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Genomic investigation of *Mycobacterium bovis* in bulk unpasteurized dairy products in Ardabil Province, Iran

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Zoonotic tuberculosis is predominantly caused by *Mycobacterium bovis*. Given the paucity of molecular data on this pathogen and the prominence of the traditional dairy industry in Ardabil Province, this study aimed to investigate the genomic presence of *M. bovis* in unpasteurized dairy products using a multi-gene targeting approach. In this cross-sectional study conducted in 2024, a total of 150 bulk unpasteurized dairy samples, comprising raw milk ($n = 50$), traditional cheese ($n = 50$), and artisanal yogurt ($n = 50$), were collected from various districts of Ardabil Province. Genomic DNA was extracted and subjected to molecular screening using conventional PCR. The detection strategy targeted the *IS6110* element for the *Mycobacterium tuberculosis* complex, followed by *16 S rRNA* and *gyrB* gene amplification for species confirmation. PCR products were validated via Sanger sequencing, and statistical analyses were performed using SPSS software (version 26.0). Out of 150 samples analyzed, PCR screening based on the *IS6110* insertion element revealed that 21 samples (14%) contained DNA belonging to the *Mycobacterium tuberculosis* complex (MTBC). Subsequent species-specific confirmation targeting the *gyrB* gene identified *M. bovis* in 12 samples (8%). The highest prevalence was observed in raw milk (14%, 7/50), followed by traditional cheese (8%, 4/50), while yogurt samples showed the lowest contamination (2%, 1/50). Statistical analysis indicated a significant association between product type and *M. bovis* contamination ($p = 0.044$), specifically in raw milk. Based on the findings of our study, the presence of *M. bovis* DNA in bulk unpasteurized dairy products from Ardabil Province indicates a potential risk of human transmission. However, the lack of culture-based confirmation limits conclusions regarding bacterial viability. These results underscore the importance of culture-based studies, enhanced veterinary surveillance, control of unpasteurized dairy consumption, and increased public awareness within a One Health framework to mitigate the risk of zoonotic tuberculosis.

Keywords *Mycobacterium bovis*, Unpasteurized dairy, Zoonosis, PCR, *gyrB*

Zoonotic tuberculosis, predominantly caused by the bacterial pathogen *Mycobacterium bovis*, stands as a formidable and often underestimated threat at the nexus of public health, animal welfare, and global economics. As a pivotal member of the *Mycobacterium tuberculosis* complex (MTBC), *M. bovis* is an acid-fast, aerobic, and slow-growing intracellular pathogen capable of causing chronic, progressive disease across an exceptionally broad range of mammalian hosts, including humans[1,2]. While concerted national and international efforts have successfully curtailed or eradicated bovine tuberculosis in the livestock of many developed countries, the disease remains deeply entrenched in pastoral and developing regions of Africa, Asia, and Latin America. The global burden of this pathogen is immense, inflicting staggering economic losses upon the agricultural sector[3]. These losses are multifaceted, arising from direct costs associated with surveillance programs, diagnostic testing, and compulsory animal culling, as well as indirect costs stemming from reduced milk and meat productivity, loss of genetic merit, and severe restrictions on international trade[4]. Consequently, the control of *M. bovis* is not merely a veterinary concern but a critical component of sustainable agricultural development and global food security[5]. The pathogenic success of *M. bovis* is intrinsically linked to its biological characteristics, which present formidable challenges for both diagnosis and control. Its intracellular lifestyle, primarily within host macrophages, allows

