Kafkas Univ Vet Fak Derg
26 (5): 647-656, 2020
DOI: 10.9775/kvfd.2020.24089

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Complete Genome Sequencing of *Mycoplasma bovis* Type Strain Ningxia-1 and Systematic Bioinformatic Characterization for Housekeeping-related Genes

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Article ID: KVFD-2020-24089 Received: 17.02.2020 Accepted: 06.08.2020 Published Online: 06.08.2020

How to Cite This Article

Sun P, Fu Y, Wan Q, Yosri M, He S, Shen X: Complete genome sequencing of Mycoplasma bovis type strain ningxia-1 and systematic bioinformatic characterization for housekeeping-related genes. *Kafkas Univ Vet Fak Derg,* 26 (5): 647-656, 2020. DOI: 10.9775/kvfd.2020.24089

Abstract

Mycoplasma bovis is a major pathogen leading to bovine respiratory disease syndrome. The worldwide prevalence of this pathogen has caused enormous economic losses in the beef industry. Substantial efforts have been made to identify and characterize the surface proteins of *M. bovis*. However, little is known regarding experimentally proved housekeeping genes, or the distribution and the number of motifs within these genes in *M. bovis*. We used Picbio SMRT technology of next-generation sequencing for *M. bovis* Ningxia-1 isolation and applied different tools (Mega X, STRING v11.0, TMHMM v2.0, MOTIF) for bioinformatics analysis. The present study compared *M. bovis* Ningxia-1 strain with another ten *M. bovis* strains with sequenced whole genome and identified 24 housekeeping genes in each strain. The phylogenetic tree indicates a close relationship between *M. bovis* Ningxia-1 with NM2012 based on BLAST results of these genes. Within the 24 housekeeping genes in *M. bovis* Ningxia-1 stain, 3/24 (12.5%) of the genes have the potential to be used as internal control genes, 2 sets of proteins have interactions which have been proved under experimental and database conditions. MetG is a putative transmembrane protein, while others are predicted to be located outside of the membrane. Additionally, there are 6 common motifs distributed among 7 of the proteins (29.17%). Our bioinformatic analysis is intended to provide new and complementary data in mining and making comparisons of housekeeping genes through *M. bovis* type strain Ningxia-1 sequencing.

Keywords: *Mycoplasma bovis, Next-generation sequencing, Housekeeping gene, Bioinformatic analysis*

Mycoplasma bovis Ningxia-1 Suşunun Tüm Genom Sekanslaması ve Housekeeping İlişkili Genlerin Sistematik Biyoinformatik Karakterizasyonu

Öz

Mycoplasma bovis, sığır solunum hastalığı sendromuna yol açan önemli bir patojendir. Bu patojenin dünya çapında yaygınlığı, sığır eti endüstrisinde büyük ekonomik kayıplara neden olmuştur. *M. bovis*'in yüzey proteinlerini tanımlamak ve karakterize etmek için önemli çabalar sarf edilmiştir. Bununla birlikte, *M. bovis*'te deneysel olarak kanıtlanmış housekeeping genleri veya bu genler içindeki motiflerin dağılımı ve sayısı hakkında çok az şey bilinmektedir. *M. bovis* Ningxia-1 izolasyonu için yeni nesil sekanslama Picbio SMRT teknolojisini kullandık ve biyoinformatik analiz için farklı araçlar (Mega X, STRING v11.0, TMHMM v2.0, MOTIF) uyguladık. Bu çalışma ile *M. bovis* Ningxia-1 suşu, sekanslanmış tam genomlu başka bir *M. bovis* suşu ile karşılaştırıldı ve her suşta 24 housekeeping geni tanımladı. Filogenetik ağaç, bu genlerin BLAST sonuçlarına dayanarak M. bovis Ningxia-1 ile NM2012 arasında yakın bir ilişki olduğunu gösterdi. *M. bovis* Ningxia-1 boyasındaki 24 housekeeping geni içinde, genlerin 3/24'ü (%12.5) internal kontrol genleri olarak kullanılma potansiyeline sahiptir, proteinlerin 2 seti deneysel ve veritabanı koşullarında kanıtlanmış etkileşim göstermektedir. MetG varsayılan bir transmembran protein olmasına rağmen, diğerlerinin membranın dışında olduğu tahmin edilmektedir. Ek olarak, 7 proteine (%29.17) dağılmış 6 yaygın motif bulunmaktadır. Biyoinformatik analizimiz, veri madenciliğinde yeni ve tamamlayıcı veriler ortaya koymuş ve *M. bovis* tipi Ningxia-1 dizileme yoluyla temel genlerin karşılaştırmalarını yapmayı sağlamıştır.

Anahtar sözcükler: *Mycoplasma bovis, Yeni nesil dizileme, Housekeeping gen, Biyoinformatik analiz*

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INTRODUCTION

Mycoplasma bovis is an important pathogen causing a variety of disease syndromes in cattle. *M. bovis* can affect a large variety of tissues and organs [1], causing mastitis [2], pneumonia [3], arthritis [4], keratoconjunctivitis [5], otitis media [6], meningitis [7], or reproductive diseases [8]. At present, the pathogen is prevalent worldwide, which causes considerable economic losses to feedlot cattle, dairy and veal calf industries [9], with hugely detrimental impacts on animal welfare [10]. *M. bovis* was reported as resistant to anti-microbial drugs including the β-lactams, polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampin [11], due to the lack of a cell wall and absence of a murein/peptidoglycan layer. It is known that *M. bovis* can be shed in the infected cattle for months or even years [12]. To date, no vaccines are commercially available, however, the macrolides, modified macrolides, tetracyclines, aminoglycosides, chloramphenicols, pleuromutilins and fluoroquinolones can treat *M. bovis* infection as potential medications [10]. Draxxin (Tulathromycin) is the only approved drug for treatment for *M. bovis* and the normal duration is 10-14 days of antibiotic therapy [13].

