05$).

Analytical performance of ONT isolate sequencing

Across partners and extraction methods, pathogen identification based on ONT data was highly reliable with accuracy (in % concordance) and reproducibility consistently reaching 100%. The accuracy for AMR marker detection based on ONT data (see Figure 3) was comparable to Illumina data (97%) and ranged between 93% - 96% (PS), 92% - 94% (HW), and 94% - 95% (BC) for samples originating from the three different extraction methods.

Partner Lab	Extraction	Identification	cgMLST*	AMR Marker Detection	Predictive AST	Plasmid Typing
AG (Illumina)	AG	100% (7/7)	66% (4/6)	97%	97% ± 5.6%	88%
A	PS	100% (7/7)	0% (0/6)	94%	95% ± 5.9%	84%
B	PS	100% (7/7)	0% (0/6)	93%	90% ± 11.8%	84%
C	PS	100% (7/7)	0% (0/6)	96%	97% ± 5.6%	90%
A	HW	100% (7/7)	0% (0/6)	94%	95% ± 5.9%	55%
B	HW	100% (7/7)	0% (0/6)	92%	96% ± 6.4%	77%
C	HW	100% (7/7)	0% (0/6)	93%	97% ± 5.6%	85%
A	BC	100% (7/7)	0% (0/6)	94%	97% ± 5.6%	84%
B	BC	100% (7/7)	0% (0/6)	95%	97% ± 5.6%	88%
C	BC	100% (7/7)	0% (0/6)	94%	97% ± 5.6%	90%
Reproducibility		100% (63/63)	0% (0/54)	97% (2489/2561)	96% (175/182)	100% (107/107)

Table 4: Accuracy and reproducibility for ONT sequencing of the validation samples.

*The only significant impact of the lower base call accuracy of ONT compared to Illumina was observed for subtyping via cgMLST.

