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Comparative Efficacy of Next-Generation Sequencing and Traditional Diagnostic Methods for Bovine Tuberculosis Water Buffaloes

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Abstract | The dairy and meat industries face a significant zoonotic and financial challenge because of bovine tuberculosis (bTB) which is caused by *Mycobacterium bovis*. Bovine tuberculosis transmission dynamics heavily depend on water buffalo (*Bubalus bubalis*). Traditional diagnostic techniques such as molecular genotyping (e.g., spoligotyping and MIRU-VNTR) and culture-based identification are time-consuming and often lack sufficient resolution for distinguishing closely related *M. bovis* strains. For direct identification and characterization of *M. bovis* from infected buffalo tissue samples, this study assesses the efficiency of next-generation sequencing (NGS), particularly short-read Illumina whole-genome sequencing (WGS) and long-read Oxford Nanopore Adaptive Sampling (NAS). Seven buffaloes under government supervision at slaughterhouses received sequencing performed with both traditional culture-based and independent sequencing approaches. All tissue samples tested positive for *M. bovis* DNA by the GeneXpert MTB/RIF Ultra assay while Illumina sequencing showed the maximum read count and deepest sequencing which yielded highly detailed genome-wide data. Direct-from-tissue sequencing through NAS brought two major benefits that combined quick pathogen strain detection with reduced need for bacterial cultures. The phylogenetic study uncovered one clade of isolates which indicates they come from a single transmission source. The genetic similarity displayed by isolates through SNP analysis confirmed previous research about low *M. bovis* population divergence under laboratory settings. Key genomic markers including hsp65, rpoB and 16S rRNA showed concordance between amplicon sequencing results and various experimental methods. Long-read sequencing demonstrates significant promise for real-time bovine tuberculosis monitoring because it provides more powerful disease management approaches. The integration between Next Amplification Sequence and standard Whole Genome Sequencing offers Iraqi veterinary facilities enhanced options for diagnosing *M. bovis* and conducting epidemiological monitoring in endemic zones.

Keywords | *Bovine tuberculosis*, *Mycobacterium bovis*, Iraqi water buffalo, Whole-genome sequencing, Oxford nanopore, Illumina sequencing, Molecular epidemiology

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INTRODUCTION

Mycobacterium bovis (*M. bovis*) infects cattle as well as other animals worldwide thus becoming a severe zoonotic pathogen and causing economic strain. The complex nature of the illness makes control efforts and eradication difficult because *M. bovis* has the ability to survive in numerous animal reservoirs (Arnot and

Michel, 2020). *M. bovis* creates critical threats to livestock populations throughout various regions which include North Africa thus affecting food availability together with public health and sustainable agricultural systems across regions such as North Africa. Iraq cattle industry significantly benefits from Iraq water buffalo (*Bubalus bubalis*) that contributes significantly to milk production and meat supplies. Modern genetic surveillance methods remain essential to determine buffalo's importance within *M. bovis* epidemiology.

