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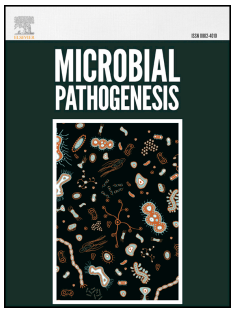

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Genotype distribution of Chinese *Mycoplasma bovis* isolates and their evolutionary relationship to strains from other countries

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Running title: Molecular characterization, genotype distribution of Chinese *Mycoplasma bovis* isolates and their global evolution

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Abstract

This study was undertaken to determine the genotypic distribution of Chinese *M.bovis* strains and their similarity to isolates from other countries. Two multilocus sequence typing (MLST) schemes (MLST-1 and MLST-2) and pulsed field gel electrophoresis (PFGE) were used to compare 44 Chinese strains and the *M. bovis* type strain PG45. The results showed a high genetic homogeneity of Chinese isolates; 43 of 44 (97.7%) Chinese isolates were identified as ST-10 and as ST-34 by MLST-1, while for MLST-2 42 of 44 (95. 5%) were identified as ST-10 with the two remaining isolates of ST-32 and ST43. PFGE clustered 42 of 44 (95.5%) of the Chinese isolates into PT-I. The overall agreement rate between the three typing methods was 97.8%（95% CI：86.8-99.9 %）. The type strain PG45 was identified as a unique type by all three methods. When the MLST-2 scheme was further used to analyze 16 isolates of Australian and Israeli origin ST-10 was more dominant among Australian isolates (7/8), compared with those from Israel (3/8). The evolutionary relationship of the 60 isolates typed in this study assessed together with 206 additional isolates retrieved from pubmlst/mbovis database analysed by geoBURST Minimum spanning tree (MST) confirmed that the Chinese, Israeli and Australian *M. bovis* isolates typed in this study that were predominantly ST-10, were clustered in CC3 with isolates originating from the USA..

Our results suggest that ST-10 is an emerging clone of *M.bovis* population. We hypothesized that the widespread distribution of this type is a result of global livestock movements. These findings will help further the understanding of the

global evolution of *M.bovis* and development of novel vaccines against *M. bovis*.

Key words: *Mycoplasma bovis*; molecular epidemiology; multilocus sequence typing (MLST); pulsed field gel electrophoresis (PFGE); cattle; evolution

Introduction

Mycoplasma bovis, the causative agent of bovine mycoplasmosis, is part of the bovine respiratory disease (BRD) complex [1]. It was first isolated from bovine mastitic milk in the USA and subsequently spread via animal movements to most countries of the world including European countries [2], China [3] Australia [4] and Israel [5]. *Mycoplasma bovis* causes pneumonia, arthritis and mastitis leading to substantial economic losses for beef and dairy cattle producers with an estimated annual loss of up to $108 million USD in the USA[2].

M. bovis related diseases are diverse among different countries and regions. Transportation is usually a main contributing factor to the disease development in fattening calves. Other factors might including, cattle species, husbandry pattern (grazing or housed), and calf feeding systems (calves which suckle or are artificially fed with pasteurized colostrum or normal milk) [2]. For example, *M. bovis* pneumonia often occurs in newly introduced fattening calves, most likely induced by transportation stress, while in newborn calves infection resulted from suckling contaminated milk of mothers with *M. bovis* mastitis[6]. To understand the

74 evolutionary relationships of *M. bovis* isolates from different countries and regions
75 of the world will help elucidate the molecular epidemiology of the organism and
76 assist in the development of prevention measures such as vaccines against this
77 pathogen. However, there remain large gaps in our understanding of the evolutionary
78 relationship of *M. bovis* isolates between different countries and globally.

79 In China the first report of mastitis from *M. bovis* was published in 1983 [7] and
80 the first case of *M. bovis* pneumonia in 2008. Since then reports of pneumonia and
81 mastitis outbreaks associated with *M. bovis* have been described frequently [3, 8]. The
82 major contributing factor to the outbreak of *M. bovis* pneumonia was thought to be the
83 stress associated with the long distance transportation of cattle between farms and
84 feedlots. The disease is difficult to control with chemotherapy due to the intrinsic and
85 induced resistance to antimicrobials [2, 9], therefore vaccination would be an ideal
86 alternative approach. An insight into the genetic diversity and population structure of
87 *M. bovis* would assist in the development of novel vaccines, as well as gaining an
88 understanding of evolutionary trends.

89 A variety of molecular typing methods have been used for epidemiological
90 characterization of *M. bovis* strains, including random amplified polymorphic DNA
91 (RAPD) analysis [10], amplified fragment length polymorphism (AFLP) analysis [11,
92 12], pulsed field gel electrophoresis (PFGE) [13, 14], insertion sequence (IS) typing
93 [15, 16] and multilocus variable number tandem repeats (VNTR) analysis [14, 17].
94 In addition, three multi-locus sequence typing (MLST) schemes were recently
95 developed to study population structure, evolution and spread of this pathogen

[18-20]. The MLST scheme developed by Manso-Silvan et al (2012) [18] is based on the four housekeeping genes *fus*A, *gyrB*, *lepA*and *rpoB* and showed a discrimination index of 0.833. Improved MLST schemes have been developed by Rosales et al (2015, 2017) (MLST-1 scheme) [20, 21] and by Register et al (2015), (MLST-2 scheme) [19]. Both schemes use seven housekeeping genes although they only have one gene in common and their discrimination powers are higher than the previous MLST scheme presented by Manso-Silvan et al (2012) [18].

The aims of the present study were to: compare MLST-1 and MLST-2 schemes with conventional PFGE for differentiating 44 Chinese isolates of *M. bovis*, assess the genetic diversity and population structure of *M. bovis* strains isolated from 2008 to 2014 using the type strain PG45 as a control, and explore the evolutionary relationship of Chinese isolates with globally diverse isolates.

Materials and Methods

Ethical approval

This study has been approved by the Hubei Province Science and Technology Department, which is responsible for experimental animal ethics (Permit Number: SYXK(er) 2005-0029) and were supervised by the animal ethics committee of Huazhong Agricultural University.

Mycoplasma bovis isolates

Forty-four Chinese isolates of *M. bovis* and one *M. bovis* type strain PG45 (ATCC 25523) were examined. These isolates had been cultured between 2008 and 2014 from nine Chinese provinces: Hubei (n=25), Anhui (n=1), Fujian (n=2), Hunan

(n=1), Jiangxi (n=3), Henan (n=8), Inner Mongolia (n=1), Guangzhou (n= 2) and Shandong (n=1). These Chinese isolates were cultured from pneumonic lungs (41); mastitic milk (1); throat swab of a case of pneumonia (1) and fluid of a joint with arthritis (1). PG45 was used as a comparative isolate in the present study.

DNA samples from eight Israeli *M. bovis* isolates were kindly provided by Prof. Dr. Inna Lysnyansky from Kimron Veterinary Institute, Israel, and had been collected in the period 2013 to 2014 from cases of pneumonia (6), stillbirth (1) and arthritis (1). These isolates had originated from seven regions in Israel: Gilboa (n=1), Beer Tuvia (n=3), Hevel Eilot (n=1), Eshkol (n=1), Mateh Yehuda (n=1) and Emek Yizrael (n=1).

