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Nanopore ultra-long read sequencing technology for antimicrobial resistance detection in *Mannheimia haemolytica*

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ABSTRACT

Disruptive innovations in long-range, cost-effective direct template nucleic acid sequencing are transforming clinical and diagnostic medicine. A multidrug resistant strain and a pan-susceptible strain of *Mannheimia haemolytica*, isolated from pneumonic bovine lung samples, were sequenced at 146× and 111× coverage, respectively with Oxford Nanopore Technologies MinION. *De novo* assembly produced a complete genome for the non-resistant strain and a nearly complete assembly for the drug resistant strain. Functional annotation using RAST (Rapid Annotations using Subsystems Technology), CARD (Comprehensive Antibiotic Resistance Database) and ResFinder databases identified genes conferring resistance to different classes of antibiotics including β-lactams, tetracyclines, lincosamides, phenicols, aminoglycosides, sulfonamides and macrolides. Resistance phenotypes of the *M. haemolytica* strains were determined by minimum inhibitory concentration (MIC) of the antibiotics. Sequencing with a highly portable MinION device corresponded to MIC assays with most of the antimicrobial resistant determinants being identified with as few as 5437 reads, except for the genes responsible for resistance to Fluoroquinolones. The resulting quality assemblies and AMR gene annotation highlight the efficiency of ultra-long read, whole-genome sequencing (WGS) as a valuable tool in diagnostic veterinary medicine.

1. Introduction

Emergence of antimicrobial resistance (AMR) among the most important bacterial pathogens is recognized as a major public health concern. Not only has AMR emerged in hospital environments, it is often identified in community settings, in livestock feedlots and in aquaculture and crop production, suggesting an ever-increasing range of reservoirs of antibiotic-resistant bacteria (FAO, 2016; FAO, 2017; Thanner et al., 2016; WHO, 2018). Bacterial response to the antibiotic "attack" is a prime example of genetic adaptation through mutations, acquisition of genetic material and alteration of gene expression with fitness consequence of the pathogen (Hughes and Andersson, 2015). As a result, understanding the genetic basis of antimicrobial resistance is of paramount importance to designing strategies to curtail its spread, as well as to devise innovative therapeutic approaches against multidrug-resistant organisms (Munita and Arias, 2016).

The increasing population of pet and farm animals has led to a higher frequency of human-animal interactions that can result in a greater probability of acquiring zoonotic infections including drug resistant bacterial infections (Ghasemzadeh and Namazi, 2015). One of the major deficiencies in veterinary medicine is the lack of validated data to determine minimum inhibitory concentration (MIC) breakpoints for drug-microbe-host combinations, based on which scientifically sound interpretations can be made regarding whether a pathogen is susceptible or resistant to a specific drug. The large diversity of domestic and exotic animal species and their numerous microbial pathogens pose significant challenges to developing reliable interpretation criteria for all antimicrobial drugs.

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Recently, whole-genome sequencing (WGS) has become an invaluable tool to characterize antibiotic resistance (Didelot et al., 2012; Fricke and Rasko, 2014; Gordon et al., 2014; Hasman et al., 2014; Joensen et al., 2014; Tagini and Greub, 2017). In addition to bench-top sequencing platforms like Illumina, in 2014 Oxford Nanopore Technologies (ONT) released a portable MinION device through an early access program (the MinION Access Program). MinION devices can be powered by a standard USB3 port and the base-calling step that turns electrical signals into nucleotides is enabled by a cloud-computing platform (Jain et al., 2016). This portability provides sequencing capacity for diagnostic microbiology in the areas where the installation of desktop genomic sequencers might not be feasible, like quarantine areas and the International Space Station (Castro-Wallace et al., 2017, Lemon et al., 2017, Li et al., 2018, Quick et al., 2015). The landmark case for diagnostic investigation of pathogen genomes was the Ebola outbreak in Guinea (Quick et al., 2016). Using three MinION devices and four laptop computers, 148 sequencing runs were conducted to cover 142 samples. A rapid turnaround was demonstrated as amplification, library preparation and sequencing was completed within 24 h (Lu et al., 2016). Currently, the sequencing capacity of MinION has reached about 450 bases per second; as the throughput and speed continue to increase, the ONT platform might become suitable for real-time pathogen surveillance and clinical diagnostic applications (Jain et al., 2018).

In veterinary medicine, approaches such as mass spectrometry and multiplexed PCR are commonly used for diagnostic applications (Singhal et al., 2015). WGS, however, has yet to be adopted as a part of routine diagnostic procedures. In this report we describe the use of WGS for evaluating AMR of *Mannheimia haemolytica,* an important bacterial pathogen of cattle causing bovine respiratory disease (BRD). The annual loss to the cattle industry from BRD is estimated to be around one billion USD in North America (Noyes et al., 2015). Increases in antimicrobial resistant *M. haemolytica* over the years have been recognized (Clawson et al., 2016; Lubbers and Hanzlicek, 2013) and the high prevalence of multidrug resistant *M. haemolytica* is being increasingly recognized (Woolums et al., 2018). Considerable evidence also shows that *M. haemolytica* are phenotypically and genetically diverse, and might be comprised of sub-species or various strain-types (Clawson and Murray, 2014). In this manuscript WGS-based detection of AMR of two *Mannheima* strains was determined using ONT's MinION devices. Potential AMR genes can be determined with assemblies constructed with as few as 5347 ONT ultra-long reads. The genetic AMR of these strains was further confirmed by the determination of MICs of the antibiotics. Using this comparative study as an example, the feasibility and effectiveness of integrating ONT's MinION technology in clinical investigations was explored.