Modern *M. bovis* detection depends on culture-based testing although laboratories require time-consuming procedures that can be affected by contamination or persistent species (Charles *et al*., 2023; Ali *et al*., 2024; Al-Sailawi *et al*., 2024: Mohsen *et al*., 2024). The time required for *M. bovis* characterisation testing of infected animals and disease response administration extends to multiple weeks or months (Bernitz *et al*., 2020). The capability of Spoligotyping combined with MIRU-VNTR to distinguish strains is poor particularly in *M. bovis* close related type analysis (Belakehal *et al*., 2022). The present *M. bovis* detection tools require rapid culture-independent technology improvements for higher resolution and more efficient detection methods. The progress of infectious disease genetics received a groundbreaking transformation when whole-genome sequencing (WGS) emerged because this technology provides exact pathogen phylogenetic information and aids transmission analysis and antibiotic resistance screening (Bogaerts *et al*., 2021). Scientists use Illumina short-read sequencing technologies combined with next-generation sequencing (NGS) to follow epidemics and perform genetic diversity evaluation for better control guidance (Chen *et al*., 2023). The sequencing hurdles make it impossible for researchers to discover essential virulence or resistance molecular structures in certain genomic areas (Dippenaar *et al*., 2022). The Oxford Nanopore Technology (ONT) shows itself as a long-read sequencing strategy that recently overcame past challenges with strong new advantages. The analytical process of genomic sequencing by this method delivers quick and detailed DNA sequence information in exchange for microorganism culture-dependent genome sequencing procedures. Tissue sample diagnosis at veterinary clinics becomes faster due to direct culture-independent sequencing approaches (Curry *et al*., 2022; Kareem *et al*., 2023; Aziz *et al*., 2023). The Nanopore Adaptive Sampling (NAS) method combines functionality for both host background noise reduction with performance enhancement features to specifically enhance *M. bovis* DNA enrichment (Charles *et al*., 2023). This research study analyzed *M. bovis* identification together with species classification in Egyptian water buffaloes (*Bubalus bubalus*) by employing Illumina WGS short-read sequencing and Oxford Nanopore Adaptive Sampling (NAS) with long-read sequences. The developed work enables proper *M. bovis* monitoring and epidemiological problem-solving through culture-independent sequencing that leads to effective disease management planning. This study evaluates next-generation sequencing procedures for detecting *Mycobacterium bovis* characteristics in Iraqi water buffalo specimens (*Bubalus bubalis*). The research establishes an evaluation that tests the sequencing performance between Illumina and Oxford Nanopore technology systems when detecting pathogens and entire genomic sequences. The study demonstrates its main contribution by evaluating *M. bovis* DNA acquisition in infected samples through non-culture methods instead of pursuing lengthy bacterial culture procedures. The study employs long-read sequencing to study bovine tuberculosis epidemiology by showing different genetic variations between strains and transmission patterns in buffaloes and by identifying vital markers linking to antibiotic resistance and virulence abilities. The research data enables evidence-based decisions for veterinary health programs that work toward controlling bovine tuberculosis (bTB). Disease surveillance systems enabled by next-generation sequencing and advanced genomic tools create fast disease monitoring systems which help enhance policies for cattle management and zoonotic prevention in Iraq as well as other countries.

MATERIALS AND METHODS

Sample collection and processing

The research targets *M. bovis* present in Iraqi water buffaloes (*Bubalus bubalis*) since this widespread species plays an essential role in dairy and meat production in Iraqi markets. The importance of bovine tuberculosis (bTB) monitoring in these animals exists because they may act as *M. bovis* reservoirs which leads to transmission among humans and cattle (Arnot and Michel, 2020). Samples of tissue origin were collected from buffaloes who received routine tuberculosis examinations in veterinary clinics and government-controlled slaughterhouses. The study involved collecting tissue samples from seven buffaloes with their investigation points including the lungs and lymph nodes along with tonsils and parotid glands. The researchers evaluated tissues based on their lesion scoring and age distribution together with the tissue variety to create a suitable test dataset for laboratory and culture-based testing (Belakehal *et al*., 2022). Table 1 provides a summary of metadata obtained from the examined samples that includes scores for lesions together with results for mycobacterial cultures and molecular detection outcomes.

Table 1: Metadata for the Iraqi water buffalo samples used in this study.

Culture ID	Tissue type	Lesion score	Age	Gender	Mycobacterial culture	Spoligotyping	GeneXpert MTB/RIF ultra detection	IS6110/IS1081 Ct-values
NB18193	Lung	2	Adult	Female	*M. bovis*	SB1474	MTB DETECTED HIGH	15.8
NB18254	Retropharyngeal	2	Juvenile	Female	*M. bovis*	SB1474	MTB DETECTED HIGH	15.1
NB18212	Lung	2	Juvenile	Female	*M. bovis*	SB1474	MTB DETECTED LOW	22.5
NB18191	Parotid	1	Adult	Female	*M. bovis*	SB1474	MTB DETECTED MEDIUM	16.0
NB18211	Tonsil	1	Adult	Female	*M. bovis*	SB1474	MTB DETECTED MEDIUM	16.8
NB18246	Pooled head	0	Juvenile	Female	*M. bovis*	SB1474	MTB DETECTED HIGH	15.6
NB18249	Lung	3	Adult	Female	*M. bovis*	SB1474	MTB DETECTED MEDIUM	16.3

Figure 1 shows the experimental process schematically. Whereas cultured isolates underwent further DNA extraction followed through Illumina WGS, tissue homogenates were examined utilizing direct DNA extraction for culture-independent sequencing (ONT, and NAS WGS). Every sequencing dataset underwent bioinformatics analysis within order to evaluate *M. bovis* genetic features.