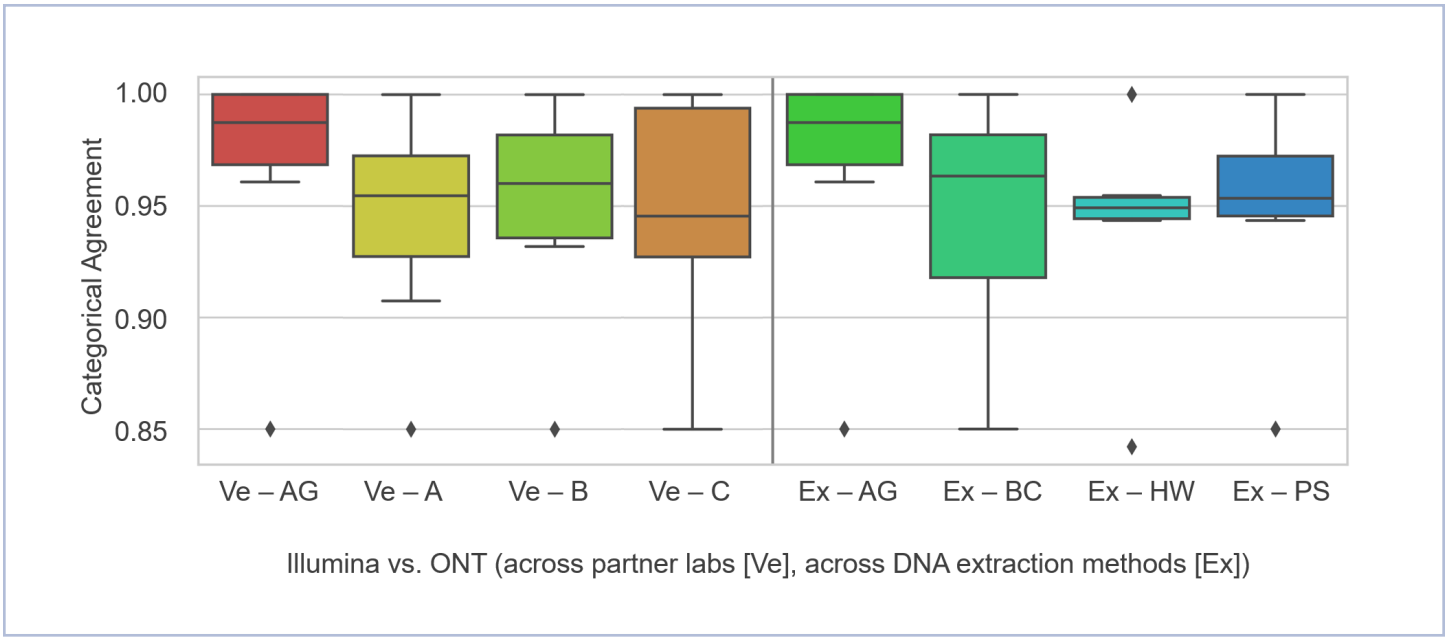

Figure 3: Categorical agreement in % of ONT based AMR marker detection with the public reference across partner labs [Ve] (left) and DNA extraction methods [Ex] (right).

Similarly, the accuracy for predictive AST based on ONT data was comparable to Illumina data (97%) and ranged between 90% - 97% (PS), 96% - 97% (HW), and 97% (BC). Also plasmid typing based on ONT data performed similar to Illumina data (88%) with accuracies of 84% - 90% (PS), 55% - 85% (HW), and 84% - 90%. The diagnostic sensitivity, specificity and positive predictive value (PPV/precision) for ONT-based predictive AST when compared to triplicate phenotypic BMD measurements reached with few exceptions 100% as shown in Table 5.

Deviations

For the reference species *Klebsiella pneumoniae* (ID246) predictive AST consistently generated false-susceptible predictions for aztreonam (a monobactam), but achieved 100% categorical agreement and a PPV of 100% for resistance predictions against the remaining compounds. Given that the false-susceptible prediction was consistently reported for ONT-derived and Illumina-derived predictive AST results as well as for the public reference genome (ATCC 700603) of this reference species, the underlying root cause remains unclear. The discrepancy could either be connected to the predictive AST model that lacks the relevant AMR marker(s), or to a discrepancy between the published ATCC reference genome and the associated phenotypic AST measurements of the ATCC culture.

For the reference species *E. coli* (ID248) sequenced on ONT by partner lab A, the specificity and PPV for predictive AST for two out of the three tested extraction methods (PS, BC) dropped to 90% and 50% respectively. The likely root cause are false positive signals due to a contamination at this partner lab with an *E. coli* strain which differs from the reference species. This assumption is supported by assembly QC, pathogen identification, and plasmid typing results (data not shown).

Reproducibility was consistently high for pathogen detection (100%), AMR detection (97%), predictive AST (96%), and plasmid typing (100%).

The only significant impact of the lower base call accuracy of ONT compared to Illumina was observed for subtyping via cgMLST (see * in Table 4). None of the ONT assemblies matched the complex type (CT) of the corresponding reference species. For six of the seven reference species the percentage of discordant loci per species compared to public ATCC/RefSeq reference genomes was above the cgMLST schema-specific CT distance above which an isolate is assigned a new CT. For one reference species, *Enterobacter cloacae* (ID250), cgMLST schema were not available.

Vendor	Extraction	Metric	ID244 (n = 4)	ID246 (n = 9)	ID247 (n = 5)	ID248 (n = 11)
ATCC	-	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	100%
		PPV	100%	100%	100%	100%
AG (Illumina)	PS	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	100%
		PPV	100%	100%	100%	100%
A	PS	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	90%
		PPV	100%	100%	100%	50%
B	PS	Sensitivity	100%	67%	100%	90%
		Specificity	100%	83%	100%	100%
		PPV	100%	67%	100%	80%
C	PS	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	100%
		PPV	100%	100%	100%	100%
A	HW	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	90%
		PPV	100%	100%	100%	50%
B	HW	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	100%
		PPV	100%	100%	100%	100%
C	HW	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	100%
		PPV	100%	100%	100%	100%
A	BC	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	100%
		PPV	100%	100%	100%	100%
B	BC	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	100%
		PPV	100%	100%	100%	100%
C	BC	Sensitivity	100%	67%	100%	100%
		Specificity	100%	100%	100%	100%
		PPV	100%	100%	100%	100%

Table 5: Performance of predictive AST – diagnostic sensitivity, specificity and positive predictive values (PPV) relative to phenotypic broth microdilution results (BMD) shown for four of the seven reference species. The total number of compounds tested is given in brackets in the column header.