2. Materials and methods

2.1. DNA extraction for bacterial isolates

M. haemolytica OADDL-1 was isolated from the lung of a 3-year-old bull and *M. haemolytica* OADDL-2 was isolated from the lung of a calf of unknown age. Both animals had a history of sudden death and a histologic diagnosis of severe acute fibrinous pleuropneumonia. The calf from which the resistant *M. haemolytica* isolate was obtained also had a history of antimicrobial treatment with tilmicosin and enrofloxacin. Bacterial isolates were cultured on 5% sheep blood agar (Hardy Diagnostics, Santa Maria, CA, Springsboro, Ohio); five to 10 colonies of bacteria were carefully selected to avoid agar contamination and then suspended in Tris EDTA buffer. Genomic DNA was extracted using the OMEGA Bio-tek EZNA® bacterial DNA kit D3350–01 protocol (OMEGA Bio-tek, Norcross, GA).

2.2. MIC (minimum inhibitory concentration)

MIC values of *M. haemolytica* isolates were determined on the Sensititre automated system (Thermo Scientific, Waltham, MA, USA), using the Bovine/Porcine panel (Thermo Scientific). Interpretations regarding susceptibility or resistance to antimicrobial were made based on CLSI VET08 standard, 4th edition (CLSI, 2018).

2.3. MinION library preparation and barcoding

Library preparation was performed following the procedures outlined for the SQK-LSK208 sequencing kit and barcoded for multiplexing using the complimentary EXP-NDB002 barcoding kit (Oxford Nanopore Technologies, United Kingdom) with the following protocol adjustments. A total of 1.5 µg of genomic DNA (gDNA) from each bacterial isolate was sheared in g-tubes (Covaris, Woburn, Massachusetts, USA) at 4200 RPM for a targeted fragment size of 20 kb. End-repair was performed following the recommended protocol of the manufacturer for Ultra II End-prep enzyme mix (NEB, Ipswich, Masschusetts). Adapter ligation reaction incubations were increased to 15 min. Bead clean-ups used 0.4× AMPureXP beads (Beckman Coulter, Brea, CA) for additional size selection, thereby removing smaller DNA fragments and maintaining longer ones; elutions were performed at 37 °C for 20 min. DNA concentration of the library was quantified using Quant-IT PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific), using a Synergy H1, hybrid multi-mode microplate reader (BioTek, Winooski, VT), so as to confirm yields above 200 ng. In total, two MinION barcoded libraries were prepared (listed as Library A and B in Table 2).

2.4. MinION sequencing

Two MinION R9.4 SpotON flow cells, labeled FAB 42646 and FAB 42472, were used for sequencing. The MinKNOW GUI application (version 1.4.2) was used to perform a platform quality check to ensure flow cell quality. Flow cells were primed as instructed prior to sequencing. MinKNOW version 1.4.2 was executed to sequence using the protocol script NC_48Hr_Sequencing_Run_FLO-MIN105_SQK-LSK208.py. Both libraries were sequenced for approximately 48 h, with no live base-calling performed.

2.5. Albacore Base-calling

Due to discontinued support of 2-D reads by ONT and Nanopolish (0.6-dev), sequenced reads were subsequently base-called using the newest version of Albacore v 2.1.0. for 1-D base-calling, where both template and complement strands were considered as individual reads.

Albacore performs base-calling on the raw reads using event detection to categorize the signal-level data, after which the event detection is compared to existing models and associated with a nitrogenous base. All raw reads were evaluated and filtered for a cut-off quality score of seven. Base-called reads were further split into their respective barcode folders of *M. haemolytica* OADDL-1 and OADDL-2 for downstream analyses. All reads were trimmed using Porechop (v0.2.1, http://github.com/rrwick/Porechop) to remove barcoded adapter sequences found in the read files.

2.6. Genome assembly using Canu

Genome assembly was performed using the software package Canu (version 1.6). Canu implements the overlap-layout-consensus method and is partitioned into three primary stages: correction, trimming and assembly (Koren et al., 2017). Default Canu command and parameters for assembly were used with a suggested genome size of 2.7 million bp. QUAST was used to evaluate assembly statistics (Gurevich et al., 2013).

2.7. Genome polishing with Nanopolish

Typically, Nanopore sequencing reads are prone to deletion errors, particularly in homopolymer regions (Sárközy et al., 2017). In this study, Nanopolish (0.6-dev), a software program that utilizes a trained hidden Markov model to improve the final consensus assembly, was used for error correction. Reads were aligned back to the Canu assembly using bwa (Li, 2013), with the *–x ont* option for ONT reads; Samtools was used to sort and index the reads (Li et al., 2009). Nanopolish was used to compute a polished consensus genome by correcting substitution, insertion and deletion errors that may have occurred during sequencing or base-calling. Following the github protocol for Nanopolish (https://github.com/jts/nanopolish), the default commands and parameters for computing a consensus sequence were used, with thread count set to four and parallel count set to eight.

2.8. RAST, CARD and ResFinder annotation

Predicted genes were called from the assembled contigs using the prokaryote gene calling software Prodigal (Hyatt et al., 2012). All amino acid sequences were annotated using a combination of homology search with NBCI blast+ (Camacho et al., 2009), and domain identification using hmmscan (Finn et al., 2015) with the PFAM v28.0 database (Finn et al., 2016). AMR gene determination was performed using NCBI blast+ with three different tools and databases: RAST (Rapid Annotation using Subsystem Technology, (Overbeek et al., 2014)), CARD (the Comprehensive Antibiotic Resistance Database, (Jia et al., 2017, McArthur et al., 2013)) and ResFinder (Zankari et al., 2012). RAST is a web-based service that offers rapid annotation of prokaryotic genomes (http://rast.nmpdr.org/rast.cgi), using an automated system that requires a genome submission. Similarly, ResFinder is also a web-based service (https://cge.cbs.dtu.dk/services/ResFinder), where AMR genes are identified with the threshold and minimum length set to 90% and 60%, respectively. Further, a second criterion was used to remove any annotated genes that were below gene coverage of 40%. The CARD database (http://arpcard.mcmaster.ca), which integrates disparate molecular and sequence data, also provides Antibiotic Resistance Ontology (ARO) (McArthur et al., 2013).