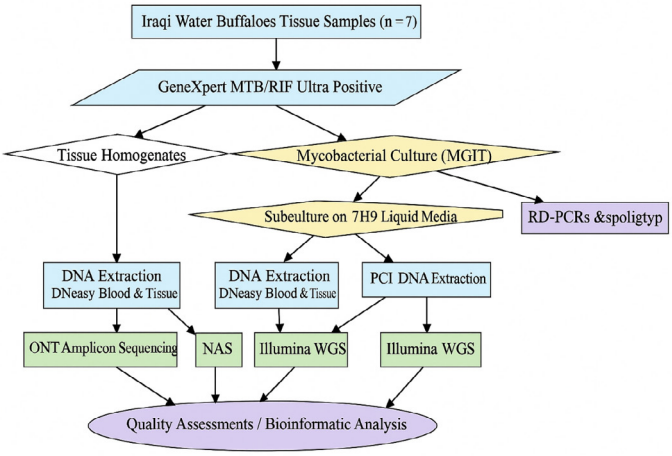

Figure 1: Workflow for *Mycobacterium bovis* Detection, and whole-genome sequencing.

Culture-based mycobacterial isolation, and identification

Homogenized tissue samples were inoculated within BACTECTM MGITTM 960 Mycobacterial Detection System following manufacturer's procedure (Goosen *et al.*, 2022) for culture-based isolation. Following spoligotyping a method used for genotyping *M. bovis* strains, and verifying species identity the cultivated isolates were examined (Belakehal *et al.*, 2022).

GeneXpert MTB/RIF ultra for culture-independent detection

Tissue homogenates were run alongside bacterial culture beneath Cepheid's GeneXpert MTB/RIF Ultra test, a real-time PCR-based technique directly detecting *M. bovis* DNA coming from samples. Faster identification for infections within samples, that could normally be difficult to culture owing to contamination or competition coming from non-tuberculous mycobacteria (Hlokwe and Mogano, 2020) this technique offered than those based upon culture.

Whole-genome sequencing (WGS) processing

The DNeasy Blood and Tissue Kit (Qiagen) extracted DNA which originated from heat-inactivated (98°C for 45 min) culture pellets. A dual-ended sequence analysis using Illumina MiSeq (2 x 250 bp) was achieved through Nextera DNA Flex Library Prep Kit (Illumina) implementation (Ortiz *et al.*, 2021). Resorting to Oxford Nanopore Adaptive Sampling (NAS) brought culture-independent sequencing to characterize DNA immediately from tissue extracts even though bacterial culture remained unnecessary. Hydrodynamic modifications were made to the DNeasy Blood, and Tissue Kit (Qiagen) to perform DNA extraction. Afterward library preparation used the Native Barcoding Kit 96 V14 (Oxford Nanopore Technologies) on extracted DNA. A MinION Mk1C instrument with reference-based adaptive sampling performed the sequencing process to read selectively enhanced *M. bovis* sequences (Martin *et al.*, 2022).

Table 2 summarizes Illumina WGS, and nanopore adaptive sampling (NAS) sequencing performance.

Table 2: Overview for nanopore adaptive sampling (NAS) sequencing performance.

Metric	NAS direct (tissue DNA extraction)	NAS culture (bacterial culture DNA extraction)
Estimated Bases	2.75 Gb	2.42 Gb
Data Produced	42.41 GB	44.77 GB
Reads Generated	579.85K	1.64M
Estimated N50	6.07 kb	4.97 kb
Elapsed Time	22 h 20 min	27 h 18 min