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it to evade the immune system, leading to the formation of characteristic granulomatous lesions, or tubercles, and establishing a persistent, long-term infection[6]. This insidious progression often means that clinical signs in cattle, such as coughing, weight loss, and reduced milk yield, only become apparent in advanced stages of the disease, long after the animal has become infectious[5,7]. The pathogen's slow growth rate further complicates detection; traditional laboratory diagnosis relies on microbial culture, a method that, while considered the gold standard for specificity, requires prolonged incubation periods of up to 12 weeks and specialized biosafety facilities, delaying confirmation and timely intervention. These biological constraints have historically hindered the efficacy of large-scale test-and-slaughter campaigns, particularly in resource-limited settings[8]. From a public health perspective, *M. bovis* represents a significant zoonotic pathogen, with epidemiological data suggesting it may be responsible for a substantial proportion of human tuberculosis cases, potentially ranging from 0.5% to as high as 7.2% globally[9,10]. The burden is disproportionately high in areas with endemic bovine TB and where specific cultural practices facilitate transmission. Human infection typically occurs through two primary routes: the inhalation of infectious aerosols from close contact with infected animals, placing farmers and slaughterhouse workers at high occupational risk, and, more commonly, the consumption of contaminated animal products[11]. Among these, unpasteurized milk and its derivatives (such as artisanal cheeses and yogurts) are recognized as the most efficient vehicles for foodborne transmission to the general population[10]. The role of dairy products as a vector is underpinned by fundamental microbiology and epidemiology. Milk's rich composition of proteins, fats, lactose, and minerals creates an ideal growth medium for a wide array of microorganisms, including pathogenic mycobacteria[12]. In infected cattle, *M. bovis* can be shed directly into the milk through lesions in the udder (tuberculous mastitis) or can contaminate otherwise clean milk via fecal or respiratory excretions during unsanitary, traditional hand-milking procedures. The pathogen demonstrates remarkable resilience, capable of surviving the fermentation and maturation processes used to create many traditional cheeses, thereby posing a prolonged risk to consumers. The resulting human disease often manifests differently from classic pulmonary tuberculosis caused by *M. tuberculosis*. Ingestion of *M. bovis* frequently leads to extrapulmonary tuberculosis, with common clinical presentations including cervical lymphadenitis (scrofula), gastrointestinal disease, and disseminated infections[8]. This clinical distinction is critical, as the disease is not only severe, particularly in children and immunocompromised individuals, but also inherently more difficult to treat, given that *M. bovis* is naturally resistant to pyrazinamide, a first-line antitubercular drug.The advent of molecular diagnostics has revolutionized the surveillance of *M. bovis*. Since the pathogen's initial molecular characterization[11], polymerase chain reaction (PCR)-based assays have emerged as a superior alternative to conventional methods for detection in clinical and environmental samples. PCR offers unparalleled advantages in sensitivity, specificity, and speed, allowing for the rapid identification of mycobacterial DNA directly from samples like milk in a matter of hours rather than weeks[13]. This technological advance is crucial for effective food safety monitoring, as it enables public health authorities to screen large numbers of samples efficiently and identify contamination hotspots in the food chain before they result in widespread human exposure. Despite the availability of these powerful tools, their application remains inconsistent, resulting in significant gaps in our understanding of regional *M. bovis* prevalence[14]. Despite the recognized public health risks, there is a critical gap in epidemiological data regarding the molecular prevalence of *M. bovis* in the food chain of northwestern Iran. Ardabil Province, characterized by extensive traditional livestock farming and a cultural preference for unpasteurized dairy consumption, represents a potential hotspot for zoonotic transmission. Therefore, this study was designed within a One Health framework to conduct the first systematic molecular survey of *M. bovis* in bulk unpasteurized dairy products in this region. We employed a multi-locus PCR strategy targeting *IS6110, 16 S rRNA*, and *gyrB* genes to determine the extent of contamination and assess the potential spillover risk to the human population.

Materials and methods

Ethical approval

This study was conducted in Ardabil University of Medical Sciences, Ardabil, Iran. The research protocol was reviewed and approved by the Ethics Committee of Ardabil University of Medical Sciences (Ethics Code: IR.ARUMS.REC.1404.243, Tracking Code: 403000604). All procedures were carried out in accordance with the ethical standards of the national research committee and with the 1964 Helsinki Declaration and its later amendments.

Study area

This cross-sectional study was conducted in Ardabil province (longitude: 48.29754649 and latitude: 38.24961279), located in the northwest of Iran, covering an area of 18,011 km². It was established in 1993. The province comprises 12 counties, 31 districts, 35 cities, and 75 rural districts. The 2016 census recorded the population of Ardabil as 1,270,420 inhabitants in 377,423 households (Fig. 1). This province was selected due to its significant domestic livestock population, including 348,150 head of cattle, as reported by the Iranian Veterinary Organization[15].