2.9. Characterization of Mobile genetic elements in the M. Haemolytica genomes

Identification of elements acquired from transformation, inserted *via* transduction, or transferred by conjugation is necessary for understanding the dynamics of the studied microbial genome. VRProfile was used to identify mobile genetic elements, like prophages and conjugative elements in the genome (Li et al., 2018a). This service uses a back-end database, MobilomeDB, a bacterial database that depends on publically available data, like ICEberg for integrative and conjugative elements (Bi et al., 2012), to identify transfer-related genes and AMR determinates. Using their web service, contigs for both strains were uploaded to VRProfile, and all available options for “Gene cluster”, “Single gene” and “Genomic island-like region” were selected. Insertion sequences were also identified, using ISfinder (Siguier et al., 2006), as part of the analysis done by VRProfile.

2.10. Genome sub-sampling for AMR gene identification

To evaluate the degree to which multiplexity would affect assembly quality for AMR detection, sub-sampling of reads was conducted. Six sub-divisions of randomly sampled reads were created from the total sequencing reads; each subdivision represented 1/10th, 1/6th, 2/6th, 3/6th, 4/6th and 5/6th of the total reads. Five replicates were generated for each sub-division of reads. Replications were created to examine the variability derived from random sampling of raw sequencing reads. AMR predictability was then assessed by the quality of the genome assemblies and annotation methods described earlier. For each sub-divided group of reads and replicates, assemblies were generated using Canu v1.6. Quality of the assemblies was evaluated using QUAST and polished with Nanopolish. And, *in silico* determination for AMR genes was conducted by CARD and ResFinder.

Table 1
Phenotypic susceptibility testing results for bacterial strains *M. haemolytica* OADDL-1 and *M. haemolytica* OADDL-2.

Antibacterial agent	Antibacterial class	*M. haemolytica* OADDL-1 (MIC -µg/mL)	*M. haemolytica* OADDL-2 (MIC -µg/mL)
Ampicillin	β-lactams	NI (≤ 0.25)	R (> 16)
Ceftiofur	β-lactams	S (≤ 0.25)	S (≤ 0.25)
Clindamycin	Lincosamides	NI (= 8)	NI (> 16)
Danofloxacin	Fluoroquinolones	S (≤ 0.12)	R (> 1)
Enrofloxacin	Fluoroquinolones	S (≤ 0.12)	R (> 2)
Florfenicol	Phenicols	S (= 0.5)	R (> 8)
Gentamicin	Aminogycosides	NI (= 2)	NI (> 16)
Oxytetracycline	Tetracyclines	S (≤ 0.5)	R (> 8)
Penicillin	β-lactams	S (= 0.25)	R (> 8)
Spectinomycin	Aminoglycosides	S (= 16)	R (> 64)
Sulfadimethoxine	Sulfonamides	NI (> 256)	NI (> 256)
Trimethoprim/ Sulfamethoxazole	Potentiated Sulfonamides	NI (≤ 2/38*)	NI (≤ 2/38*)
Tilmicosin	Macrolides	S (≤ 4)	R (> 64)
Tulathromycin	Macrolides	S (= 4)	R (> 64)
Tylosin	Macrolides	NI (= 32)	NI (> 32)

R = Resistant, S = Susceptible, NI = No interpretive criteria
* 2 µg/mL Trimethoprim and 38 µg/mL Sulfamethoxazole (1:19 ratio).

3. Results

3.1. AMR Phenotyping by MIC

Details of the MIC results are listed in Table 1. Overall, *M. haemolytica* OADDL-1 was found to be susceptible to the antimicrobials that have CLSI interpretative criteria. *M. haemolytica* OADDL-2 was resistant to most antimicrobials except ceftiofur. For antimicrobials with no CLSI interpretative criteria (NI), the MIC values for OADDL-2 were higher than those of OADDL-1. However, both OADDL-1 and OADDL-2 showed similar MIC levels for sulfadimethoxine and sulfamethoxazole-trimethoprim (Table 1). MIC values for sulfadimethoxine were high for both OADDL-1 and OADDL-2 indicative of resistance; as for sulfamethoxazole-trimethoprim the MIC values were lower for both *M. haemolytica* strains.

3.2. Sequencing and base-calling

Initial MinION sequencing yielded 138,689 and 116,073 raw reads for flow cell FAB 42646 and FAB 42472, respectively, for a total of 254,762 reads. As shown in Table 2, Albacore 1-D base-calling resulted in 53,478 reads that passed the quality filter for OADDL-1, and 28,162 total reads for OADDL-2. The mean read length for OADDL-1 was 7562 bp for flow cell FAB 42646 and 6969 bp for FAB 42472 with an N50 value at 6807 for flow cell FAB 42646 and 8796 for flow cell FAB 42472. The mean read length for *M. haemolytica* OADDL-2 was 11,611 for FAB 42646 and 10,629 for FAB 42472 with an N50 of 17,018 bp for FAB 42646 and 15,722 bp for FAB 42472 (Table 2).

3.3. Genome assemblies and genome polishing

Genome assembly with Canu v1.6 of quality sequencing reads for OADDL-1 resulted in a single contig of 2,644,744 bp with a mean GC content of 41.19%. The draft genome assembly of OADDL-2 was

Table 2
1D base-called read statistics from Albacore for both *Mannheimia haemolytica* strains.