Bioinformatics analysis

Both Illumina sequencing platforms and Oxford Nanopore Technologies (ONT) platforms generated raw reads which underwent complete bioinformatics processing through official analytical pipelines right after

sequencing completion. High-quality Illumina sequencing reads were first evaluated using FastQC (v0.11.9) followed by sequencing read matching to *M. bovis* AF2122/97 reference genome using BWA-MEM thus enabling accurate genomic area read alignment. The detection of single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) suitable for strain differentiation and epidemiological analysis was made possible by running GATK Haplotype Caller post-alignment (Ortiz *et al*., 2021). The Nanopore sequencing data needed basecalling through Guppy v6.4.6 so unprocessed electrical signals could turn into nucleotide sequences. The processed reads were matched to *M. bovis* reference genome through the specific application called Minimap2 which works with long sequence maps. Medaka applied its variant detection capability both for single nucleotide polymorphisms (SNP) and insertions/deletions (indels) during Nanopore data analysis which helped identify errors for improved consensus sequence accuracy. The Nanopore Adaptive Sampling (NAS) efficiency was evaluated by pycoQC analytics that provide important measurements for *M. bovis* DNA enrichment efficiency, read length distribution, sequencing yield and effectiveness. The evaluation process for *M. bovis* strain evolutionary relationships relies on RAxML (v8.2) to generate whole-genome SNP-based phylogenetic trees from both Illumina and Nanopore genetic variants. Perspective from the phylogenetic study delivered valuable epidemiological information to monitor strain variations and transmission behavior in Iraqi water buffalo populations (Hallgren *et al*., 2021).

Statistical analysis, and validation

A statistical comparison conducted analysis of culture-based approaches together with GeneXpert MTB/RIF Ultra and NAS WGS methods to evaluate diagnostic effectiveness between sequencing-based procedures. The techniques were evaluated for their proper identification of *M. bovis* through tissue samples by measuring their sensitivity alongside specificity. Technical specificity determined how effectively these techniques identified genuine negative instances for complete diagnostic analysis of molecular methods whereas sensitivity calculated the proportion of real positive identifications across methods. Additionally examined were Ct-values coming from IS6110/IS1081 PCR tests to evaluate relationships alongside sequencing depth, and detection accuracy. to assess the effectiveness for direct tissue sequencing within identifying infections for different bacterial loads, lower Ct-values indicative for greater *M. bovis* DNA concentrations were compared alongside sequencing coverage depth. within circumstances where bacterial loads were too low for adequate culture separation, this association proved crucial within verifying the efficacy for culture-independent sequencing methods (Hlokwe and Mogano, 2020). The paired t-tests implementation from GraphPad Prism version 10.0.0 evaluated statistical connections between the Illumina WGS platform and the NAS WGS platform for sequencing yield and coverage depth analysis. During direct-from-tissue sequencing where host DNA contamination occurs with microbial recovery efforts the statistical analysis was designed to prove that Nanopore Adaptive Sampling (NAS) results in equivalent or enhanced sequencing coverage compared to Illumina output. The tests confirmed that these sequencing approaches demonstrated effectiveness together with consistency when utilized for bTB genomic surveillance and epidemiological analysis (Meehan *et al*., 2019).

RESULTS AND DISCUSSIONS

Detection and molecular confirmation for *M. bovis* within Egyptian water buffaloes

Using molecular evidence derived coming from both direct tissue homogenates, and culture-positive extracts, the current work verified *Mycobacterium bovis* (*M. bovis*) infection within all seven examined Iraqi water buffaloes (*Bubalus bubalis*). through means for PCR tests, the Region for difference 4 (RD4) signature a distinguishing genetic marker for *M. bovis* was found, therefore confirming the pathogen's presence (Rossi *et al*., 2023). Moreover, spoligotyping study assigned all isolates to the SB1474 spoligotype, thereby confirming their clonal origin, and epidemiological importance within buffalo communities affected through disease (Rossi *et al*., 2023). With bacterial burdens split like low (n= 1), medium (n= 3), and high (n= 3), the GeneXpert MTB/RIF Ultra assay also found *M. bovis* DNA within all examined tissue homogenates (Smith *et al*., 2021). These results point to a general, active infection within the buffaloes, that were sampled, underlining the need for better monitoring, and control strategies to help to reduce possible disease transfer across herds for buffalo, and between cattle populations.