Sample size determination and collection

In this cross-sectional study, a total of 150 bulk unpasteurized dairy products (50 each of milk, cheese, and yogurt) were collected from January to December 2024. The sample size was determined using a standard statistical formula for prevalence studies: $\mathrm{n} = \frac{Z^2 * P(1-\mathrm{P})}{\mathrm{d}^2}$, where n is the required sample size, Z is the value corresponding to a 95% confidence level ($\mathrm{Z} = 1.96$), P is the expected prevalence, and d is the desired precision. An expected prevalence of 13%, based on previous studies[16], and a 5% margin of error were used to calculate the minimum required sample size ($n = 150$). The samples were comprised of 50 raw milk, 50 traditional cheese, and 50 artisanal yogurt samples, due to the limited availability and declining commercial distribution of unpasteurized dairy products, a convenience sampling approach was employed. Retail availability of unpasteurized products

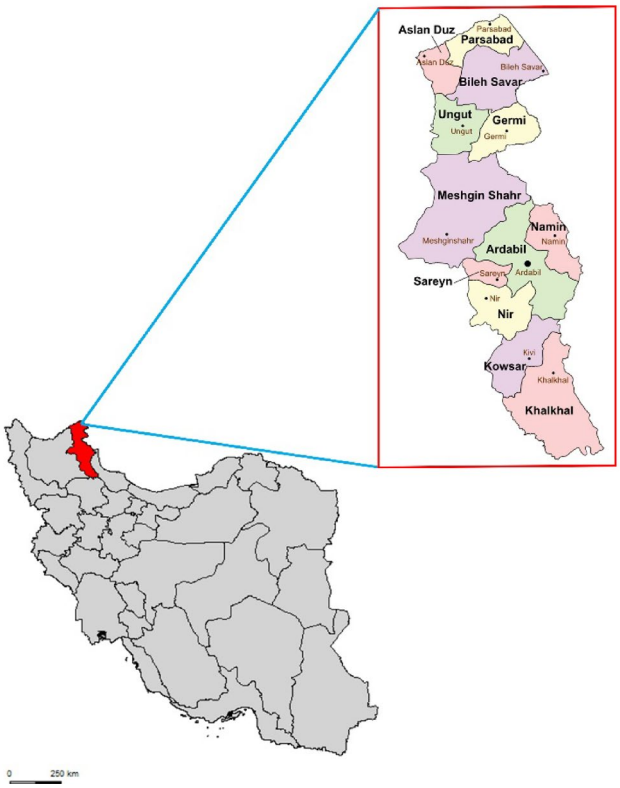

Fig. 1. Map showing Ardabil Province in northwestern Iran, highlighting the 12 counties where sampling was conducted. The map was generated using ArcGIS software (version 20), with county boundaries and sampling locations accurately plotted to visualize the geographic distribution of the study area.

in urban centers is strictly limited due to sanitary regulations and short shelf life. Consequently, a convenience sampling strategy was employed, focusing on direct procurement from rural producers and collection centers to ensure sample authenticity. Each sample was aseptically transferred into a sterile 50-mL conical centrifuge tube (Falcon®, Cat. No. 352070; Corning, NY, USA). Samples were immediately placed in a portable cooler maintained at 4 °C with ice packs and were transported to the Microbiology Research Laboratory at Ardabil University of Medical Sciences. The transport time from collection to laboratory processing did not exceed 4 h (Fig. 2).