In addition, whole genome sequences of eight Australian isolates were retrieved from GenBank. These had originated from mastitis cases (4), lungs (1), nasal swab (1), joint fluid (1) and semen (1) from five Australian regions (New South Wales 2, Queensland 1, Tasmania =3, South Australia 1 and Victoria 1) with accession numbers of SAMN05444185, SAMN05444199, SAMN05444228, SAMN05444239, SAMN05444243, SAMN05444247, SAMN05444250, and SAMN05444261 respectively (Table 1).

Growth conditions, species identification and DNA extraction

Isolates were confirmed as *M. bovis* by a species-specific PCR [22]. The *M. bovis* samples were grown in PPLO broth (BD Difco^{Tm}, US California) supplemented with 0.5% (w/v) sodium pyruvate (Biosharp, China), 0.09% (w/v) yeast extract (BD Biosciences, San Jose, CA, USA)，0.004% (w/v) phenol red, 1% (v/v) 10× minimum

essential medium (MEM) (Sigma-Aldrich, Saint Louis, MO, USA), 20% (v/v) Hyclone donor horse serum (Invitrogen, Carlsbad, CA, USA) and penicillin G 80,000 IU/100 mL and the final pH was adjusted to 7.6 [23]. DNA from each isolate was extracted using a bacterial genomic DNA extraction kit (Tiangen, Beijing, China).

Multilocus sequence typing (MLST)

The 44 Chinese isolates and type strain PG45 were characterized using the MLST-1 scheme [20] which is based on partial sequencing of *dnaA*, *metS*, *recA*, *tufA*, *atpA*, *rpoD* and *tkt* genes. After amplification, PCR products were further purified using a Magnetic Beads PCR Products Extraction Kit (Enriching Biotechnology, LTD, Wuhan, China) and sequenced. Sequencing reactions were performed by the company Tianyi Hui Yuan Biological Technology Private. Ltd., Wuhan, China. The quality of chromatograms was checked visually and sequence data were assembled and edited using SeqMan software (DNASTAR Inc., Wisconsin, USA). The assembled MLST-1 sequences were compared using the non-redundant database (NRDB) comparison tool (http://pubmlst.org/analysis/) together with 10 previously analyzed strains to assign allele and sequence type number [20].

The 44 Chinese strains and PG45 were also assessed by the MLST-2 scheme which is based on a partial sequencing of *adh-1*, *gltX*, *gpsA*, *gyrB*, *pta-2*, *tdk* and *tkt* genes [19]. The PCR products were sequenced and categorized as for MLST-1. The assembled sequences of all isolates were compared to the http://pubmlst.org/mbovis/ database to assign allele numbers and STs. Novel allele sequences were submitted to

the database for confirmation and allocation of novel sequence type.

For the evolutionary assay, an additional eight Israeli strains were typed by the same method MLST-2. The whole genome of eight Australian isolates was annotated using prokka 1.11 rapid prokaryotic genome annotation software [24] (http://www.vicbioinformatics.com). Each locus sequence was extracted from the annotated genome.

Pulsed Field Gel Electrophoresis (PFGE) analysis

PFGE of the 44 Chinese *M. bovis* field strains and type strain PG45 was performed as previously described [13, 25] with some modifications for agarose block preparation. Briefly, macro-restriction analysis was performed with the restriction enzyme *Sma*I as follows: 15 ml of the *M. bovis* culture was centrifuged at 15000 ×*g* for 20 min at 4°C, The pellet was then washed three times with Tris-EDTA buffer and resuspended in 400 µl of cold Tris-EDTA buffer (pH 8.0). Agarose plugs were prepared from a 1:1 mixture of the above cell suspension and 2% low melting point agarose (Bio-Rad, Wuhan, Hubei, China). They were then incubated in a lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% lauroyl sarcosine, and 1mgml^{-1} of proteinase K) for 48 h at 56°C. The plugs were then washed for 6 h with eight to ten changes of Tris-EDTA buffer at 4°C, then cut aseptically into 2 mm sections and equilibrated in 120 µl restriction buffer (Promega, Shanghai, China) for 30 min at 4°C. Subsequently plugs were digested with 30 U of *Sma*I (Promega, Shanghai, China) at 24°C for 4 h prior to loading on 1% pulsed-field-certified agarose gel (Bio-Rad) and run on a CHEF-DRIII system (Bio-Rad), at 6 V/cm with an angle of 120° in 0.5× TBE buffer at 14°C. The

initial pulse time was 5 s, with a final pulse time of 40 s with a running time of 24 h. The lambda DNA ladder PFGE marker (Bio-Rad) was used as a reference marker. PFGE fragments in the gel were stained with ethidium bromide (EB) (1mg/ml) for 20 min, and destained in distilled water for 1.5 h and visualized under a UV transilluminator. Pulsotypes (PT) were assigned numbers consecutively based on differences of more than one band in PFGE patterns upon visual inspection. The banding patterns were analyzed using Dice coefficients with 1% band position tolerance with GelJ software [26]. The clustering of patterns was performed using unweighted pair group matching algorithm (UPGMA) [13, 27].

Allelic sequence variance analysis

The Sequence Type Analysis and Recombinational Test Version 2 (START2) [28] were used to analyze polymorphic sites, construct UPGM dendrograms and calculate non-synonymous to synonymous ratios (d_N/d_S). The genetic diversity (H) of each locus and the Index of Association (I_A) were calculated with LIAN 3.5 [29] (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian. cgi.pl/query).

Global evolution and minimum spanning tree (MST) analysis

The evolutionary relationship between isolates and the *M. bovi*s population structure were determined using PHYLOViZ [30] and evaluated by minimum spanning tree (MST) created using eBURST (geoBURST) algorithm [31]. The MST for MLST-2 was performed for 266 isolates from 11 countries including the 60 strains (44 Chinese, 8 Israeli and 8 Australian isolates) typed in this study and 206 isolates retrieved in January 2017 (Supplementary Table 3) from the *M. bovis* MLST-2

206 database, including the type strain PG45 (www.pubmlst.org/mbovis).

207 **Statistical analysis**

208 The discriminatory ability of both MLST methods and PFGE was calculated using
209 Simpson's index of diversity [32]. Congruence between both typing techniques was
210 measured using the adjusted Rand Coefficient and Wallace Coefficient [33]. All
211 statistical analyses were performed using the freely available online tool
212 (http://darwin.phyloviz.net/ComparingPartitions/).

213

214 **Results**

215 **The characterization of Chinese *M. bovis* isolates with three typing methods**

216 **MLST-1 analysis**

217 The mean GC contents of the seven gene fragments ranged from 29.15% *(dnaA)* to
218 37.23% *(tufA)* while it was 37.4% for the whole *M. bovis* HB0801 genome [34]. The
219 number of polymorphic sites per locus ranged from 4 (6.2%) in *recA* to 19 (29.6 %)
220 in *dnaA*, and a total of 64 polymorphic sites for all seven genes were identified
221 (Table 2). The number of alleles observed ranged from 2 (*metS*, *recA*, *tufA*, *atpA*, and
222 *tkt)* to 3 *(dnaA* and *rpoD)*. The genetic diversity (H) for each locus was 0.0879 for
223 *dnaA* and 0.0444 for *metS, recA, tufA, atpA* and *tkt*. The d_N and d_S substitutions
224 ranged from 0.0000 to 0.0605.