The LOD of ONT sequencing

We examined the effect of ONT sequencing depth (measured as fold coverage of the assembled genomes with ONT reads) on sensitivity. A lower limit of detection (LOD) was defined here as the coverage at which the sensitivity reaches ≥ 90% which occurred on average at ~20x ONT coverage.

The LOD thresholds determined for pathogen identification, AMR marker detection, and predictive AST results were 10x, 20x, and 20x coverage respectively.

When predicting or detecting antibiotic resistance, the avoidance of false-susceptible calls also referred to as "very major errors" (VME) is of particular importance. At 100% sensitivity the rate of VME reaches 0%. Figure 4 therefore explores the coverage at which the sensitivity of AMR marker detection approaches 100%. The average sensitivity of AMR marker detection across partner labs and DNA extraction methods reaches ≥ 99% at approximately 40x ONT coverage.

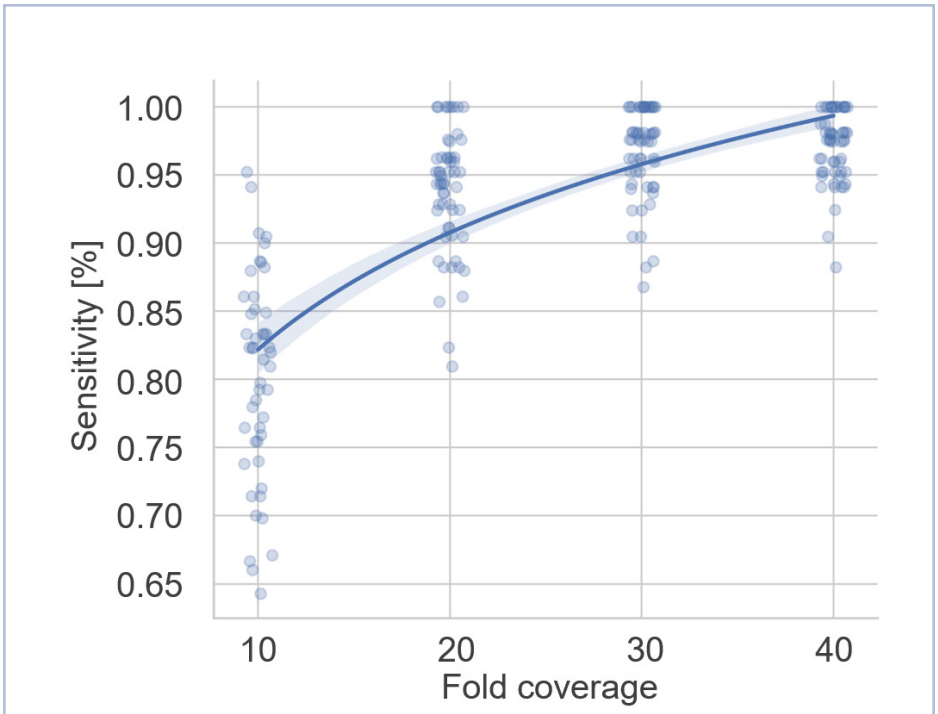

Figure 4: AMR marker profiling, sensitivity as a function of ONT coverage.

5. Conclusion

We have validated the performance of ONT sequencing of clinical isolates for the detection and surveillance of AMR pathogens.

Despite a significantly reduced base call accuracy compared to Illumina data, the workflows presented here for ONT sequencing have proven highly accurate and reproducible when coupled with the AI-powered AREScloud analysis platform to deliver critical clinical information including pathogen identity, AMR marker profiles, predictive AST based on genomic data, and plasmid type profiles.

The workflows validated here for ONT sequencing of bacterial isolates represent a faster, more cost effective, accurate and reproducible alternative to the de-facto standard NGS platforms for genomic antimicrobial surveillance.