Sample preparation and DNA extraction

For liquid samples (milk and yogurt), a 40 mL aliquot was used for analysis. For solid cheese samples, a 10 g portion was homogenized in 30 mL of sterile Phosphate-Buffered Saline (PBS) before analysis. The resulting suspensions were then centrifuged using an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany) with an A-4–81 rotor at 3,000 × g for 15 min at 4 °C to pellet microbial cells. The supernatant was discarded, and the sediment was retained for DNA extraction. Total genomic DNA was isolated using the Sambio Genomic DNA Purification Kit (Cat. No.: K3031; Sambio, South Korea). Given the robust cell wall structure of mycobacteria, an enzymatic pre-treatment was applied to ensure efficient lysis before using the kit. The sediment was resuspended in a lysis buffer containing lysozyme (20 mg/mL) and incubated at 37 °C for 2 h to digest the peptidoglycan layer. Following this pre-treatment, the extraction proceeded strictly according to the manufacturer's specified protocol. The quantity and purity of the extracted DNA were determined using a Thermo Scientific NanoDrop™ One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples with an A260/280 absorbance ratio of 1.8–2.0 were considered sufficiently pure for downstream analysis. All extracted DNA samples were stored at − 20 °C until required for PCR amplification[17]. A positive extraction control (BCG DNA) was processed alongside the samples to ensure successful DNA recovery, and a negative extraction control (sterile distilled water) was included to monitor for potential contamination during extraction (Fig. 3).

PCR amplification

The presence of *M. bovis* DNA was determined by targeting the insertion sequence (*IS6110*, *16 S rRNA*, and *gyr B)* via conventional PCR. The primer set used was described in Table 1. PCR amplification was performed in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total reaction volume of 25 µL. Each reaction contained: 12.5 µL of 2x Master Mix (Ampliqon, Odense, Denmark), 1.0 µL of Forward primer (10 µM), 1.0 µL of Reverse primer (10 µM), 2.0 µL of template DNA (~ 50–100 ng), and 8.5 µL of nuclease-free water. The final concentration of each primer in the reaction was 0.4 µM. Genomic DNA extracted from the *M. bovis*