225 The 44 Chinese isolates were divided into two STs by MLST-1 (ST-10 and
226 ST-34, Table 1). The ST-10 (with allelic profile of 2, 6, 2, 2, 2, 5, 3) was most
227 numerically dominant, comprising 97.7%（43/44）of the isolates including HB0801

(Fig. 1). ST-34 (allelic profile of 11, 6, 2, 2, 2, 5, 3) contained only one strain SZ, while ST-1 (allelic profile of 1, 1, 1, 1, 1, 1, 1) was represented only by strain PG45 (Table 1). Based on the genetic relatedness, the 44 Chinese strains could be classified into two clades, A and B. Clade A contained the majority (97.7%) of the isolates (43/44) including HB0801, while clade B contained only SZ (ST-34). PG45 type strain was an outlier of these two clades (Fig. 1).

MLST-2 analysis

The mean GC contents of seven gene fragments after examination by MLST-2 ranged from 28.76% *(tdk)* to 35.61% *(gyrB).* The number of polymorphic sites per locus ranged from 0 to 14 (15.5%) in *gpsA* and a total of 90 polymorphic sites were identified (Table 2). Three alleles were identified for *gyrB and gltX*, two for *adh-1*, *gpsA*, *pta2 and tkt*, but only a single allele for *tdk*. The genetic diversity varied from 0.000 to 0.879 within the locus (Table 2).

The Chinese strains were distributed into three different sequence types. ST-10 with allelic profile 4, 3, 3, 3, 5, 3, 4 was the most numerically dominant type, comprising 95.4% (42/44) of Chinese isolates; and two novel sequence types ST-32 (isolate EZ-8-NHD0962) and ST-43 (NHD0986). All isolates tested in this study were clustered into two major clades, A and B, based on genetic relatedness by UPGMA. Clade A comprised 95.4% (42/44) of the Chinese isolates, including HB0801. Clade-B contained the remaining two Chinese isolates (EZ-8-NHD0962 and NHD0986). PG45 type strain was again an outlier of these two clades (Fig. 2).

PFGE typing

All 44 Chinese isolates and strain PG45 were typeable by *Sma*I with production of 6 to 10 bands (from <48.5 kb to 450 kb in size). PFGE revealed 3 distinct pulsotypes: pulsotype (PT)-I contained 95.5% (42/44) of the Chinese strains, including HB0801; PT-II contained two Chinese strains (SD-130626-NHD0969 and F150niu-NHD0954); and PT-III only contained the PG45 strain (Table 1). The results of the UPGMA analysis of all PTs and their relatedness to the two MLST sequence types are displayed in Fig. 3.

Discriminatory power of MLST schemes and PFGE

Based on the data of 44 Chinese strains and PG45, the overall agreement rate among the three typing methods was 97.8% (44/45) (95% CI：86.8-99.9%). The Simpson's Index of Diversity (D) discrimination was highest for PFGE (D = 0.160) with (D = 0.130) for MLST-2 and (D = 0.088) for MLST-1 schemes. Housekeeping loci of both MLST schemes had a very low dN/dS ratio (<1). The standard index of association (IA) for the MLST-1 scheme was 0.87($p \leq 0.001$), and for the MLST-2 scheme 0.623 ($p \leq 0.001$). Isolates that had the ST-10 and ST-34 by MLST-1 and ST-10, ST-43 and ST-32 by MLST-2 belonged to the PFGE-PT-I pulsotype. The PFGE differentiated two Chinese isolates belonging to ST-10 in both MLST schemes into PT- II. The three typing methods similarly identified the PG45 reference strain as a unique type: ST-1(MLST-1) / ST-17(MLST-2) / PT-III (PFGE).

geoBURST MST analysis by MLST2

The eight Israeli strains typed in this study were shown to have five different STs with ST-10 being the most dominant type (n=3) compared with ST-5 (n=2 isolates)

and three novel STs (ST-42, ST-44, ST-45) each had one isolate. The 8 Australian isolates were divided into two sequence types ST-10 (n=7) and the novel ST-41 (n=1) (Table 1).

The MST comparisons of all 266 isolates identified six clonal complexes (CC1 to CC6) based on the maximum distance between the nodes. Interestingly, the Chinese, Israeli and Australian *M. bovis* isolates in this study that were predominantly ST-10, were clustered in CC3 with isolates originating from the USA (Fig. 4). Other Israeli and Australian isolates belonging to ST-41, ST-42, ST-45, ST-5 and ST41 were grouped in CC1, CC2 and CC5 and are shown together with remaining clonal complexes (C4 and C6) containing isolates representing different countries origin in Figure 4.

Discussion

An understanding of the *M. bovis* population structure is critical in elucidating the epidemiology of *M. bovis* associated diseases and to help develop efficient control measures including development of vaccines and accurate diagnostic tools. Both MLST schemes and the PFGE analysis demonstrated a high degree of homology among the 44 Chinese isolates, indicating the clonal nature of the disseminated isolates. MLST-1 and MLST-2 classified 97.7% and 95.5% of the Chinese isolates, respectively, as ST10, despite only one of the seven loci in common between the two schemes (Table 1)., while PFGE also classified 95.5% (42/44) of them as PT-I (Fig. 4). This high homology supports the known distribution and movement pattern of Chinese cattle. In mainland China, the dominant beef cattle raising areas are

294 concentrated in the north and west. These areas are either pasture areas for grazing
295 cattle or agricultural areas with adequate supplies of maize grain, and stubble. Beef
296 calves and stockers are usually transported over a period of 2 to 3 days from the north
297 to the south of China. Although the isolates tested were originally isolated from calves
298 in Hubei and other provinces, the calves would most likely have originated from more
299 northern provinces. The findings of the current study highlight the circulation of a
300 single dominant clone in all provinces of China, supporting the hypothesis that a
301 single clone has spread through animal movement. A similar finding has recently been
302 observed in France [35] where loss of diversity within *M. bovis* isolates over the last
303 35 years and spread of a single clone throughout the country were hypothesized to be
304 linked to selection and distribution of an antimicrobial resistant clone. A single clonal
305 population of *M. bovis* was also detected by VNTR analysis of 29 isolates sourced
306 from distant geographic origins in Austria [17]. In our previous study[36] we found
307 Chinese, *M. bovis* isolates were resistant to levofloxacin, lemofloxacin, and
308 ciprofloxacin and intermediate resistance for norfloxacin and nalidixic acid, the set of
309 same isolates [36] were typed in this study found as same ST-10. So it might be
310 possible that the clonal spread of *M. bovis* linked to antimicrobial treatments.
311 To assess the evolutionary relatedness between Chinese strains and those from other
312 countries, 266 strains were compared. Of the 60 strains typed with MLST-2, a
313 dominant type, ST-10 in CC3, was found in the isolates from China, Israel and
314 Australia suggesting a possible common source. Based on the high prevalence (60%)
315 of ST-10 in dairy herds in Israel, a link between *M. bovis* strains through the