	Total Reads	Mean (bp)	Median (bp)	Min (bp)	Max (bp)	N25	N50	N75
M. haemolytica OADDL-1								
FAB 42646	27,304	7562	5267	210	77,585	19,083	11,974	6444
FAB 42472	26,174	6969	4818	69	65,212	17,903	10,837	5831
M. haemolytica OADDL-2								
FAB 42646	14,132	11,611	9132	241	75,460	23,851	17,018	9985
FAB 42472	14,030	10,629	8665	249	68,470	22,657	15,722	8982

Table 3
Assembly statistics generated by Canu and improved by Nanopolish.

	Number of contigs	Total length	Total length polished	N50	GC content (%)
M. haemolytica OADDL-1	1	2,644,744 bp	2,656,520 bp	–	41.19
M. haemolytica OADDL-2	5	2,804,188 bp	2,815,485 bp	2,381,899 bp	41.24

composed of five contigs totaling 2,804,188 bp with an average GC content of 41.24% (Table 3). Polishing (Nanopolish) resulted in increased genome length for both OADDL-1 (2,656,520 bp) and OADDL-2 (2,815,485 bp) (Table 3).

A global alignment to identify homologous genomic regions between OADDL-1 and OADDL-2 was performed using Mauve (Darling et al., 2004), and visualized using Circos (Krzywinski et al., 2009). In Fig. 1, regions with shared colors indicate sequence homology. Additionally, to confirm the genome alignment performed by Mauve, BLAST was performed aligning OADDL-2 contigs to OADDL-1. BLAST results showed that contig 1 covered in total 7% of OADDL-1 with an identity score of 99%, contig 2 covered 87% of OADDL-1 with an identity of 99%, contig 3 covered 9% of OADDL-1 with an identity of 99% and contig 4 covered only 1% of OADDL-1 with an identity score of 99%. Contig 5 yielded no alignment. These BLAST results correspond with the Mauve alignment as seen in Fig. 1. A BLAST search of the unaligned contig 5 returned a 99% identity and 80% coverage to a known plasmid *Pasteurella multocida* pCCK411, strain U—B411.

3.4. Genome annotation and AMR gene identification

Annotation by RAST for OADDL-1 revealed 3593 coding sequences, which are further categorized under subsystems such as carbohydrates metabolism, DNA metabolism, and subsystem of virulence, disease and defense for genes that confer resistance to antibiotics and toxic compounds as well as bacterocins, and ribosomally synthesized antibacterial peptides (Overbeek et al., 2014). Detailed information of RAST annotations is provided in Table 1S. The RAST annotation for OADDL-2 detected 4030 coding sequences, of which 45 were related to virulence, disease and defense (Table 2S). Among these 4030 coding sequences, the RAST annotation for OADDL-2 revealed multiple genes that confer resistance; these include β-lactamase (*blaROB-1*), Aminoglycoside N6'-acetyltransferase (*aph(6)-Id*), and Streptomycin 3"-O-adenylyltransferase (*aadA25*) (Table 2S).

It should be noted that the numbers of the coding sequences identified using MinION sequencing in this study were greater than other *M. haemolytica* genomes (Gioia et al., 2006; Klima et al., 2016), potentially due to the pseudogenes resulted from small frameshifting indels (Lerat and Ochman, 2005). For example, fig|6666666.262833.peg.19 and fig|6666666.262833.peg.20 both encode for a Glutathione s-transferase (EC 2.5.1.18), as seen in Table 1S, and fig|6666666.262838.peg.9 and fig|6666666.262838.peg.10 both encode for a Tfp pilus assembly protein (Table 2S).

ResFinder, which by default searches resistance genes that share 98% identity to the genes within the databases, was used to identify 14 AMR genes in OADDL-2 strain (Table 4); examples of these AMR genes are *blaOXA-2* and *floR*, on the contig 1 and contig 3, respectively (Fig. 2). AMR genes commonly found in *M. haemolytica* resistant strains (Clawson et al., 2016; Kehrenberg et al., 2005), such as *tet(H)* (Tetracyclines) and *aph(3')-Ia* (Aminoglycosides), were also identified in OADDL-2. Two *blaROB-1* genes were identified on the contig 5 (Fig. 2), which showed no alignment with OADDL-1 but, an 80% coverage with a known plasmid *Pasteurella multocida* pCCK411 from strain U—B411. The susceptible OADDL-1 returned no results for AMR genes on ResFinder.

Alternatively, the AMR gene homology search using CARD identified 14 AMR genes in the OADDL-2 resistant strain; similar AMR genes like *blaROB-1*, *tet(H)* and *aph(3')-Ia* and *aph(6)-Id* were present and have also been identified in two other published strains 89,010,807 N (GenBank accession no. CP011098) (Heaton et al., 2015) and M42548 (GenBank accession no. CP005383) (Eidam et al., 2013) (Table 5). AMR gene *aadA25* on contig 1 was only identified by CARD (Fig. 2). CARD search also returned a *macB* that confers resistance to macrolide antibiotics for both OADDL-1 and OADDL-2 (Table 4); however, this *macB* annotation only showed a sequence identity of 10.65% for OADDL-1, a value too low to identify as a gene.

3.5. Characterization of inserted elements in M. Haemolytica genomes

Identification of mobile elements by VRProfile identified 15 putative gene clusters on the chromosome of OADDL-2 (shown in Fig. 1). Eleven of these clusters were identified as being "prophage-like", while the remaining clusters were putatively identified as part of the type IV secretion system, responsible for DNA and protein transport. Two clusters of insertion sequences were found in OADDL-2 by ISfinder. Shown in Fig. 1, cluster 1 contains two insertion sequences, while cluster 2 has three (Fig. 1). Results for strain OADDL-1, identified 7 putative "prophage-like" clusters, one type IV secretion system cluster and no insertion sequences (Fig. 1).