The first complete genome of *M. bovis* PG45 was published in 2010, and genomes of two other Chinese isolates (Hubei-1 and HB0801) were reported in 2011 and 2012. With the development of sequencing technology, next-generation sequencing (NGS) for microbes using Picbio SMRT technology is rapidly becoming a common method for characterization of the whole genome of all living species, allowing for much higher levels of detail [14]. It is an invaluable tool for comparative genomic studies through the analysis of single nucleotide polymorphisms (SNPs) and gene-based comparative methods [15]. To date, the genome of 11 *M. bovis* has been fully sequenced and are available from NCBI Genbank. We collected these 11 different *M. bovis* genomes together with their biosample information and compared these strains using 24 housekeeping genes presented by a phylogenetic tree.

The genome of *M. bovis* Ningxia-1 contains a single circular chromosome of 1,033,629 bp, with a GC content of 29.3% [16]. Housekeeping genes are a group of constitutive genes that are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and patho-physiological conditions [17]. Additionally, housekeeping genes are instrumental for calibration in many computational applications and genomic studies and are used widely as internal controls for experiments [18,19]. To date, less information is known about *M. bovis* housekeeping genes. Thus, we collected all published housekeeping genes from the Pubmed database to compare and analyze these genes using bioinformatic approaches. Systematic analysis-based software, such as Mega X, STRING v11.0, TMHMM v2.0, MOTIF, were used to identify relationships of molecular evolution on gene sequence alignment, protein-protein interactions, membrane distribution and all motifs respectively in these housekeeping genes. Our urpose of these sequence-based methods was to identify various housekeeping genes in *M. bovis* Ningxia-1 strain and to define these 24 genes using different KEGG pathways, cellular location, motif distribution and the potential clinical detection markers. We identified 3/24 (12.5%) potential genes which could be used as clinical detection markers for further molecular experimental study. 4/24 (16.67%) of the proteins had protein-protein interactions under experimental and databases conditions, 23/24 (95.8%) proteins are an outside membrane protein, and 6 common motifs were distributed in 7/24 (29.17%) proteins.

MATERIAL and METHODS

Strain and Culture

The Ningxia-1 strain was isolated from the lung of an infected calf and stored at the College of Agriculture, Ningxia University, China. The strain was cultured in a pleuropneumonia-like organisms (PPLO) broth (1 g glucose, 21 g PPLO, 100 mL 25% yeast, 2 g sodium pyruvate, 200 mL calf serum (BD, Waltham, MA, USA), 0.1g of 80,000 IU/ mL penicillin-G, and 4.5 mL 0.4% phenol red). Type 2 water (Millipore, Burlington, MA) was added to make 1 L of broth. The inoculation amount was v/v 1:10 at 37°C for 3 days on an orbital shaker.

Genomic DNA Preparation

The cultured *M. bovis* was harvested from 1 L of broth by centrifugation at 12.000×g under 4°C for 30 min, the supernatant was discarded and pelleted cells were sent to the GeneDenovo Guangzhou, China for genome sequencing. Genomic DNA was extracted using commercial kits according to the manufacturer's instructions. The DNA quality was detected using Qubit (Thermo Fisher Scientific, Waltham, MA) and Nanodrop (Thermo Fisher Scientific, Waltham, MA) accordingly.

Sequencing

Qualified genomic DNA was fragmented with G-tubes (Covaris) and end-repaired to prepare SMRTbell DNA template libraries (with fragment size of >10 Kb selected by bluepippin system) according to the manufacturer's specifications (PacBio, Menlo Park, CA). A total of library quality was detected by Qubit and average fragment size was estimated on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Single Molecule Real Time (SMRT) sequencing was performed on the Pacific Biosciences RSII sequencer (PacBio, Menlo Park, CA) according to standard protocols (MagBead Standard Seq v2 loading, 1 × 180 min movie) using the P4/C2 chemistry [20].

De Novo Genome Assembly

Continuous long reads were attained from three SMRT

sequencing runs. Reads longer than 500 bp with a quality value over 0.75 were merged together into a single dataset. Next, the hierarchical genome-assembly process (HGAP) pipeline was used to correct for random errors in the long seed reads (seed length threshold 6 Kb) by aligning shorter reads from the same library against them [21]. The resulting corrected, preassembled reads were used for de novo assembly using Celera Assembler with an overlap-layout-consensus (OLC) strategy [22]. Since SMRT sequencing features very little variations of the quality throughout the reads, no quality values were used during the assembly [23]. To validate the quality of the assembly and determine the final genome sequence, the Quiver consensus algorithm was used [21]. Finally, the ends of the assembled sequence were trimmed to have the genome circularized. The depth of final sequencing is 1262 fold.

Extraction and Mining for Housekeeping Genes

Mycoplasma bovis strain PG45 (CP002188.1) and *M. bovis* Ningxia-1 24 housekeeping genes were selected from Pubmed (US National Library of Medicine and the National Institutes of Health) for STRING, trans-membrane and motif analysis. The flowchart is shown in *Fig 1*. Literature published between 2010 and 2018 was scanned using the following search terms: *M. bovis* and housekeeping genes. A list of 7 published articles was retrieved *(https://www.ncbi.nlm.nih.gov/pubmed)*. Articles were reviewed and analyzed for diagnostic methods by amplification and molecular methods, as well as for clinical diagnosis markers.