316 importation of calves from Australia, which have been shown to possess the similar
317 dominant ST-10 genotype, was previously suggested [5]. In the current study this ST
318 was also identified from seven of the eight isolates originating from five different
319 Australian states investigated. Whole genome SNP was recently used to show that a
320 single homogenous strain was widely distributed in Australia[37]. Interestingly,
321 ST-10 the most prevalent ST found in current is of a similar type to that reported in
322 previous studies [19] for strains isolated from cattle in the USA. Previously, the
323 international spread of contagious bovine pleuropneumonia (CBPP), caused by
324 *Mycoplasma mycoides subspecies mycoides* (formerly small colony, (Sc), was
325 shown to be linked to the movement of cattle [38, 39]. It is probable that a similar
326 transfer of *M. bovis*, and in particular of the ST-10 type, through the international
327 movement of cattle has resulted in it becoming the dominant type in China as well as
328 Australia, Israel and USA.
329 Over the last few decades the MLST and PFGE approaches have been considered to
330 be the gold standards for investigating the molecular epidemiology and population
331 structure of microorganisms. MLST is a reproducible and highly discriminatory
332 method that can easily differentiate isolates based on housekeeping gene sequences
333 and can be easily compared between laboratories [40]. Two MLST schemes with
334 reportedly similar discriminatory powers have been developed for *M. bovis* [19, 20].
335 In the present study we have demonstrated that two different MLST schemes,
336 identified a similar number of alleles per locus despite having only one common loci,
337 suggesting that the seven loci examined in each scheme is likely to be sufficient to

differentiate strains of *M. bovis*. However, both schemes had very low d_N/d_S ratios (<1). A similar finding had also been reported by other researchers [19], indicating stable selection of all loci. The standardized index of association is used to investigate the linkage equilibrium of frequent recombination events. In the current study both MLST schemes loci had an I_A of >1, indicating a linkage disequilibrium between the alleles of the tested *M. bovis* population and suggesting that their evolution is free of recombination. Our study showed very limited genetic diversity in the number of alleles (Table 2). Similar findings have been reported for analysis of *M. bovis* and the gnomically related *M. agalactiae* populations previously studied by MLST [20, 41] . Low values confirms the clonal nature of *M.bovis* population in this study (1-2 isolates were different). The clonal relationship between the Chinese isolates was also confirmed using PFGE. Based on the data of the 44 Chinese strains and PG45 the agreement rate among the three typing methods was 97.8% (44/45)（95% CI：86.8-99.9%). PFGE results obtained in this study with *SmaI* were in agreement with the results reported by others who reported that this endonuclease was one of the best for discriminating *M. bovi*s [13, 14, 27]. Of the three typing methods, PFGE was found to be the most discriminatory. No large significant difference was observed in the discriminatory power of the two MLST schemes. However, as the method is sequenced based MLST offers the advantage of being able to characterize strains when culture is not possible, a disadvantage of PFGE. Analysis of Chinese *M. bovis* isolates by both MLST schemes and PFGE has been useful in determining their clonality and in helping to understand their molecular epidemiology and

population structure. In the future more isolates from geographically diverse locations should be characterized to provide further information on the population structure of *M. bovis*.

Conclusions

This study has revealed *M. bovis* population in China exists as one single dominant which is also present in Israel, Australia and USA, suggesting that Chinese *M. bovis* strains might have originated in Australia or the USA as a result of livestock introductions. The three *M. bovis* genotyping methods applied in this study showed a high level of agreement. These results may help further our understanding of the global evolution of *M. bovis* and provide information that may be useful for the development of novel vaccines.

Conflict of interest statement

The authors declare that they have no competing interests.

Author Contributions:

Conceived and designed the project: HM, AG. Performed the experiments: HM FAK MF MZ. Contributed reagents/materials/analysis tools: HC ZH GZ XZ YC. Analysis data: HM AR MAR AMM IR AR Writing the paper: HA AG AR, IR

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535 **Figure Captions**

536 **Figure 1. UPGMA dendrogram of MLST-1**

537 UPGMA derived dendrogram showing the genetic relatedness among *M. bovis*

538 isolates using the MLST-1 scheme**.**

539 **Figure 2. UPGMA dendrogram of MLST-2**

540 UPGMA dendrogram developed after differentiation by the MLST-2 scheme of 44

541 Chinese isolates and PG45

542

543 **Figure 3**. **PFGE Dendrogram**

544 Dendrogram derived from *Sma*I PFGE patterns of 44 Chinese *M. bovis* isolates and

545 the PG45 reference strain.

546

547 **Figure 4. Minimum spanning tree of MLST-2**

548 The Minimum spanning tree analysis of 266 *M. bovis* isolates with 45 STs. Isolates

549 from China, Australia and Israel with ST-10 grouped in CC3 with isolates originating

550 from the United States. ST-26 and ST-28 in CC3 from China and Israel were already

551 listed in MLST database. Each color represents the country of origin of isolates.

552 (Red circles are isolates of this study)

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Table 1 *M. bovis* strains used in this study and their genotypes obtained by two MLST schemes and PFGE.

Country	Province	City / Village	Isolate identification	Year of isolation	Specimen	PFGE pulsotype (PT)	Allelic numbers according to the MLST-1							ST	Source	Allelic numbers according to the MLST-2							ST	CC
							dnaA	metS	recA	tufA	atpA	rpoD	tkt			adh1	gltX	gpsA	gyrB	pta2	tdk	tkt		
China	Hubei	Yingcheng	HB0801	2008	Lung	PT-I	2	6	2	2	2	5	3	10	Rosales et al., (2015)	4	3	2	3	5	3	4	10	CC3
(n=44)	(n = 25)																							
		Jingshan	JS1075-NHD0955	14/05/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Suizhou	SZ-NHD0960	07/06/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Ezhou	1834-NHD0953	10/06/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Ezhou	EZ-3-NHD0947	10/07/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Ezhou	EZ-8-NHD0962	10/07/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	5	2	3	5	3	4	32	CC3
		Ezhou	NHD0986	10/07/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This Study	4	3	2	**12***	5	3	4	**43***	CC3
		Xinzhou	XZ-1-NHD0981	11/07/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Xinzhou	XZ-2-NHD0946	11/07/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Fangxian	FX-NHD0970	05/12/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Hongan	NNH-NHD0956	01/05/2010	Throat	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Huanggang	HG1007	16/05/2010	Milk	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Zhongxiang	ZhX	30/06/2010	Lung	PT-I	2	6	2	2	2	5	3	10	Rosales et ., (2015)	4	3	2	3	5	3	4	10	CC3
		Daye	DY-NHD0963	23/07/2010	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Tongshan	TY-120615-NHD0952	15/06/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Jiangxia	JX-NHD0966	15/07/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Yingcheng	F150tu-NHD0954	15/07/2012	Lung	PT-II	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Yingcheng	F150niu-NHD0949	15/07/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Daye	Dyrengong-NHD0951	11/10/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
		Yingcheng	HB0801-rengong NHD0989	11/10/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3