3.6. Genome assembly quality assessments and AMR gene identification

To assess adequate amounts of long reads for microbial genome assembly and subsequent AMR gene detection, subsampling was conducted at 1/10th (5347 reads for OADDL-1 and 2816 for OADDL-2), 1/6th (8913 for OADDL-1, 4693 for OADDL-2), 2/6th (17,826 for OADDL-1, 9386 for OADDL-2), 3/6th (26,739 for OADDL-1, 14,079 for OADDL-1), 4/6th (35,652 for OADDL-1, 18,772 for OADDL-2), and 5/6th (44,565 for OADDL-1, 23,465 for OADDL-2) of the total reads used for original assembly. In OADDL-1, the base pair length was consistent across all replicates and partitions (mean = 2,649,246 bp), with standard deviations ranging from 9575 bp (1/6th) to 36,605 bp (1/10th). Mean GC content was also stable across all samples ranging from 41.16% to 41.23% (Table 5). Assembly quality began to decline at 2/

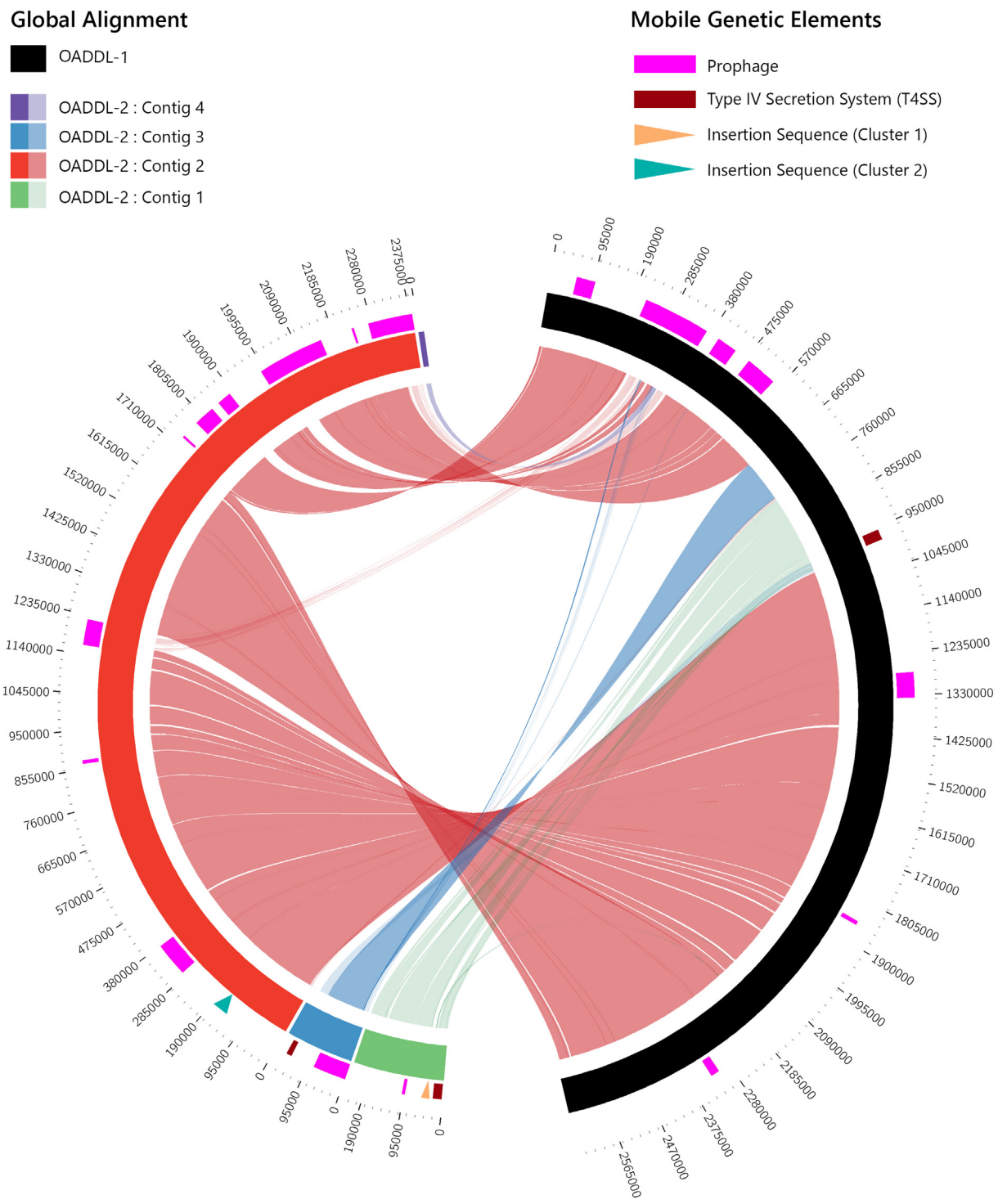


Fig. 1. Shared global genome alignment between *M. haemolytica* OADDL-1 and OADDL-2. The black semi-circle on the right represents the whole genome of OADDL-1 while the colored semi-circles on the left represent the individual assembled contigs for OADDL-2. Genomic regions that were aligned are connected by colored ribbons between the two genomes. A lighter color hue in the colored ribbons represents a reverse-complement match while the darker hue represents a normal match. Only 4 out of the 5 contigs assembled for OADDL-2 provided global alignment information. Additionally, 3 types of mobile genetic elements: prophages, insertion sequences (two clusters), and type IV secretion systems are visualized on their respective genomic positions; the track of these mobile elements is placed outside of the respective genomes.