Genomic Prediction and Evolutionary Position

The reference sequence NZ_CP023663.1 was derived from CP023663. Annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline (released 2013). Information about the Pipeline can be found at *https://www.ncbi.nlm.nih.gov/genome/annotation_prok/*. Meanwhile, we identified 11 *M. bovis* and one *Mycoplasma agalactiae (M. agalactiae)* genomes with whole genome sequencing. Twenty-four selected housekeeping genes were aligned by MUSCLE [25]. DNA sequences of 11 orthologous mycoplasma species and one *M. agalactiae* were aligned using a maximum likelihood (ML) method with Mega X *(http://www.megasoftware.net)* [25]. The following parameters were used: 1000 replications for bootstrap analysis, "Tamura and Nei model" for the substitution model, "use all site" for the proportion of gaps/missing data treatment, "Nearest-Neighbor-Interchange (NNI)" for ML heuristic method, and "BIONJ" for starting tree(s) [25]. EvolView is a comprehensive tool for tree visualization and annotation after obtained the original phylogenetic tree for Mega [26].

BLAST Analysis

A DNA sequence comparison tool BLAST *(http://www.ncbi.nlm.nih.gov/blast/blast.cgi)* was using to compare the similarity of 24 genes in *M. bovis* Ningxia-1 strain and *M. agalactiae*. Nucleotide of 24 genes in *M. bovis* Ningxia-1 were placed as the query sequence and the DNA genome sequence of *M. agalactiae* was to deposited into the subject sequence box. More dissimilar sequences (discontiguous

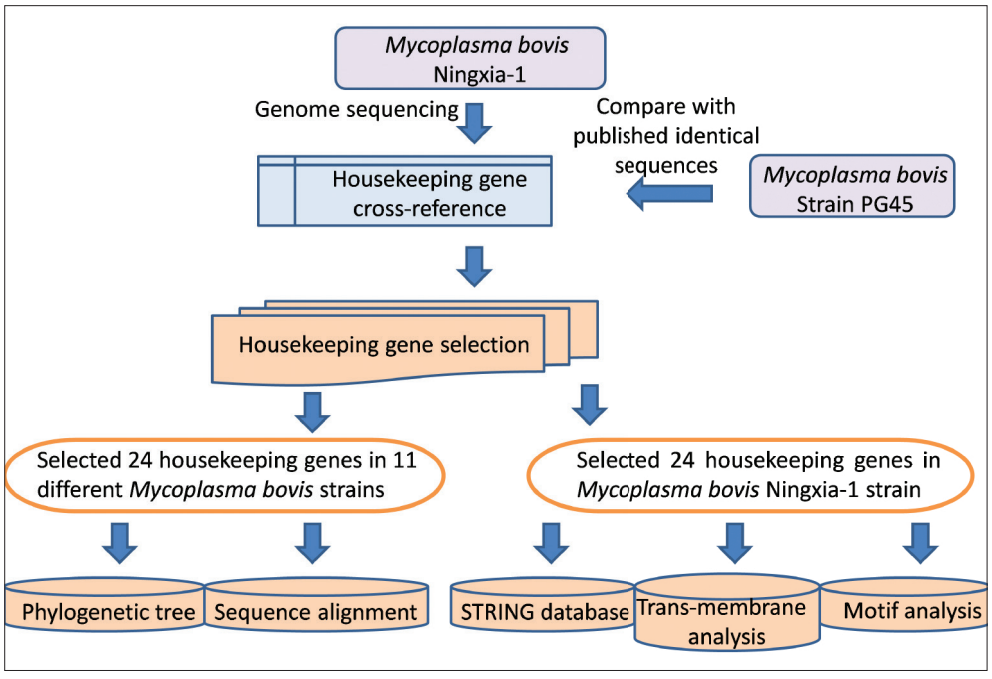


Fig 1. A systematic workflow of selecting and evaluating housekeeping genes in *M. bovis*. To compare essential housekeeping genes after the next generation sequencing of *M. bovis* Ningxia-1 isolate. *M. bovis* was searched for housekeeping genes studies in NCBI database. For each housekeeping gene collected, the PMID of the corresponding peer-reviewed articles was recorded, comparing with *M. bovis* PG45. Gene information was then identified from the NCBI Gene website, and the protein sequence was extracted for further analysis

Table 1. *The list of 11 M. bovis strain selected bioinformation in this study*

No	*Mycoplasma bovis* Strain	Isolation Source	Isolate Location	Time
1	PG45	Bovine mastitis	USA	1965
2	Ningxia-1	Lung of a beef calf (10 d after birth)	Pengyang, Ningxia, China	2013
3	Hubei-1	Lung of a calf	Hubei, China	2008
4	HB0801	Lesioned lung of an infected beef	Yingcheng, Hubei, China	2008
5	NM 2012	Synovial fluid	China:Inner Mongolia	2012
6	CQ-w70	Lung	China:Yunyang County, ChongQing Municipality	2009
7	08M	The lung of a calf with pneumonia	China	2008
8	JF4278	mastitic milk	Switzerland	2008
9	HB0801-115	Lesioned lung of an infected beef cattle, *in vitro* subculture	Yingcheng city in Hubei province, China	2012
10	HB0801-150	Lesioned lung of an infected beef cattle, *in vitro* subculture	Yingcheng city in Hubei province, China	2012
11	HB0801-180	Lesioned lung of an infected beef cattle, *in vitro* subculture	Yingcheng city in Hubei province, China	2012