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	Shanyang	SY-NHD0950	24/01/2013	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Wuxue	WX-NHD0964	12/03/2013	Synovial fluid	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Yichang	YC-NHD0967	15/03/2013	Milk	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Huangshi	HS-130614-NHD0990	14/06/2013	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Shiyan	SY-141210	10/12/2014	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
Anhui (n=1)	Bozhou	BZ-NHD0982	10/06/2008	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
Fujian (n=2)	Xianmen	XM	17/10/2009	Lung	PT-I	2	6	2	2	2	5	3	10	Rosales et al., (2015)	4	3	2	3	5	3	4	10	CC3
	Xinanmen	XMrengong-NHD0985	11/10/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
Hunan (n=1)	Lianjiang	LJ1225-NHD0945	22/12/2009	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
Henan (n=8)	Yanling	YL-NHD0941	25/02/2009	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Kaifeng	KF	10/10/2009	Lung	PT-I	2	6	2	2	2	5	3	10	Rosales et al., (2015)	4	3	2	3	5	3	4	10	CC3
	Yanling	YL0724-NHD0957	12/11/2009	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Yanjing	YJ0719-NHD0958	03/02/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Keerqin	KEQ-NHD0988	17/07/2010	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Keerqin	KLQ	28/04/2010	Lung	PT-I	2	6	2	2	2	5	3	10	Rosales et al., (2015)	4	3	2	3	5	3	4	10	CC3
	Zhumadian	ZMD	28/05/2011	Lung	PT-I	2	6	2	2	2	5	3	10	Rosales et al., (2015)	4	3	2	3	5	3	4	10	CC3
	Yanling	YLrengong-NHD0968	11/10/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
Inner Mangolia (n=1)	Neimeng Yuliang	YL2086	19/07/2012	Lung	PT-I	2	6	2	2	2	5	3	10	Rosales et al., (2015)	4	3	2	3	5	3	4	10	CC3
Jiangxi (n=3)	Xinyu	JXXY	06/10/2012	Lung	PT-1	2	6	2	2	2	5	3	10	Rosales et al., (2015)	4	3	2	3	5	3	4	10	CC3
	Gaoan	GA-NHD0984	19/07/2012	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Jian	JX-140526	26/05/2014	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
Guangzhou	Shenzhen	SZ	27/05/2012	Lung	PT-I	11	6	2	2	2	5	3	34	Rosales et al.,	4	3	2	3	5	3	4	10	CC3

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	(n=2)														(2015)								
		Shenzhen	SG-NHD0983	01/04/2013	Lung	PT-I	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
	Shandong (N) (n=1)	Shandong	SD-130626-NHD0969	24/06/2013	Lung	PT-II	2	6	2	2	2	5	3	10	This study	4	3	2	3	5	3	4	10	CC3
USA	Reference Type strain		PG45	1961	Milk	PT-III	1	1	1	1	1	1	1	1	Rosales et al., (2015)	3	2	4	2	1	3	2	17	CC3
Israel (n=8)	Gilboa	M	204996	2014	Lung	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3
	Beer Tuvia	BT	200579	2014	Lung	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	1	2	1	2	5	1	2	***42**	CC5
	Beer Tuvia	A	214734	2014	Lung	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	3	3	2	3	6	**5**	CC5
	Hevel Eilot	Y	168689	2013	Stillbirth	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	3	3	2	3	6	5	CC3
	Eshkol	NO	213874	2014	Lung	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3
	Beer Tuvia	AZ	211790	2014	Lung	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	7	3	***11**	5	3	4	***44**	CC3
	Mateh Yehuda	Z	211029	2014	Synovial fluid	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	**10**	CC3
	EmekYizrael	G	197326	2014	Lung	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	6	6	***10**	7	5	9	***45**	CC3
Australia (n=8)	New South Wales		Mb02	26/02/2007	Milk	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3
	Queensland		Mb16	09/09/2009	Milk	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3
	New South Wales		Mb47	30/10/2014	Lung	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3
	Tasmania		Mb61	13/09/2013	Semen Culture	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	**5***	3	5	3	4	**41***	CC2
	Tasmania		Mb67	24/09/2013	Nose Swab	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3
	Tasmania		Mb72	05/11/2013	Joint fluid	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3
	South Australia		Mb76	02/05/2014	Milk	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3
	Victoria		Mb90	14/06/2011	Milk	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	3	2	3	5	3	4	10	CC3

ST - sequence type; CC: clonal complex; PFGE: Pulsed filed gel-electrophoresis; PT: Pulsotype determined by PFGE; NT – not tested, Bold number with * represent novel Sequence type and novel allele number.

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Table 2 Sequence polymorphic analysis of the housekeeping genes used in MLST-1 and MLST-2 schemes.

Scheme	Genes	Start position in HB0801*	H[b]	Allele number	Polymorphic sites	d_N/d_S[c]	G+C% content
MLST-1	atpA	85827	0.1066	2	12	0.000	34.04%
	dnaA	1	0.087-	3	19	0.0623	29.15%
	metS	257222	0.0444	2	7	0.0406	30.46%
	recA	376937	0.444	2	4	0.0000	34.41%
	rpoD	353661	0.0444	2	5	0.0605	30.51%
	tkt	245945	0.0444	2	12	0.0505	30.29%
	tufA	56138	0.0444	2	5	0.0000	37.23%
MLST-2	adh-1	420995	0.0444	2	8	0.1884	33.34%
	gltX	774205	0..0879	3	12	0.0506	31.44%
	gpsA	66919	0.0444	2	14	0.1431	29.50%
	gyrB	964083	0.0879	3	9	0.0805	35.61%
	pta-2	669100	0.0044	2	10	0.1179	34.39%
	tdk	921251	0.000	1	0	0.0000	28.76%
	tkt	245945	0.0444	2	13	0.0448	30.06%

[a] Based on *M. bovis* HB0801 complete genome. [b] H: genetic diversity. [c] d_N/d_s statistical test for purifying selection.

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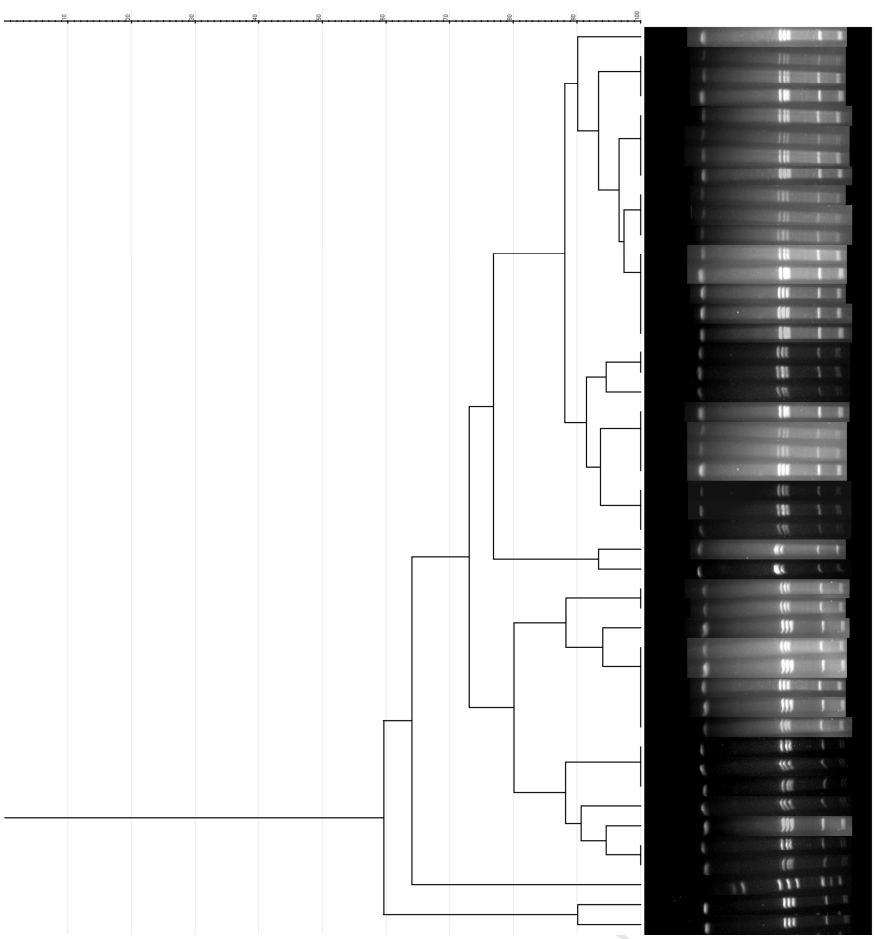