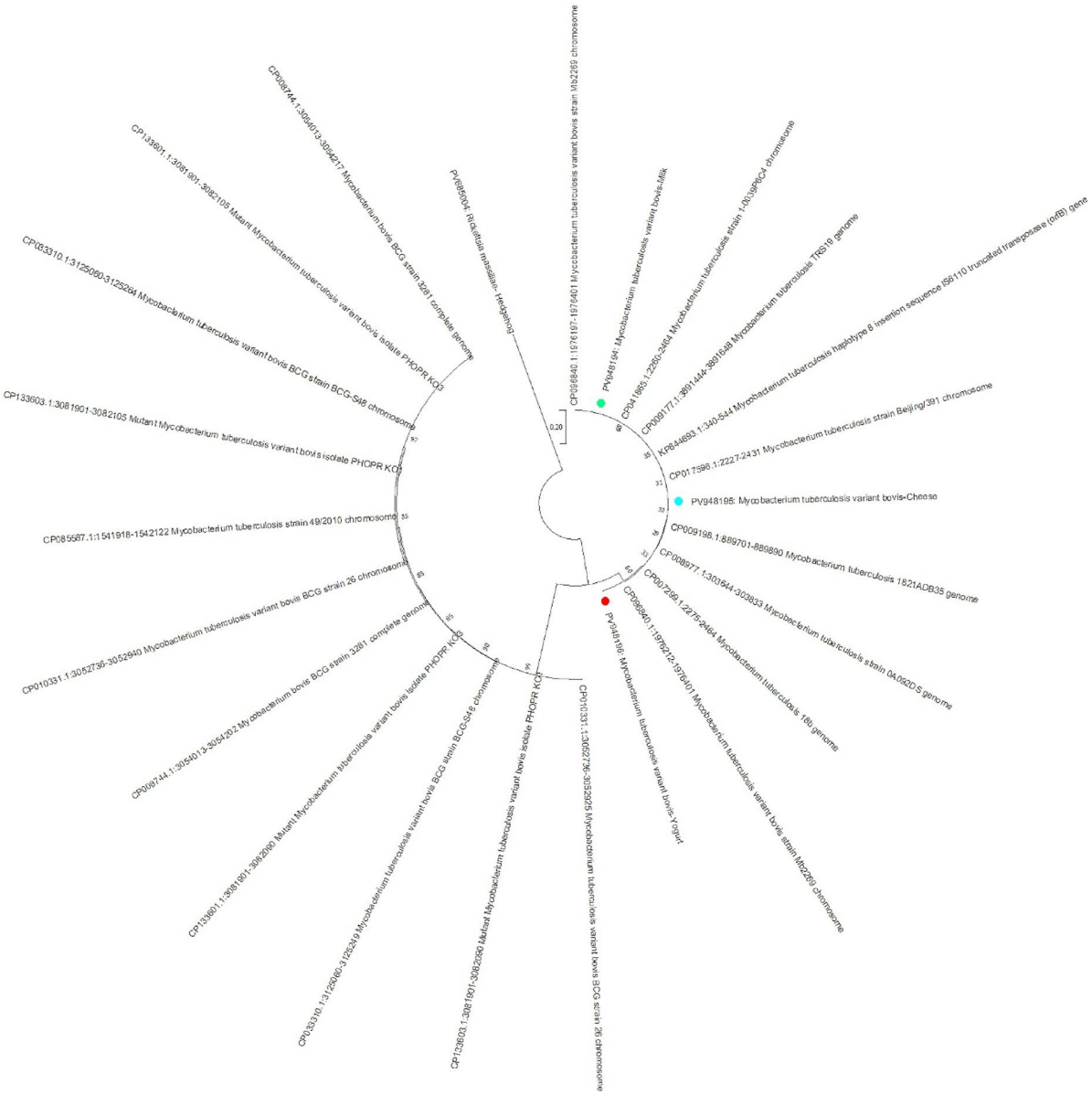

Fig. 2. Phylogenetic analysis of the *IS6110* gene sequences based on partial sequences illustrating the genetic diversity of *M. bovis* isolates recovered from unpasteurized dairy products in Ardabil. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The isolates are color-coded: Green (Milk), Blue (Cheese), and Red (Yogurt). *Rickettsia rickettsii* was used as the outgroup.

Bacille Calmette-Guérin (BCG) vaccine (Danish strain 1331), supplied by the Razi Vaccine and Serum Research Institute (Karaj, Iran), was used as the positive control. Nuclease-free sterile water served as the negative control.

Agarose gel electrophoresis and visualization

The PCR amplicons were resolved by electrophoresis on a 1% (w/v) agarose gel (Cat. No. 1.01603; Merck, Darmstadt, Germany). The 0.5× TBE buffer was prepared in-house by diluting a 10× TBE stock solution (890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.3) with nuclease-free water. A volume of 5 µL of each PCR product, mixed with 1 µL of 6× DNA loading dye, was loaded into each well. The gel was stained with Safe Stain (Cat. No. DM003-R500; Smobio, Hsinchu, Taiwan) at a concentration of 5 µg/mL. Electrophoresis was conducted at 90 V for 50 min. A 100 bp DNA Ladder (Cat. No.: PS-100BL; Pishgam Co., Tehran, Iran) was run in parallel to estimate amplicon size. DNA bands were visualized and captured using a Vilber Lourmat Fusion FX7 Gel Documentation System (Vilber, France).