Table 2. *Genome comparison in 11 selected M. bovis strains*

No.	Name	Species	INSDC	Size	GC%	Protein	rRna	tRna	Other RNA	Gene	Pseudogene
1	PG45	*Mycoplasma bovis*	CP002188.1	1,003,404	29.3	779	6	34	3	870	48
2	Hubei-1	*Mycoplasma bovis*	CP002513.1	948,121	29.3	731	4	34	3	813	41
3	HB0801	*Mycoplasma bovis*	CP002058.1	991,702	29.3	764	6	34	3	845	38
4	CQ-W70	*Mycoplasma bovis*	CP005933.1	948,516	29.3	740	4	34	3	813	32
5	NM 2012	*Mycoplasma bovis*	CP011348.1	990,348	29.3	763	6	34	3	843	37
6	08M	*Mycoplasma bovis*	CP019639.1	1,016,753	29.3	770	6	34	3	867	54
7	Ningxia-1	*Mycoplasma bovis*	CP023663.1	1,033,629	29.3	750	6	34	3	887	94
8	JF4278	*Mycoplasma bovis*	LT578453.1	1,038,531	29.3	787	6	34	3	894	64
9	HB0801-P115	*Mycoplasma bovis*	CP007589.1	977,322	29.3	738	6	34	3	834	53
10	HB0801-P150	*Mycoplasma bovis*	CP007590.1	977,304	29.3	714	6	34	3	835	78
11	HB0801-P180	*Mycoplasma bovis*	CP007591.1	977,257	29.3	694	6	34	3	835	98

megablast) were selected by a BLAST algorithm.

STRING Analysis

In order to predict interaction networks between the 24 housekeeping genes in *M. bovis* Ningxia-1 strain, the annotated genome was downloaded from the Genebank database *(https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP023663.1)*. The protein sequences of these genes were stored in a FASTA format document and then input into the latest version STRING 11.0 *(https://string-db.org/)*. The *M. bovis* PG45 organism was selected. For generating the figures, a confidence cutoff of 0.4 was used. Under evidence view, a network map was downloaded. To identify different genes involved in KEGG/MAPK pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database *(https://www.kegg.jp/kegg-bin/show_organism?menu_type=pathway_maps&org=mbv)* was used basing on enrichment function. To identify the experiment conformed genes among these 24, we chose confidence view which indicating the strength of the data support with highest confidence score of 0.9 (experiments and databases condition was chosen for analysis).

Transmembrane Analysis

To determine transmembrane probability for each housekeeping gene, their protein sequences were pasted to TMHMM Server v. 2.0 *(http://www.cbs.dtu.dk/services/TMHMM/)*. The library was set up in a FASTA format (Supplement data) and analyzed using standard settings to determine transmembrane helices in the 24 proteins.

Motif Analysis

To further expand our analysis and sort the housekeeping genes based on their expression of functional domains, the 24 housekeeping genes protein sequences were queried against the Motif Search Library *(https://www.genome.jp/tools/motif/)* with an E-value cut off score of 1.0 in Pfam database [27].

RESULTS

Bioinformatics Study and Genome Comparison

Bioinformation of 11 selected *M. bovis* strains was collected in this study *(Table 1)*. With the updated annotation information from NCBI, 750 open reading frames (ORFs) were identified in *M. bovis* Ningxia-1 genome (total 920,475 bp, max 9,981 bp and min 114 bp) which occupied 89.05% of the whole genome, with an average length of 1,227 bp and a mean GC content of 29.74%. 629 out of 750