Pulsotypes	MLST-1	MLST-2	Isolates Names
PT-I	ST-10	ST-10	GA-NHD0984
PT-I	ST-10	ST-10	FX-NHD0970
PT-I	ST-10	ST-10	YC-NHD0967
PT-I	ST-10	ST-10	WX-NHD0964
PT-I	ST-10	ST-10	XM
PT-I	ST-10	ST-10	SY-141210
PT-I	ST-10	ST-10	HG1007
PT-I	ST-10	ST-10	YL0724-NHD0957
PT-I	ST-10	ST-10	YLrengong -NHD0968
PT-I	ST-10	ST-10	ZhX
PT-I	ST-10	ST-10	SZ-NHD0960
PT-I	ST-10	ST-43	NHD0986
PT-I	ST-10	ST-10	SG-NHD0983
PT-I	ST-10	ST-10	JX-NHD0966
PT-I	ST-10	ST-10	YJ0719-NHD0958
PT-I	ST-10	ST-10	NNH-NHD0956
PT-I	ST-10	ST-10	TY-120615-NHD0952
PT-I	ST-10	ST-10	Dyrengong-NHD0951
PT-I	ST-10	ST-10	SY-NHD0950
PT-I	ST-10	ST-10	JX-140526
PT-I	ST-10	ST-10	KEQ-NHD0988
PT-I	ST-10	ST-10	ZMD
PT-I	ST-10	ST-10	XMrengong -NHD0985
PT-I	ST-10	ST-10	LJ1225-NHD0945
PT-I	ST-10	ST-10	KF
PT-I	ST-10	ST-10	JX-14026
PT-II	ST-10	ST-10	SD-130626-NHD0969
PT-II	ST-10	ST-10	f150niu-NHD0954
PT-I	ST-10	ST-10	HS-130614-NHD0990
PT-I	ST-10	ST-10	DY-NHD0963
PT-I	ST-10	ST-10	SD-130626-NHD0989
PT-I	ST-10	ST-10	BZ-NHD0982
PT-I	ST-10	ST-10	XZ-1-NHD0981
PT-I	ST-10	ST-10	KLQ
PT-I	ST-10	ST-32	EZ-8-NHD0962
PT-I	ST-10	ST-10	JS1075-NHD0955
PT-I	ST-10	ST-10	F150niu-NHD0949
PT-I	ST-34	ST-10	SZ-0527
PT-I	ST-10	ST-10	EZ-3-NHD0947
PT-I	ST-10	ST-10	YL2086
PT-I	ST-10	ST-10	1834-NHD0953
PT-I	ST-10	ST-10	JXXY
PT-I	ST-10	ST-10	YL-NHD0941
PT-III	ST-1	ST-17	PG45
PT-I	ST-10	ST-10	XZ-2-NHD0946
PT-I	ST-10	ST-10	HB0801