6th of the original total reads (8913 reads); average N50 reduced to 1,864,819 bp, and the average number of mismatches with original assembly increased to 17,233 bp. At 1/10th of the total reads, the number of contigs increased 10 fold (19 to 24 contigs) and the N50 of the assemblies dropped to 351,563 bp.

For OADDL-2, subsampling of the reads generated comparable results. The average genome size of all subsampled assemblies was 2,777,982 bp, with a standard deviation of 24,724 bp. The mean GC content remained consistent among these assemblies with a range of 41.22% to 41.33% (Table 6). The putative plasmid identified through BLAST search in the final alignment was found in all assemblies. Partitioning of sequencing reads at 1/10th showed a rapid degradation in

Table 4
Identification of AMR genes with ResFinder and CARD databases.

AMR Genes	Database	Antibiotic class	*M. haemolytica* OADDL-2	*M. haemolytica* OADDL-1	*M. haemolytica* 89,010,807 N	*M. haemolytica* M42548
aadA25	CARD	Aminoglycosides	Present	–	–	–
aadA8b	ResFinder	Aminoglycosides	Present	–	–	–
ant(2″)-Ia	ResFinder/CARD	Aminoglycosides	Present	–	–	–
aph(3′)-Ia	ResFinder/CARD	Aminoglycosides	Present	–	–	Present
aph(3″)-Ib	ResFinder/CARD	Aminoglycosides	Present	–	–	Present
aph(6)-Id	ResFinder/CARD	Aminoglycosides	Present			Present
blaOXA-2	ResFinder/CARD	β-lactams	Present	–	–	–
blaROB-1	ResFinder/CARD	β-lactams	Present	–	–	–
erm(42)	ResFinder/CARD	MLSB	Present	–	–	–
floR	ResFinder/CARD	Phenicols	Present	–	–	–
macB	CARD	Macrolides	Present	–	–	–
mphD	CARD	Macrolides	Present	–	–	–
mph(E)	ResFinder	Macrolides	Present	–	–	–
msrE[a]	CARD	Macrolides	Present	–	–	–
msr(E)[a]	ResFinder	MLSB	Present	–	–	–
tet(H)	ResFinder/CARD	Tetracyclines	Present	–	Present	Present
sul2	ResFinder/CARD	Sulfonamides	Present	–	–	Present
strA	ResFinder	Aminoglycosides	–	–	–	Present

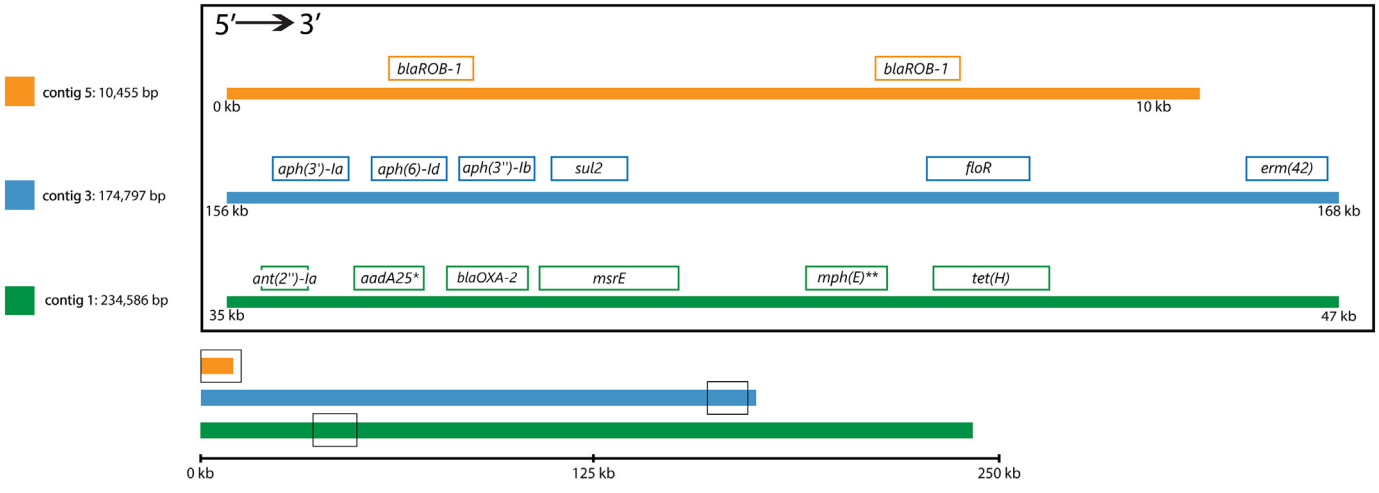
[a] In the CARD database, *msr(E)* is a synonym to m*srE*. Further, the *msrE* gene in the ResFinder is referenced to (Bonnin et al., 2013), whereas the *msr(E)* of CARD is referenced to (Zarrilli et al., 2008).

assembly quality: the number of contigs increased to at least 29 (maximum at 40), and the N50 value dropped to an average of 133,657 bp (Table 6).

Following annotation of all sub-sampled assemblies the results indicate that there were no observable changes compared to the original annotation for AMR genes found in OADDL-2. Using ResFinder and CARD, a total of 14 AMR genes were annotated for OADDL-2 with the original genome assembly that used the entire flow call capacity (Table 4). Shown in Table 6, the total number of sequencing reads required to arrive at the same AMR gene annotation was only as little as one-tenth of the total reads (2816 reads). The minor variation observed in the number of AMR genes annotated was due to the low gene coverage at AMR gene sites (see section 2.9 in the Materials and Methods). CARD AMR annotation varied slightly due to the low sequence identity of the *macB* gene. With the exception of the *macB* gene present at low coverage, there were no resistance genes found in OADDL-1 (Table 5).