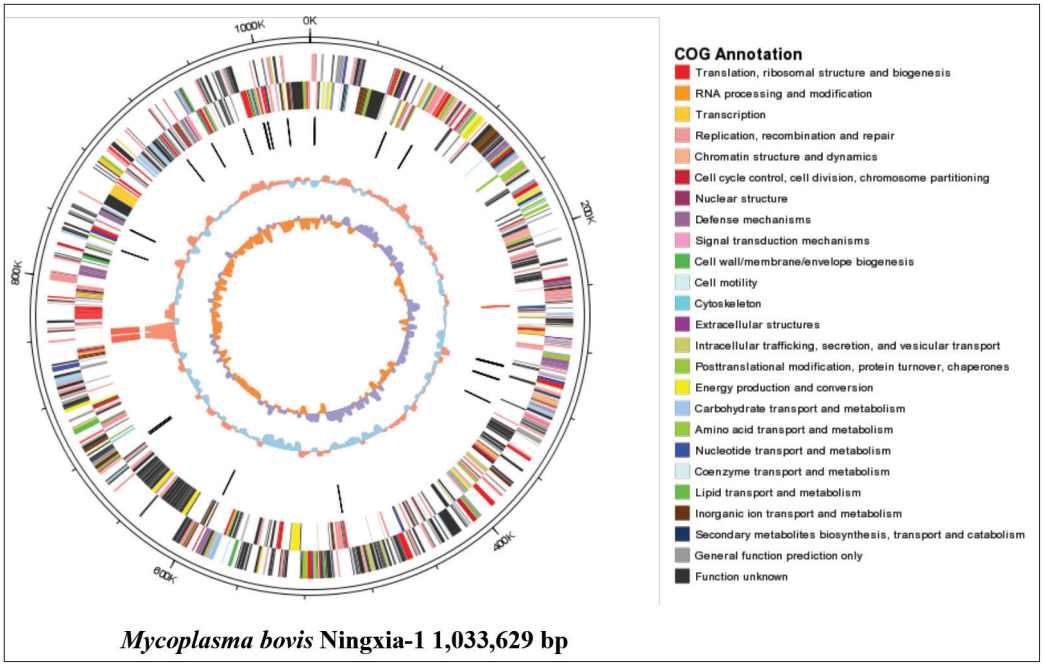


Fig 2. Circular Diagram of the *M. bovis* Ningxia-1 Genome Structure. The genome of *M. bovis* Ningxia-1 contains a single circular chromosome of 1,033,629 bp, with a GC content of 29.3%. A total of 887 genes and 750 open reading frames (ORFs) were identified. Ninety-four pseudogenes were predicted by GeneMarkS+. The genome encodes 6 rRNA and 34 tRNA genes, which representing all 20 amino acids. The outer black circle shows the whole genome length. Moving inside, the first and second circles show predicted coding sequences (CDSs) on the plus and minus strands respectively. Gold for translation, ribosomal structure and biogenesis; orange for RNA processing and modification; light orange for transcription; dark orange for DNA replication, recombination and repair; antique white for cell division and chromosome partitioning; pink for defense mechanisms; red for signal transduction mechanisms; peach for cell envelope biogenesis and outer membrane; deep pink for intracellular trafficking, secretion and vesicular transport; pale green for post-translational modification, protein turnover and chaperones; royal blue for energy production and conversion; blue for carbohydrate transport and metabolism; dodger blue for amino acid transport and metabolism; sky blue for nucleotide transport and metabolism; light blue for coenzyme metabolism; cyan for lipid metabolism; medium purple for inorganic ion transport and metabolism; aquamarine for secondary metabolites biosynthesis, transport and catabolism; and gray for general function prediction only; black for unknown function). The third circle shows tRNA (black) and rRNA (red). The fourth circle shows the content of G+C (red: above mean, blue: below mean). The fifth circle shows G+C skew (purple: above mean, orange: below 0)

(83.9%) of these genes could be classified into Clusters of Orthologous Groups (COG) families which have 19 functional categories *(Fig. 2)*. The genome encodes 6 rRNAs and 34 tRNAs genes which represent all 20 amino acids. The comparison of 11 selected *M. bovis* strain genomes is shown in *Table 2*.

Identification of 24 Housekeeping Genes through Literature Annotation and Evolutionary Position

Through manual literature annotation, we identified reports on 24 housekeeping genes that have been determined and applied in experiments. For each gene in the list, the following information was recorded: (1) paper PMID; (2) gene symbol; (3) nucleotide sequence; (4) protein sequence; and (5) identifier. The 24 housekeeping genes identified in this study were all found in STRING database (Supplement Table S1).

We identified 24 genes in 11 orthologous strains, *adk*, *atpA*, *dnaA*, *dnaK*, *dnaN*, *efp*, *fusA*, *gltX*, *gmk*, *gpsA*, *gyrB*, *lepA*, *metG*, *polC*, *pta_1*, *recA*, *rpoB*, *rpoD*, *tdk*, *tkt*, *tpiA*, *tuf* and *uvrC*. Phylogenetic trees were created for each of the 24 genes between 11 *M. bovis* stains and *M. agalactiae* strain to examine evolutionary position, which indicated a close relationship between *M. bovis* Ningxia-1 and NM2012 *(Fig. 3)*.

BLAST Homology Between M. bovis Ningxia-1and M. agalactiae

Each of the 24 identified genes was BLASTed against *M. bovis* Ningxia-1 strains along with the *M. agalactiae* whole genome. The homology between *fusA*, *dnaK*, *tkt* and *tuf* genes is over 90%, which indicates a high similarity between *M. bovis* Ningxia-1 and *M. agalactiae*, whereas the similarity of *adk*, *gpsA*, *polC* and *uvrC* is below 83%, which indicates the possibility for those genes to be used as a clinical detection marker to differentiate *M. bovis* and *M. agalactiae (Table 3)*. *adh-1*, *atpA*, *dnaA*, *dnaN*, *efp*, *gltX*, *gmk*, *gyrB*, *lepA*, *metG*, *pta_1*, *recA*, *rpoB*, *rpoD*, *tdk* and *tpiA* of *M. bovis*, fall between 83-90% homology range also making them potential candidates for use in diagnosis.

STRING Protein-Protein Analysis of M. bovis Ningxia-1

In order to predict interaction networks between the 24 housekeeping genes in *M. bovis* Ningxia-1 strain each