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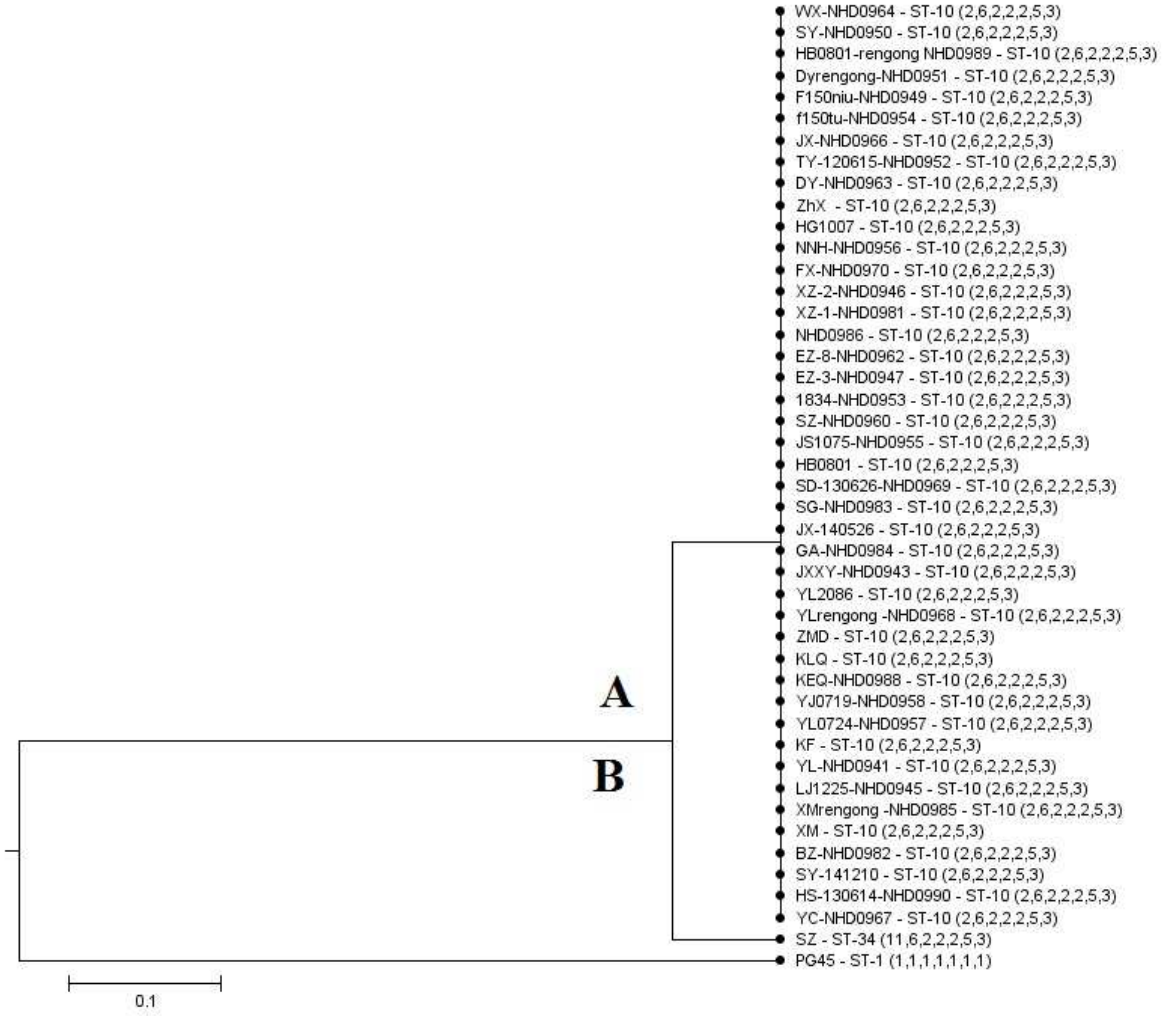
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Dyrengong-NHD0951 - ST-10 (2,6,2,2,2,5,3)
F150niu-NHD0949 - ST-10 (2,6,2,2,2,5,3)
f150tu-NHD0954 - ST-10 (2,6,2,2,2,5,3)
JX-NHD0966 - ST-10 (2,6,2,2,2,5,3)
TY-120615-NHD0952 - ST-10 (2,6,2,2,2,5,3)
DY-NHD0963 - ST-10 (2,6,2,2,2,5,3)
ZhX - ST-10 (2,6,2,2,2,5,3)
HG1007 - ST-10 (2,6,2,2,2,5,3)
NNH-NHD0956 - ST-10 (2,6,2,2,2,5,3)
FX-NHD0970 - ST-10 (2,6,2,2,2,5,3)
XZ-2-NHD0946 - ST-10 (2,6,2,2,2,5,3)
XZ-1-NHD0981 - ST-10 (2,6,2,2,2,5,3)
NHD0986 - ST-10 (2,6,2,2,2,5,3)
EZ-8-NHD0962 - ST-10 (2,6,2,2,2,5,3)
EZ-3-NHD0947 - ST-10 (2,6,2,2,2,5,3)
1834-NHD0953 - ST-10 (2,6,2,2,2,5,3)
SZ-NHD0960 - ST-10 (2,6,2,2,2,5,3)
JS1075-NHD0955 - ST-10 (2,6,2,2,2,5,3)
HB0801 - ST-10 (2,6,2,2,2,5,3)
SD-130626-NHD0969 - ST-10 (2,6,2,2,2,5,3)
SG-NHD0983 - ST-10 (2,6,2,2,2,5,3)
JX-140526 - ST-10 (2,6,2,2,2,5,3)
GA-NHD0984 - ST-10 (2,6,2,2,2,5,3)
JXXY-NHD0943 - ST-10 (2,6,2,2,2,5,3)
YL2086 - ST-10 (2,6,2,2,2,5,3)
YLrengong -NHD0968 - ST-10 (2,6,2,2,2,5,3)
ZMD - ST-10 (2,6,2,2,2,5,3)
KLQ - ST-10 (2,6,2,2,2,5,3)
KEQ-NHD0988 - ST-10 (2,6,2,2,2,5,3)
YJ0719-NHD0958 - ST-10 (2,6,2,2,2,5,3)
YL0724-NHD0957 - ST-10 (2,6,2,2,2,5,3)
KF - ST-10 (2,6,2,2,2,5,3)
YL-NHD0941 - ST-10 (2,6,2,2,2,5,3)
LJ1225-NHD0945 - ST-10 (2,6,2,2,2,5,3)
XMrengong -NHD0985 - ST-10 (2,6,2,2,2,5,3)
XM - ST-10 (2,6,2,2,2,5,3)
BZ-NHD0982 - ST-10 (2,6,2,2,2,5,3)
SY-141210 - ST-10 (2,6,2,2,2,5,3)
HS-130614-NHD0990 - ST-10 (2,6,2,2,2,5,3)
YC-NHD0967 - ST-10 (2,6,2,2,2,5,3)
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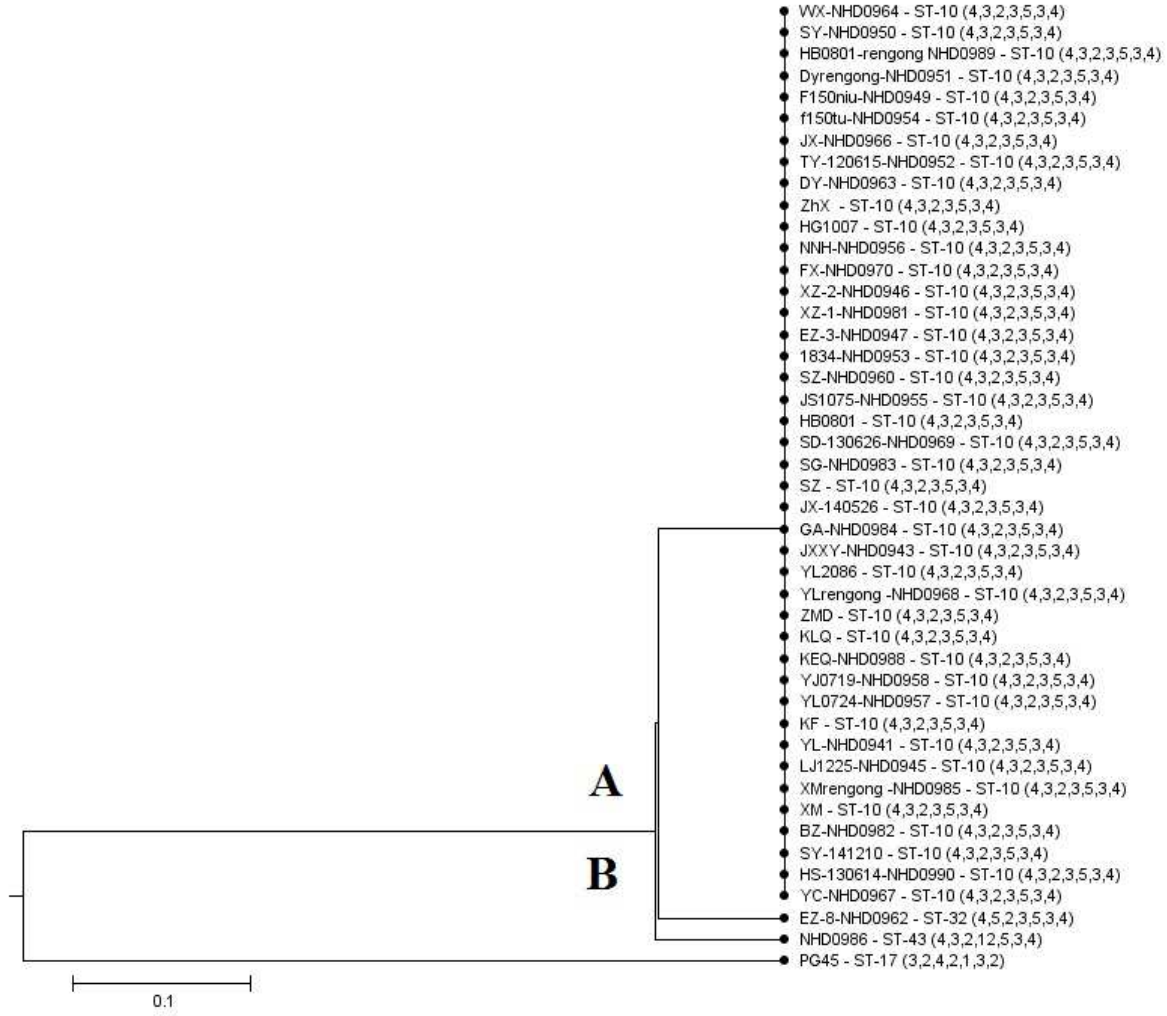
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Dyrengong-NHD0951 - ST-10 (4,3,2,3,5,3,4)
F150niu-NHD0949 - ST-10 (4,3,2,3,5,3,4)
f150tu-NHD0954 - ST-10 (4,3,2,3,5,3,4)
JX-NHD0966 - ST-10 (4,3,2,3,5,3,4)
TY-120615-NHD0952 - ST-10 (4,3,2,3,5,3,4)
DY-NHD0963 - ST-10 (4,3,2,3,5,3,4)
ZhX - ST-10 (4,3,2,3,5,3,4)
HG1007 - ST-10 (4,3,2,3,5,3,4)
NNH-NHD0956 - ST-10 (4,3,2,3,5,3,4)
FX-NHD0970 - ST-10 (4,3,2,3,5,3,4)
XZ-2-NHD0946 - ST-10 (4,3,2,3,5,3,4)
XZ-1-NHD0981 - ST-10 (4,3,2,3,5,3,4)
EZ-3-NHD0947 - ST-10 (4,3,2,3,5,3,4)