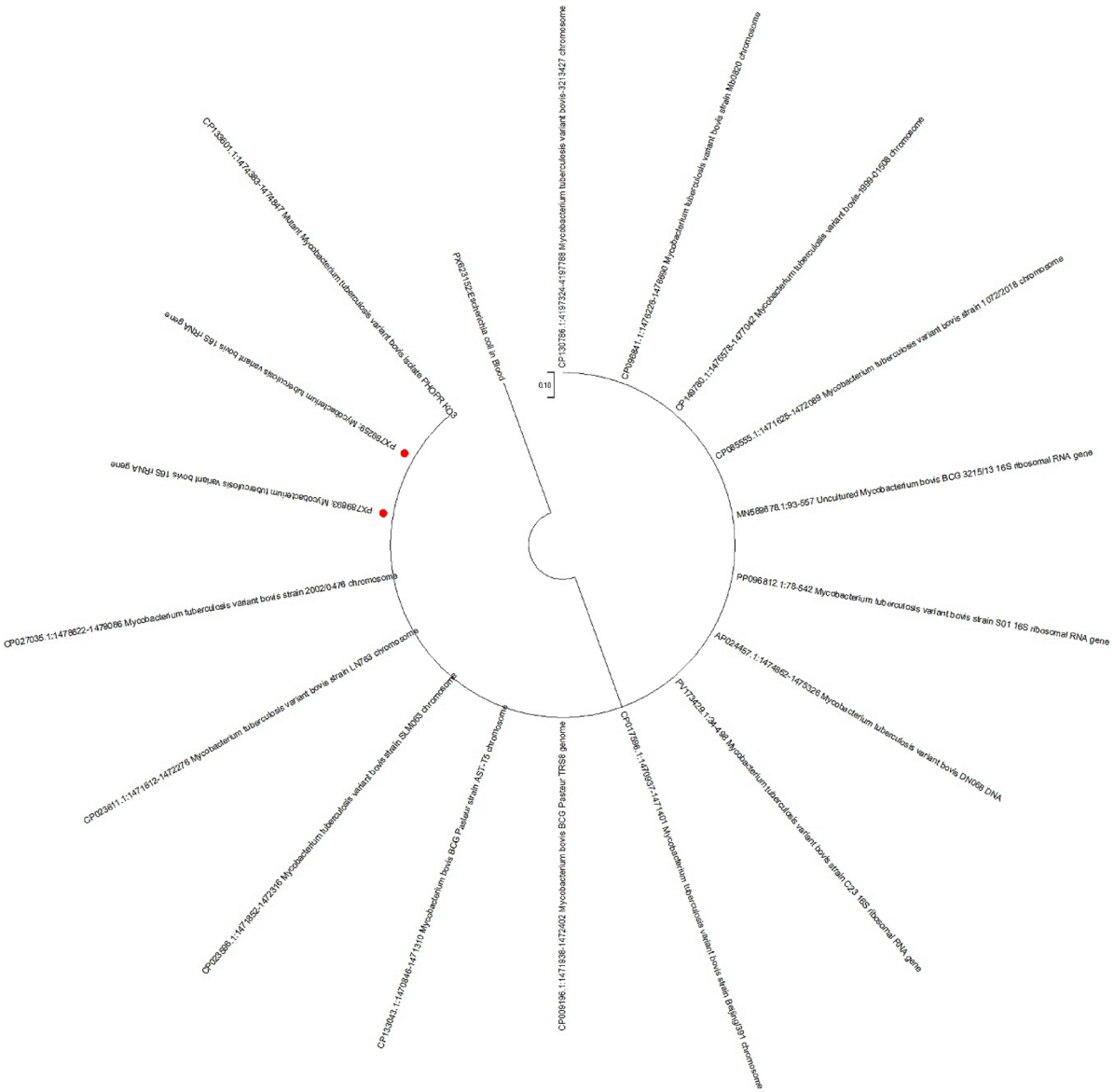

Fig. 3. Phylogenetic analysis of the *16 S rRNA* gene sequences of *M. bovis* isolates obtained in this study (marked with red circles) alongside reference strains from GenBank. The tree was constructed using the Neighbor-Joining algorithm with 1000 bootstrap resamplings. *Escherichia coli* (PX623152) was included as an outgroup to root the tree. The clustering pattern confirms the genus-level identification of the local isolates within the *Mycobacterium tuberculosis* complex.

Sequence confirmation and phylogenetic analysis

All samples that produced a PCR band of the expected size were subjected to sequence confirmation. Before sequencing, PCR products were purified using the QIAquick PCR Purification Kit (Cat. No.: 28104; Qiagen, Hilden, Germany). The purified products were sent for bidirectional Sanger sequencing at Pishgam Company (Tehran, Iran). Resulting sequences were analyzed using Chromas v2.6.6 for quality control, and consensus sequences were compared against the NCBI nucleotide database using BLASTn to confirm identity. The confirmed sequences were submitted to the GenBank database under accession numbers PV948194–PV948196, PX789259, PX789893, and PX789366–PX789367. Phylogenetic trees were constructed using MEGA software (Version 11) with the Neighbor-Joining method and Kimura 2-parameter model, evaluated by 1000 bootstrap replicates.