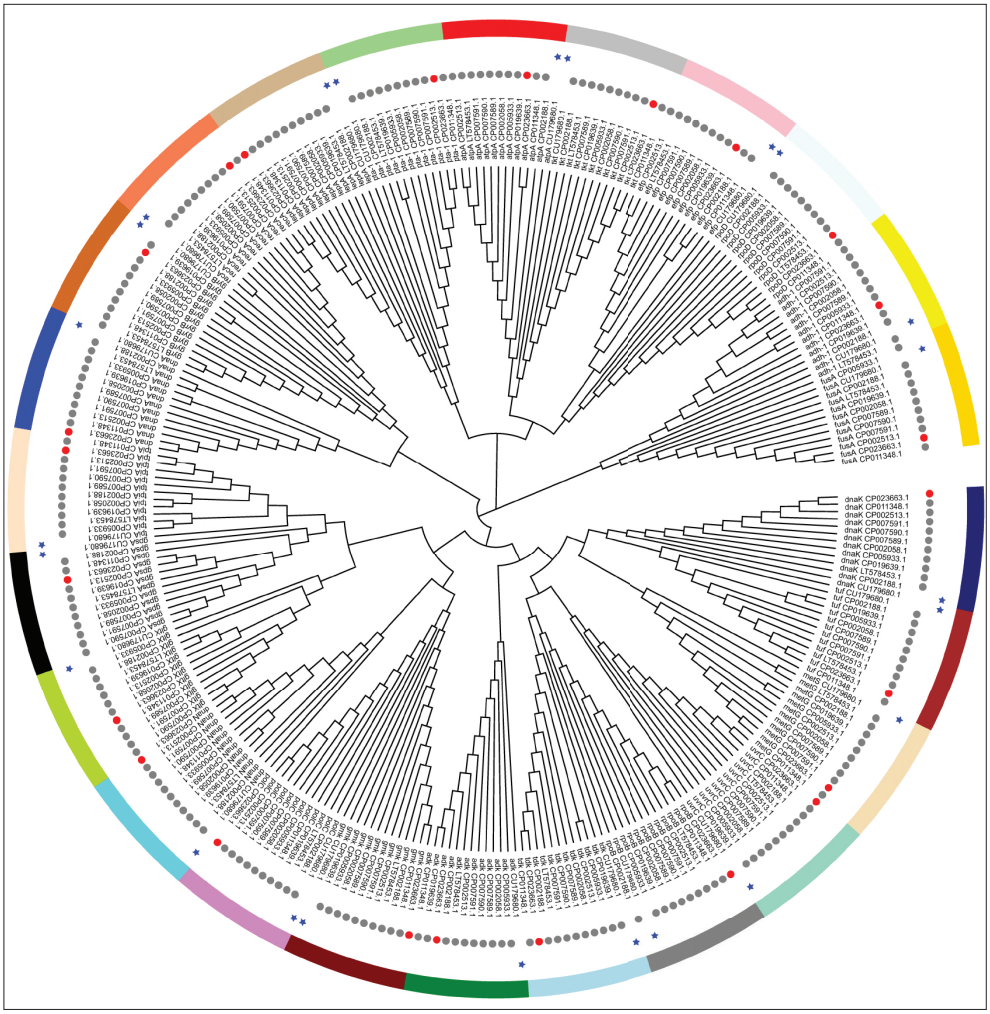
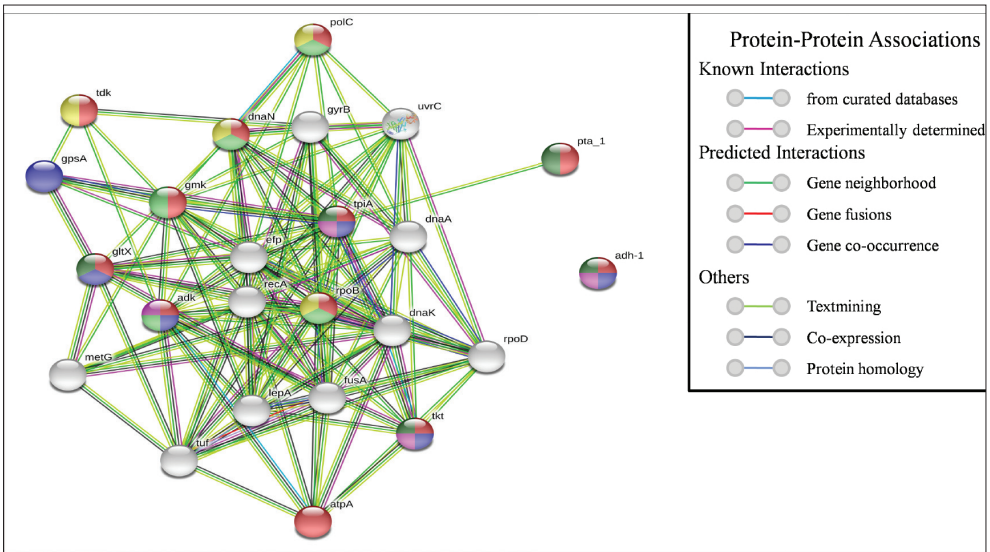


Fig 3. Phylogenetic position of 24 selected housekeeping genes from 11 selected *M. bovis* strains and 1 exogenous reference strain *M. agalactiae*. *M. bovis* Ningxia-1 has a close relationship with NM2012, PG45, JF4278 and Hubei-1. The red dot represented *M. bovis* Ningxia-1strain, the grey indicated other homology *M. bovis*. *M. agalactiae* was marked by blue stars. The different strip color represented 24 genes, yellow is for *adh-1*, green is for *adk*, red is for *atpA*, blue is for *dnaA*, navy is for *dnaK*, cyan is for *dnaN*, pink is for *efp*, gold is *fusA*, greenyellow is for *gltX*, maroon is for gmk, black is for *gpsA*, chocolate is for *gyrB*, tan is for *lepA*, wheat is for *metG*, violet is for *polC*, lightgreen is for *pta_1*, coral is fro *recA*, gray is for *rpoB*, azure is for *rpoD*, lightblue is for *tdk*, silver is for *tkt*, bisque is for *tpiA*, brown is for *tuf*, aquamarine is for *uvrC*

was analyzed using STRING. These networks include 130 interactions, with expected number of edges to be 90 based on the evidence view. Such enrichment indicates that the proteins are at least partially biologically connected, as a group. 12 proteins including *adh-1, adk, atpA, dnaN, gltX, gmk, polC, pta_1, rpoB, tdk, tkt* and *tpiA*, are involved in metabolic pathways. 6 proteins, *adh-1, adk, gltX, gpsA, tkt* and *tpiA*, are involved in biosynthesis of secondary metabolites. 4 proteins including *adh-1, adk, tkt and tpiA* may be involved in the biosynthesis of antibiotics. 5 proteins, *adh-1, gltX, pta_1, tkt* and *tpiA*, are involved in microbial metabolism in diverse environments while 2 sets of genes are involved in nucleotide metabolism process: 5 proteins, *adk, dnaN, gmk, polC* and *rpoB* perform in purine metabolism and 4 proteins, *dnaN, polC, rpoB* and *tdk* have function in pyrimidine metabolism *(Fig. 4)*. Under the confidence view with active interaction sources of experiments and databases, only four proteins *adk, atpA, rpoB* and *rpoD* are present in the results. *Adk* has 4 functions in KEGG pathways (pathway ID: mbv01100, mbv01110, mbv00230, mbv01130), including metabolic pathways, biosynthesis of secondary metabolites, purine metabolism, biosynthesis of antibiotics. *AtpA* is only involved in metabolic pathways. *Adk* and *atpA* participated in curated pathways in relevant datasets in *M. bovis*: adenosine ribonucleotides de novo biosynthesis, bioCyc/ecoCyc Pathways *(www.biocyc.org)*. RpoB has 3 functions in KEGG pathways (pathway ID: mbv01100, mbv00230 and mbv00240), including metabolic pathways, purine metabolism and pyrimidine metabolism. No experimental/biochemical data was shown, but putative homologs were found interacting in other species (score 0.990). RpoB and rpoD show relevant information with *Escherichia coli K12* MG1655.