4. Discussion

Massive parallel sequencing reactions, such as those generated by Illumina platforms (Goodwin et al., 2016), have remarkably increased the read numbers per run and transformed DNA molecular readers into diagnostic tools in virtually every field of biomedical research (Buermans and den Dunnen, 2014; Katsanis and Katsanis, 2013; Vrijenhoek et al., 2015). Among all available technologies, there is growing interest in the field of long-read sequencing, because of its capacity to span repetitive regions and facilitate contiguous assemblies (Goodwin et al., 2016; Tyson et al., 2018). Currently, the dominant platforms for direct typing long read fragments are Pacific Biosciences (PacBio) and Oxford Nanopore, both employing a single-molecule real-time approach. In addition to the improved assembly when kilobase sized reads are available (English et al., 2012), single molecular technologies are amplification free and thereby not hampered by PCR-based artifacts or GC-bias, giving a much more uniform coverage and span across GC-rich regions (Loomis et al., 2013). The feasibility of ONT has been studied in clinical plasmid isolates of *Escherichia coli* (Lemon et al., 2017), *Salmonella typhimurium* (Li et al., 2018a, 2018b), *Vibrio parahaemolyticus* (Li et al., 2018a, 2018b) and *Klebsiella pneumoniae* (Lemon et al., 2017; Li et al., 2018a, 2018b). Nanopore-only assembly yielded > 99% (0.2% mismatches and 0.5–0.6% gaps) consensus



Lorem ipsum

Fig. 2. AMR genes identified by ResFinder and CARD annotation for *M. haemolytica* OADDL-2 contigs. Out of the five contigs that were assembled, contigs 1, 3 and 5 contained genes coding for AMR and contig 2 and 4 procured no annotations.

Table 5
Assembly quality assessment of sequencing read subsets; results of *M. haemolytica* OADDL-1.

Subsample	Average base-pair length (std. dev.)	Contig count	Average N50 (std. dev.)	GC content (%)	Ave. Tot. mismatches (std. dev.)
1/10th of reads	2,628,537 (36,605)	19–24	351,563 (224,531)	41.2–41.3	30,993.6 (531.6)
1/6th of reads	2,624,083 (9575)	4–10	1,292,922 (518,613)	41.2–41.2	20,988.0 (131.9)
2/6th of reads	2,668,633 (13,312)	2–5	1,864,819 (652,239)	41.2–41.2	17,233.4 (231.7)
3/6th of reads	2,662,841 (13,994)	1–4	2,610,430 (41,359)	41.2–41.2	15,802.6 (365.1)
4/6th of reads	2,660,579 (14,754)	1–4	2,576,912 (71,524)	41.2–41.2	14,632.6 (185.2)
5/6th of reads	2,650,805 (16,078)	1–3	2,589,962 (111,922)	41.2–41.2	13,939.0 (181.4)
Mean	2,649,246 (25,931)	–	–	–	18,931.5 (271.2)

Table 6
Assembly quality assessment of sequencing read subsets and AMR gene identification; results of *M. haemolytica* OADDL-2.

Subsamples	Average base-pair length (std. dev.)	Contig count	Average N50 (std. dev.)	GC content (%)	AMR genes ResFinder (mean)	AMR genes CARD (mean)	Ave. Tot. mismatches (std. dev.)
1/10th of reads	2,619,204 (43,898)	29–40	133,657 (9800)	41.2–41.3	13–15 (13.6)	13–15 (14)	37,875.2 (599.8)
1/6th of reads	2,800,552 (16,129)	6–20	606,852 (471,233)	41.3–41.3	13–15 (13.6)	13–15 (14)	25,032.6 (559.5)
2/6th of reads	2,806,768 (16,792)	4–6	2,178,862 (407,453)	41.2–41.3	13–14 (13.4)	13–14 (13.6)	17,772.2 (292.8)
3/6th of reads	2,792,790 (14,509)	3–6	2,197,805 (532,021)	41.2–41.3	13–15 (13.6)	13–14 (13.6)	15,962.6 (145.8)
4/6th of reads	2,844,871 (40,342)	3–8	2,254,699 (412,158)	41.2–41.3	12–14 (13)	13–14 (13.6)	14,807.2 (221.7)
5/6th of reads	2,803,710 (16,678)	3–4	2,602,409 (349,320)	41.3–41.3	13–14 (13.2)	13–14 (13.4)	14,064.8 (173.2)
Mean	2,777,982 (24,724)	–	–	–	13.4	13.7	20,919.1 (332.1)

accuracy compared to Illumina MiSeq, and with polishing using MiSeq paired-end reads, assembly accuracy increased to ~ 99.9% (Lemon et al., 2017). However, when multiplexing 12 plasmids by Oxford's Rapid Barcoding Sequencing kit, the average accuracy of two plasmids compared to the known reference assembly turned out to be considerably lower (87% in (Li et al., 2018a, 2018b)). In Wick et al. (2017a, 2017b), 12 isolates of *K. pneumoniae* were barcoded, multiplexed and sequenced with 1-D technology on a single flow cell. The most accurate ONT-only assembly had an estimated 0.349% error rate after polishing. These results have recommended an alternative approach of using both Illumina and ONT reads for hybrid assemblies (Wick et al., 2017a, 2017b).

To study AMR detection capacity of ONT, we sampled reads from MinION R9.4 flow-cells. When using the full capacity, one *M. haemolytica* strain was fully assembled while the other produced 5 contigs with N50 that covered over 85% of the complete genome. The averaged genome coverage was at ~146× and ~111× for OADDL-1 and OADDL-2, respectively. Both assemblies showed a GC content (Table 3) similar to other published strains (~ 41% in (Eidam et al., 2013, Harhay et al., 2013, Heaton et al., 2015, Kidanemariam Gelaw et al., 2015)). The results from our barcoded, multiplexed *M. haemolytica* strains suggest that consistent taxonomic status and genome adaptability could be correctly determined (Canchaya et al., 2004; Lassalle et al., 2015; Mann and Chen, 2010). Capacity of ONT's single molecule long-read sequencing platform for strain identification is thus confirmed.