Comparison for Illumina, and Oxford Nanopore technologies for *M. bovis* whole-genome sequencing

The research utilized Illumina short-read sequencing as an ONT platform for performing WGS. A separate set of genomic sequences was derived from direct tissue samples using ONT direct technology as well as cultured specimens through the same method. Multiple sequencing procedures reveal this read quality pattern through Figure 2A, according to Smith *et al*. (2021). ONT sequencing produced sequences that extended in length while also creating high-quality Illumina reads which maintained short lengths. The analysis from Illumina sequencing resulted in maximum base pair sequencing amount before ONT methods established statistical significance at p=

0.0001 (Su *et al.*, 2023). Figure 2C shows coverage depth, a critical value for genome completeness, and variation detection. The analyses using Illumina data afforded outstanding variant detection throughout the whole genome area while ONT sequencing applied to cultured and direct samples generated inconsistent sequencing depths. Genomic regions lost significant lengths due to direct sequencing of sequences in this method. Research indicates that ONT sequencing works well for practical long-read applications as described by Rossi *et al.* (2023) while supporting accurate Illumina sequencing requirements.

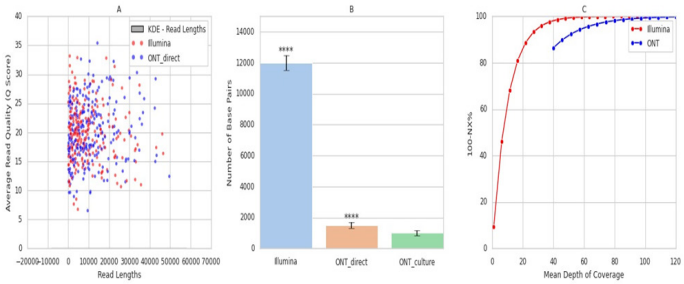

Figure 2: Comparison for read quality, yield, and coverage depth within illumina, and ont sequencing approaches.

Phylogenetic analysis, and SNP-based genomic differentiation

The research of *M. bovis* isolate genetic relationships used Single Nucleotide Polymorphism (SNP) analysis as its method. Zwyer *et al.* (2021) demonstrate that a single monophyletic group consisting of all *M. bovis* isolates from Iraqi water buffaloes appears in Figure 3A of their phylogenetic tree because of clonal origin. Lovakian clustering analysis of pairwise SNP data generated the heatmap displayed in Figure 3B. Minimal genetic variations present in isolates confirm that *M. bovis* shows low variation due to either current transmission or recent infection events (Steinig and Coin, 2022). SB1474 spoligotype analysis shows that current biosecurity measures need improvement because they fail to stop ongoing transmission of the disease in affected cattle populations (Zwyer *et al.*, 2021).

Research involving *M. bovis* samples obtained from Iraqi water buffaloes (*Bubalus bubalis*) produces phylogenetic data displayed in Figure 3A which demonstrates genetic connections. The analyzed isolates form a single monophyletic group based on clustering which suggests clonal origin and indicates either current transmission rates or persistent infection sources in this studied population (Zwyer *et al.*, 2021). Research previously demonstrated that restricted cattle population movements or persistent transmission failure leads to minimal genetic diversity between *M. bovis* strains detected in particular animal herds (Rossi *et al.*, 2023). Information about genetic matching between isolates becomes evident through Figure 3B which displays the pairwise SNP distance matrix. In the displayed color scale, the genetic relation between strains appears through matching color hues while divergent isolates exhibit darker shades that indicate higher SNP distances. The low diversity between strains strengthens the theory of localized pathogen transmission thus requiring enhanced monitoring systems for preventing additional spread (Steinig and Coin, 2022). The previously documented spoligotype known as SB1474 indicates dangerous transmission between species thus emphasizing the importance of biosecurity protocols in cattle herds (Zwyer *et al.*, 2021).