Table 3. *Homology of 24 housekeeping genes between M. bovis and M. agalactiae*

Gene Name	Search Sequence	Query Sequence	Identity	Size	Different	E Value	Total Score
adh-1	CP023663.1:430952-432001	CU179680.1	84	1050	168	0	1137
adk	CP023663.1:c751508-750858	CU179680.1	82.642	651	113	0	665
atpA	CP023663.1:c528856-527270	CU179680.1	85.381	1587	232	0	1817
dnaA	CP023663.1:1000-2400	CU179680.1	85.796	1401	199	0	1630
dnaN	CP023663.1:2535-3644	CU179680.1	84.054	1110	177	0	1205
efp	CP023663.1:592716-593279	CU179680.1	88.652	564	64	0	729
fusA	CP023663.1:c840005-837912	CU179680.1	92.311	2094	161	0	3051
gltX	CP023663.1:820782-822173	CU179680.1	86.135	1392	193	0	1641
gmk	CP023663.1:272425-273012	CU179680.1	84.014	588	94	0	637
gpsA	CP023663.1:c69556-68558	CU179680.1	80.08	999	199	0	905
gyrB	CP023663.1:1005729-1007696	CU179680.1	85.018	1969	293	0	2214
dnaK	CP023663.1:185269-187065	CU179680.1	90.095	1797	178	0	2439
lepA	CP023663.1:698096-699889	CU179680.1	87.786	1793	219	0	2247
metG	CP023663.1:c268839-267289	CU179680.1	84.521	1544	239	0	1708
polC	CP023663.1:83679-88055	CU179680.1	81.536	4387	791	0	4223
pta_1	CP023663.1:173625-174581	CU179680.1	88.506	957	110	0	1231
recA	CP023663.1:783965-784948	CU179680.1	86.154	975	135	0	1150
rpoB	CP023663.1:c873691-870056	CU179680.1	89.741	3636	373	0	4876
rpoD	CP023663.1:c365396-363867	CU179680.1	88.374	1531	176	0	1952
tdk	CP023663.1:c957009-956437	CU179680.1	83.877	552	89	4.94E-171	595
tkt	CP023663.1:256009-257955	CU179680.1	93.066	1947	135	0	2903
tpiA	CP023663.1:c743511-742729	CU179680.1	87.101	783	100	0	954
tuf	CP023663.1:c578649-577459	CU179680.1	96.725	1191	39	0	1973
uvrC	CP023663.1:725657-727372	CU179680.1	82.761	1717	294	0	1755



KEGG Pathways

#Pathway ID	Pathway description	Observed gene count	False discovery rate	Colour	Matching proteins in your network (labels)
mbv01100	Metabolic pathways	12	0.00068		adh-1,adk,atpA,dnaN,gltX,gmk,polC,pta_1,rpoB,tdk,tkt,tpiA
mbv01110	Biosynthesis of secondary metab	6	0.0086		adh-1,adk,gltX,gpsA,tkt,tpiA
mbv00230	Purine metabolism	5	0.0086		adk,dnaN,gmk,polC,rpoB
mbv00240	Pyrimidine metabolism	4	0.0394		dnaN,polC,rpoB,tdk
mbv01130	Biosynthesis of antibiotics	4	0.042		adh-1,adk,tkt,tpiA
mbv01120	Microbial metabolism in diverse	5	0.042		adh-1,gltX,pta_1,tkt,tpiA

Fig 4. Analysis of 24 housekeeping genes is to define the essential genes in *M. bovis* strain Ningxia-1 compared to PG45 isolate, to prevent cross reaction during identification. 24 selected protein sequences were put into the STRING database to generate potential protein-protein interactions, using the type strain of *M. bovis* PG45 as the database default reference. Different colored lines show different types of interactions. Different colored lines show different types of interactions e.g., textmining (emerald green), experiments (violet), databases (sapphire), neighborhood (green), gene fusion (red), co-occurrence (purple), co-expression (black) and etc. In evidence view, all possible interactions are shown

Table 4. *Most frequent Motifs among M. bovis Ningxia-1 strain housekeeping genes*

Motif	Genes	Description
MMR_HSR1	*dnaA, fusA, lepA, tuf*	PF01926, 50S ribosome-binding GTPase
GTP_EFTU_D2	*fusA, lepA, tuf*	PF03144, Elongation factor Tu domain 2
GTP_EFTU	*fusA, lepA, tuf*	PF00009, Elongation factor Tu GTP binding domain
ABC_tran	*adk, dnaA, recA*	PF00005, ABC transporter
AAA_14	*atpA, dnaA, recA*	PF13173, AAA domain
AAA	*adk, dnaA, recA*	PF00004, ATPase family associated with various cellular activities (AAA)

Transmembrane Analysis of Housekeeping Genes of M. bovis Ningxia-1

To determine transmembrane probability for each housekeeping gene, their protein sequences were pasted into TMHMM. TMHMM v 2.0 is the most popular software in the field [28], with the ability to distinguish cytoplasmic membrane and outer domains in a hidden Markov model [29]. Our results demonstrate that metG is very likely to be a transmembrane protein and the total probability that the N-term is on the cytoplasmic side of the membrane. The rest of 23 housekeeping genes are outer membrane-associated protein in *M. bovis*.