We also examined different aspects that could impact the cost-effectiveness for broader applicability of ONT to for AMR detection. Aside from the issues associated with the biased input of barcoded DNA samples (Wick et al., 2017a, 2017b), at a greater degree of multiplexity assembly quality could be compromised, which might lead to misidentification of AMR genes. Current ONT R9.4.1 flow cell technology with SQK-LSK 109 sequencing kits averages over 10 billion bp per flow cell. When read coverage and assembly quality are not the major concerns, our results indicate that rapid AMR diagnosis using ONT long-read sequencing technology could provide sufficient capacity for genotyping of over 12 microbial genomes simultaneously within 48 h. The limitation, in principle, is due to the capacity of ONT barcoding kit (EXP-NBD103), which only allows 12 barcoded genomes to be sequenced simultaneously. Here we also should note that, unlike Wick et al. (2017a, 2017b), our simulated genome assemblies listed in Tables 5 and 6 were assembled using ONT-only reads, but polished on the assembled genome of OADDL-1. There are other proven cases that demonstrate how superior assembly quality can be obtained through hybrid assembly by ONT-Illumina (Ashton et al., 2015; Walker et al., 2014) and PacBio-Illumina (Chin et al., 2013; Utturkar et al., 2017). Based on our ONT-only read assemblies, AMR genes identified in *M. haemolytica* strains were congruous to the MIC results (Table 1). Studies focusing on comparisons of phenotypic susceptibility testing with sequence-based AMR prediction have already been performed for a variety of bacterial pathogens (Gordon et al., 2014; Köser et al., 2014; Stoesser et al., 2013; Zankari et al., 2013). Among these, promising results of 97% sensitivity and 99% specificity for AMR detection was reported from a large study conducted in U.K. for *Staphylococcus aureus* strains (Gordon et al., 2014).

Equipped with the described sequencing capacities, we believe direct genome sequencing approaches for AMR detection has started to shed light on genomic surveillance for disease control. At present, routine drug susceptibility testing is undertaken using phenotype-based methods, including disc diffusion, gradient diffusion and broth dilution methods that have been automated in a number of commercial platforms (Reller et al., 2009; Stoesser et al., 2013). This susceptibility testing would require up to 96 h of laboratory procedure, but is still subject to common operation errors and result misinterpretation (Andrews, 2001; Bulik et al., 2010; Jorgensen and Ferraro, 2009). Further, standard susceptibility testing protocols can only be done for culturable bacteria, creating significant challenges for risk assessment when the entire AMR reservoir needs to be investigated for both culturable and unculturable pathogens (Wang and Yu, 2012). Bacterial unculturability is therefore the most problematic for complete AMR detection, as it is possible that only about 1% of what is present in a sample can be grown with current cultivation techniques (Adam et al., 2018; Staley and Konopka, 1985). Determination of AMR in anaerobic bacteria and other difficult to grow fastidious bacteria is also challenging. Genomic approaches (Chitsaz et al., 2011; Rasko et al., 2011), on the other hand, can provide sufficient information to re-assemble genomics of AMR for “unculturable” samples (Chitsaz et al., 2011; Hasman et al., 2014). To fulfill the need for timely response in disease control, WGS approaches that rely on growing, isolating and purifying bacterial isolates might still fall short. To effectively reduce the response time and expand genomic surveillance for “unculturable” pathogens, direct sequencing approaches should be considered.

This culture-independent, direct sequencing approach is further encouraged by the recent successes in meta-genomics (sequencing all

genomes in a sample) (Afshinnekoo et al., 2017; Fang et al., 2012; Rasko et al., 2011). In an environmental metagenomic analysis, a synthetic mixture of DNA community was used to access the applicability of the MinION platform. At the current data yielding capacity and sequence quality, 91% of reads were assigned to the correct species, 93% to the correct genus and > 99% to the correct family (Brown et al., 2017; Forbes et al., 2017). With a real-time bioinformatics pipeline, viral pathogen reads from human blood samples could be directly detected in just 40 min using MinION, despite an average individual error rate of 24% (8–49%) (Greninger et al., 2015). Similar success in rapid identification of *E. coli* and acquired AMR genes has also been achieved using real-time MinION platform within a time frame similar to PCR (4 h from sample to result) (Schmidt et al., 2017). Despite its considerable potential for clinical microbiology, several challenges need to be addressed prior to the technological adoption of WGS in diagnostic laboratories (Forbes et al., 2017). For example, both Brown et al. (2017) and Greninger et al. (2015) pointed out unreliable detection for low biomass DNA species in high complex community samples. Also, owing to MinION's sequencing error rate, among 55 acquired AMR genes detected by Illumina, only 51 were found by MinION in (Schmidt et al., 2017). Predicting AMR with mere annotation approaches might therefore be insufficient. As a result, to overcome the limitation to all sequence-based approaches, a comprehensive understanding of genotypic mechanism conferring AMR might be of the most important priority (Tyson et al., 2015). For examples, in our cases annotation approach for AMR gene detection was not successful for predicting OADDL-2's resistance to Fluoroquinolones, an antibiotic class that the resistance might be manifested by mutations in genes coding for required physiological functioning. To effectively identify genotype-phenotype relationship of AMR, genome-wide association study (GWAS) that considers all regions of the genomes would be a potential approach (Alam et al., 2014; Guérillot et al., 2018; Jaillard et al., 2018).

Accession number(s)

The MinION data obtained in this study have been deposited in the NCBI Sequence Read Archive under BioProject PRJNA486393.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.03.001.

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