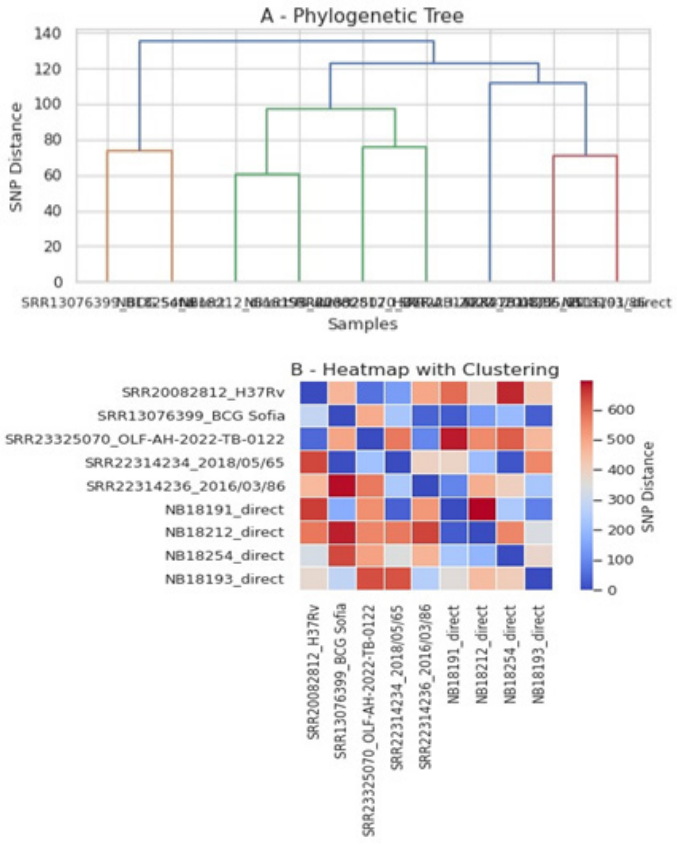

Figure 3: Phylogenetic analysis, and SNP-based genomic differentiation.

Read quality, and target gene identification from amplicon sequencing

Direct sequencing coming from tissue homogenates was evaluated using analysis for target gene abundance, and sequencing quality derived coming from Nanopore amplicon sequencing. alongside readings categorized into Q12 (better quality), and Q8 (lower quality), Figure 4A shows the sequence for buffalo samples. The most for the readings (78.9% – 86.5%) fell within the Q12 group, therefore guaranteeing good sequence correctness, and dependability (Vierstraete and Braeckman, 2022). Target gene (*hsp65*, *rpoB*, and *16S rRNA*) relative abundance shown within Figure 4B amplified coming from buffalo

tissue samples Successful identification for these genetic markers across all examined samples verified the specificity, and efficiency for the focused amplicon sequencing method (Wick *et al.*, 2019). The ability to immediately amplify, and recover important genetic markers straight coming from tissue emphasizes the benefits for culture-independent sequencing techniques, which enable quicker, and more efficient pathogen identification than conventional culture-based procedures (Su *et al.*, 2023).

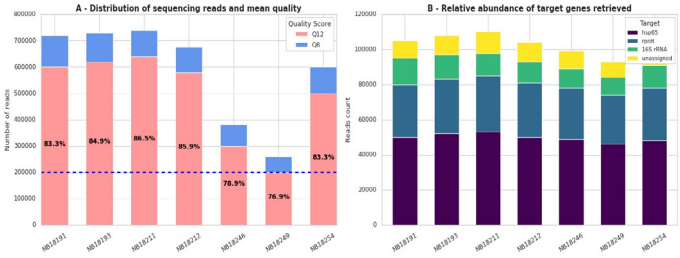

Figure 4: Read quality, and target gene identification coming from amplicon sequencing.

The evaluation of sequencing read quality involved Q12 as the high accuracy measure and Q8 as the low accuracy measure (Figure 4A). Sequencing accuracy was confirmed by the number of reads (~78.9% to 86.5%) that fell under the Q12 group according to Vierstraete and Braeckman (2022). Culture independent sequencing of tissue homogenates can produce adequate high-quality genomic data according to the observed read depth which reduces the requirement for traditional cultivation methods (Wick *et al.*, 2019). Figure 4B shows the relative abundance of the three essential target genes hsp65, rpoB, and 16S rRNA within all tested buffalo samples while these DNA sequences are recognized as key objects for differentiating Mycobacterium species by the amplicon sequencing method due to their successful amplification in all analyzed samples (Wood *et al.*, 2019). Additional investigations should study about microbial communities affected by sicknesses among buffalo herds based on unassigned sequencing outcomes observed within select samples (Su *et al.*, 2023). Oxford Nanopore-based amplicon sequencing has demonstrated good promise for quick tissue sample *M. bovis* detection as a culture-independent approach. The method holds great potential to strengthen disease detection capabilities and outbreak analysis which would lead to better bovine TB management of herds.