Motif Analysis of Housekeeping Genes

Table 4 lists the top common motifs in the housekeeping gene proteins of *M. bovis* Ningxia-1 strain. The 50S ribosome-binding GTPase family motif PF01926 is present in 4 housekeeping gene proteins *(dnaA, fusA, lepA* and *tuf)* and is the most common motif among all housekeeping genes. Concurrent with the high frequency motif of elongation factor Tu domain 2 and elongation factor Tu GTP binding domain are also found in *fusA, lepA* and *tuf*. ABC transporter and ATPase family associated with various cellular activities (AAA) motifs are found in *adk, dnaA* and *recA*. Additionally, AAA domain is found in proteins encoded by *atpA, dnaA* and *recA* of *M. bovis* Ningxia-1. Finally, we analyzed a less frequent but equally interesting set of genes linked by two genes for example the 3-hydroxyacyl-CoA dehydrogenase (NAD binding domain), AAA ATPase domain and UDP-glucose/GDP-mannose dehydrogenase family (NAD binding domain), are three motifs found in many proteins of *M bovis (adh-1, gpsA, dnaA* and *recA)*. tRNA synthetases class I (C) catalytic domain is only shown to have less impact on metG.

DISCUSSION

Mycoplasma. bovis is one of actively evolving mycoplasmas [30]. *M. bovis* PG45 strain was identified in the USA six decades ago, whereas the Hubei-1 and HB0801 strains were detected in China during 2008. An inversion has been found in the two strains isolated in China. It is assumed that a long interval and the geographical variation may be a cause for this inversion [30]. In contrast with the *M. bovis* genome, housekeeping genes are stable and perform basic fundamental functions and evolve more slowly in terms of both coding and core promoter sequences [17].

Many efforts have been made to identify and characterize the surface proteins in *M. bovis* [31]. However, little is known regarding hierarchy among experimentally proven housekeeping genes, or the distribution and the number of motifs within these genes in *M. bovis*. To date, there is no consensus among the current databases for *M. bovis* and from our understanding many of these predicted surface membrane proteins are yet to be definitively identified and the functions of most of them have not been determined [32]. Despite this though, several membrane proteins and lipoproteins are used in diagnostic assays for detection of antibodies specific for *M. bovis* [32].

16S rRNA gene is a small subunit within prokaryotic ribosomes, commonly used for bacterial identification [33] [Yang, 2016 #1128][Yang, 2016 #1128]. *M. bovis* infections are typically diagnosed by isolation and identification of causative agent and confirmed by the presence of the 16S rRNA gene using PCR [5]. Despite the 16s rRNA gene-based PCR possessing great specificity, cross-amplification of *M. agalactiae* can still occur [34]. A highly stable gene, uvrC, encodes an enzyme essential for replication and involved in DNA repair, known as deoxyribodipyrimidine photolyase [35]. The *uvrC* gene has no cross amplification with non-*M. bovis* species including *M. agalactiae,* proving it is a well conserved and much more specific target gene than 16S rRNA gene [36,37]. However, point mutations in the *M. bovis uvrC* gene have been identified in recent studies, making false negative PCR results identify *M. bovis* strains [38]. Meantime, many novel genes such as *fusA* (encodes for elongation factor G and require in the translation process of mRNA into proteins) genes were also developed to use as detection markers [39]. *RpoB* (encoding the β-subunit of RNA polymerase), is another core gene candidate for phylogenetic analyses and identification of bacteria, especially of closely related isolates [40]. But from our BLAST results, *fusA* has 92.3% and *rpoB* has 89.7% identity with *M. agalactiae*, may not contribute as a diagnosis marker to differentiate *M. bovis* and *M. agalactiae*. Our findings on the 24 housekeeping genes show *adk*, *gpsA*, *polC* also could be used as potential

detection genes to differentiate between *M. bovis* species and *M. agalactiae*.

STRING is more liberal with assigning interactions, as it uses data from homologous protein interactions in different schemas. These interactions suggest that these proteins might function together, and thus, based on bioinformatics methods, we can have a further analysis for these genes. A homology comparison feature is incorporated into STRING, which makes it easier to determine the function of un-identified *M. bovis* genes. In addition to these features which we used in this study, STRING also has other useful features, including a feature that allows for homology comparisons in a phylogenetic context, and protein family analysis. TMHMM utilizes a hidden Markov model to determine transmembrane domains on proteins, with the ability to distinguish cytoplasmic and outer domains and is currently one of the most accurate membrane protein topology prediction methods [29]. Motif analysis could provide us the path to determining the motifs present in the housekeeping genes.

Identification of the essential specific genes and their motifs in the host could benefit us to develop drugs and vaccines against *M. bovis* infection [41,42]. Therefore, future work should focus on identifying these housekeeping genes, especially for the *adk*, *gpsA* and *polC*, which have great potential to benefit the insight of *M. bovis*, and be used as an improved detection tool for clinical diagnosis, *metG* gene could be an important virulence gene based on in silico prediction as a trans-membrane gene and *adk*, *dnaA*, *fusA*, *lepA* and *recA* would extend the treatment method.

Funding

This study was supported by the Science and Technology Agent of Ningxia Province (Grant No. 412-0164 to HH) and the Natural Science Foundation of China (NSFC) (Grant No. 31960708).

Acknowledgment

The authors wish to acknowledge help from Yangyang Wei of GeneDenovo Guangzhou, China and Lingfeng Zeng from PTM Biolabs, for data analysis. The authors also acknowledge Dr. Donglei Sun and Ashley B. Strickland (Division of Immunology, Virginia-Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, Maryland, USA) for providing useful suggestions for our paper.

Conflict of Interest

The authors have declared that no conflict of interest exists.

Author Contributions

P.S. and Y.F. designed and performed experiments, analyzed results, and wrote the manuscript. Q.W. and M.Y. provided advice. S.H. and X.S. supervised the study and wrote the manuscript.

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