1834-NHD0953 - ST-10 (4,3,2,3,5,3,4)
SZ-NHD0960 - ST-10 (4,3,2,3,5,3,4)
JS1075-NHD0955 - ST-10 (4,3,2,3,5,3,4)
HB0801 - ST-10 (4,3,2,3,5,3,4)
SD-130626-NHD0969 - ST-10 (4,3,2,3,5,3,4)
SG-NHD0983 - ST-10 (4,3,2,3,5,3,4)
SZ - ST-10 (4,3,2,3,5,3,4)
JX-140526 - ST-10 (4,3,2,3,5,3,4)
GA-NHD0984 - ST-10 (4,3,2,3,5,3,4)
JXXY-NHD0943 - ST-10 (4,3,2,3,5,3,4)
YL2086 - ST-10 (4,3,2,3,5,3,4)
YLrengong -NHD0968 - ST-10 (4,3,2,3,5,3,4)
ZMD - ST-10 (4,3,2,3,5,3,4)
KLQ - ST-10 (4,3,2,3,5,3,4)
KEQ-NHD0988 - ST-10 (4,3,2,3,5,3,4)
YJ0719-NHD0958 - ST-10 (4,3,2,3,5,3,4)
YL0724-NHD0957 - ST-10 (4,3,2,3,5,3,4)
KF - ST-10 (4,3,2,3,5,3,4)
YL-NHD0941 - ST-10 (4,3,2,3,5,3,4)
LJ1225-NHD0945 - ST-10 (4,3,2,3,5,3,4)
XMrengong -NHD0985 - ST-10 (4,3,2,3,5,3,4)
XM - ST-10 (4,3,2,3,5,3,4)
BZ-NHD0982 - ST-10 (4,3,2,3,5,3,4)
SY-141210 - ST-10 (4,3,2,3,5,3,4)
HS-130614-NHD0990 - ST-10 (4,3,2,3,5,3,4)
YC-NHD0967 - ST-10 (4,3,2,3,5,3,4)
EZ-8-NHD0962 - ST-32 (4,5,2,3,5,3,4)
NHD0986 - ST-43 (4,3,2,12,5,3,4)
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A
B
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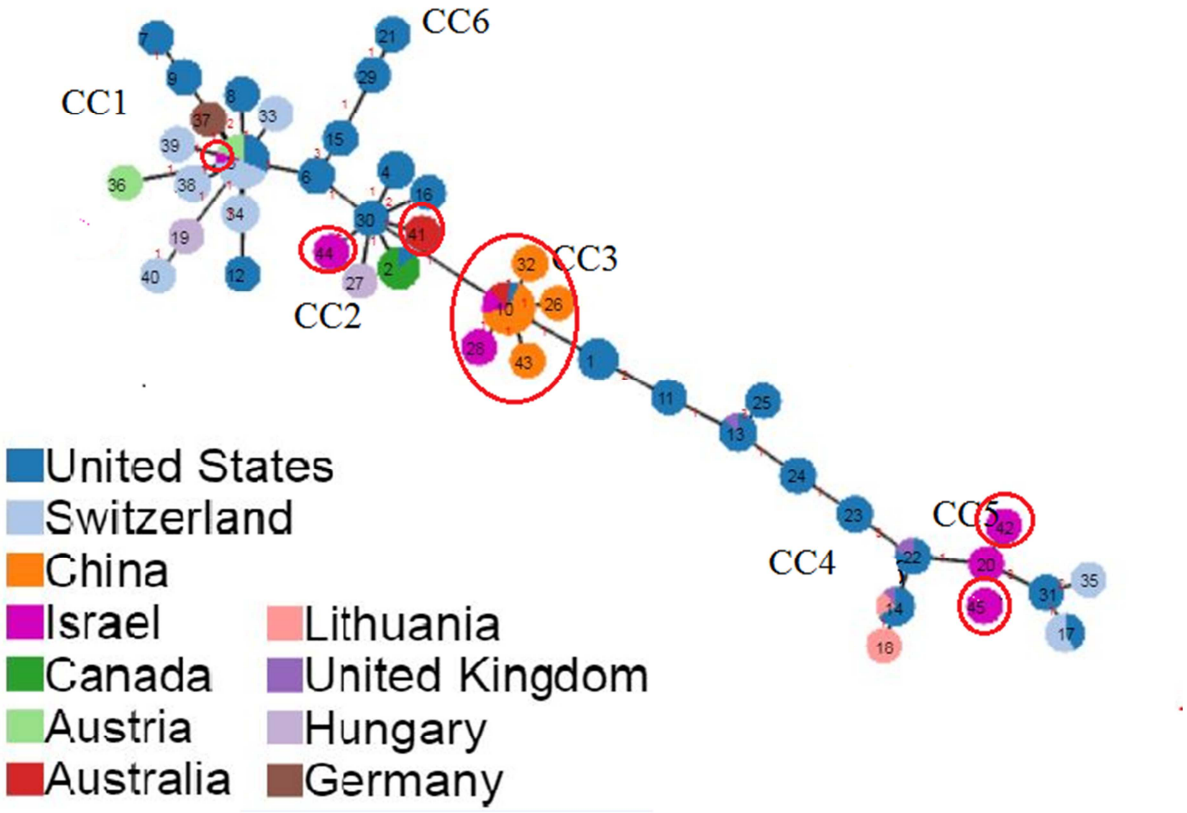

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Highlights

- A total of 44 Chinese *M.bovis* isolates collected form nine different provinces of China during 2008-2014 and a reference type strain PG45 were genotyped using two *M.bovis* MLST schemes and PFGE.
- The 43 of 44 (97.7%) Chinese isolates were identified as ST-10 by MLST-1, while 42 of 44 (95. 5%) as ST-10 by MLST-2.
- PFGE clustered 42 of 44 (95.5%) of the Chinese isolates into PT-I and reference strain PG45 was unique type by all the three techniques
- *M. bovis* population in China exists as one single dominant ST-10 clone, which is also present in Israel, Australia and USA.
- Five novel sequence types (ST-43, ST-41, ST-42, ST-44 and ST-45) related to one Chinese, one Australian and three Israeli isolates were identified.
- It is hypothesized that the widespread distribution of this type is a result of global livestock movements. Development of novel single strain vaccine may be effective in controlling *M.bovis* associated diseases.

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