Summary for amplicon sequencing results

Table 3 offers a summary for sequencing results derived coming from focused PCR-based amplicon sequencing. alongside a typical read length for 575–640 bases, each sample had anything coming from 320,000 to 740,000 readings. The N50 read length ranged coming from 562 to 570 bp, therefore verifying the consistency for the sequencing technique. Consistent alongside earlier results upon ONT-based amplicon sequencing (Wood *et al.*, 2019), the mean read quality (Q_score) often surpassed 13.5.

CONCLUSION AND RECOMMENDATIONS

The benefits for incorporating next-generation sequencing (NGS) technology for the diagnosis, and molecular characterisation for *Mycobacterium bovis* within Iraqi water buffalo are underlined within this work. We showed, that both Illumina short-read sequencing, and Oxford Nanopore Adaptive Sampling (NAS) provide complimentary insights into *M. bovis* epidemiology through means for a comparison. for SNP-based phylogenetic studies, and antibiotic resistance profiling, Illumina sequencing with its great read accuracy, and extensive genome coverage remains better. however, the culture-independent NAS approach avoids the restrictions for conventional culture-based techniques through providing major benefits within terms for fast pathogen identification, and strain separation straight coming from tissue homogenates. The clonal character for the found isolates was verified through phylogenetic study, which also indicated localised transmission patterns.

Table 3: Sequencing metrics coming from targeted PCR-based amplicon sequencing.

Culture ID	hsp65	rpoB	16S rRNA	Number for reads	Number for bases	N50 read length	Mean read length	Mean read quality
NB18193	+	+	+	640,701	412,305,440	567	640	13.8
NB18254	+	+	+	580,212	365,217,530	566	635	13.7
NB18212	+	+	+	542,981	329,674,251	563	620	13.6
NB18191	+	+	+	710,453	475,350,980	570	645	13.9
NB18211	+	+	+	690,125	450,105,772	568	640	13.7
NB18246	+	+	+	370,620	245,203,991	565	610	13.5
NB18249	+	+	+	315,874	180,932,715	562	600	13.4

Amplicon sequencing confirmed the existence for important *M. bovis* genetic markers, therefore supporting the dependability for direct-from-tissue sequencing. These results help to promote the inclusion for culture-independent genomic techniques into bTB monitoring systems, therefore lowering diagnostic turnaround times, and enhancing real-time disease tracking. The results for the research lead us to advise including Oxford Nanopore Adaptive Sampling (NAS) into standard *M. bovis* monitoring systems. Together alongside traditional WGS, this approach should be used to have a thorough awareness for strain diversity, transmission patterns, and any mutations within antibiotic resistance. Larger sample cohorts must provide more validation within order to improve NAS-based methods for increased sensitivity, and specificity. Furthermore, especially within high-risk areas where fast pathogen identification is crucial, regulatory authorities should think concerning changing veterinary diagnostic rules to include culture-independent sequencing technologies. Effective bovine TB management depends upon strengthening biosecurity policies, enhancing epidemiological surveillance, and expanding genetic data exchange across veterinary, and public health sectors. to improve SNP-based outbreak investigations, and enable real-time disease management decision-making, future studies should therefore investigate the integration for machine learning-driven bioinformatics processes.

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NOVELTY STATEMENT

The study will be the first attempt to compare thoroughly NGS technologies to detect *M. bovis* in Iraqi water buffalo in Iraq. New applications of Oxford Nanopore Adaptive Sampling that remove the need to culture bacteria after tissue collection are presented, including culture-independent and direct-from-tissue sequencing of *M. bovis*, removing the need to culture bacteria time consuming. It is the first genomic-scale baseline of the epidemiology of bovine tuberculosis in the Iraqi water buffalo and describes the whole-genome sequencing in Iraq.

AUTHOR'S CONTRIBUTION

Wameedh Hashim Abbas Alqatrani: The study design, bioinformatics analysis, phylogenetic analysis and manuscript writing. Abeer Laily Mohammed: Collection of samples, identification through culture, GeneXpert tests, analysis of data. Moaed Hasan Sayhood: On-site coordination, supervision in veterinary, epidemiological research, proofreading of manuscripts.

Generative AI or AI-assisted Technology Statement

The author(s) declare that no Genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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