In this study, culture-based methods for *M. bovis* were not performed. Isolation and culture of *M. bovis* require Biosafety Level 3 (BSL-3) laboratory facilities due to the zoonotic risk and slow-growing nature of the pathogen. Consequently, the findings are based solely on molecular detection of mycobacterial DNA and do not provide information regarding bacterial viability or infectious potential. Future studies incorporating culture methods in appropriately equipped BSL-3 facilities are warranted to further validate and expand upon these results.

Gene	Gene Oligonucleotide sequence (5′ to 3′)	Size (bp)	PCR condition	Accession number (gene bank, NCBI)	Ref.
IS6110	F: CGTGAGGGCATCGAGGTGGC R: GCGTAGGCGTCGGTGACAAA	245	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94◦C for 1 min Annealing at 60◦C for 1 min Extensionat 72◦ C for 1 min (34 cycles)	PV948194 PV948195 PV948196	18
16 S rRNA	F: AGTGGCGAACGGGTGAGTAAC R: AACCACTACGAGCTCTTTACGCC	485	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94◦C for 1 min Annealing at 63◦C for 1 min Extensionat 72◦ C for 1 min (34 cycles)	PX789259 PX789893	19
gyr B	F: TACGAGTGGTCTCAGGTTT R: CTTGTTCACAACGACTTTCG	798	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94◦C for 1 min Annealing at 62◦C for 1 min Extensionat 72◦ C for 1 min (34 cycles)	PX789366 PX789367	20

Table 1. Primers and PCR conditions used for amplification of *IS6110*, *16 S rRNA*, and *gyrB* genes.

County	Sample type			
	Milk (positive/total)	Cheese (positive/total)	Yogurt (positive/total)	Total (positive/total)
Ardabil	1/8 (0.6%)	1/4 (0.6%)	0/4 (0%)	2/16 (1.3%)
Namin	0/2 (0%)	0/6 (0%)	0/5 (0%)	0/13 (0%)
Khalkhal	2/6 (1.3%)	1/7 (0.6%)	1/7 (0.6%)	4/20 (2.6%)
Meshkinshahr	2/5 (1.3%)	0/2 (0%)	0/5 (0%)	2/12 (1.3%)
Kowsar	0/4 (0%)	0/3 (0%)	0/4 (0%)	0/11 (0%)
Sarein	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/15 (0%)
Germi	0/4 (0%)	0/6 (0%)	0/5 (0%)	0/15 (0%)
Parsabad	1/4 (0.6%)	1/3 (0.6%)	0/2 (0%)	2/9 (1.3%)
Nir	0/5 (0%)	0/4 (0%)	0/3 (0%)	0/12 (0%)
Aslanduz	0/2 (0%)	1/5 (0.6%)	0/2 (0%)	1/9 (0.6%)
Bilehsavar	1/3 (0.6%)	0/3 (0%)	0/5 (0%)	1/11 (0.6%)
Angut	0/2 (0%)	0/2 (0%)	0/3 (0%)	0/7 (0%)
Total	7/50 (4.6%)	4/50 (2.6%)	1/50 (0.6%)	12/150 (8%)

Table 2. Geographic distribution of *M. bovis*.

Data analysis

Statistical analyses were conducted using Chi-square and Fisher's exact tests. All computations were performed in SPSS version 26.0, with a significance level of $p < 0.05$.

Result

Prevalence of *Mycobacterium bovis* in bulk unpasteurized dairy products

A total of 150 bulk unpasteurized dairy products (50 raw milk, 50 yogurts, and 50 cheese samples) were analyzed in this study. Given that cows are the most common livestock for dairy production in this region, all of our samples were from cows, and we avoided collecting mixed samples or samples from sheep and goats. Initial molecular screening revealed that 14% (21/150) of the samples were positive for the *IS6110* insertion sequence, a marker specific to the *Mycobacterium tuberculosis* complex. However, definitive identification of *M. bovis* was confirmed in 8% (12/150) of the total samples based on the amplification of the species-specific *gyrB* gene. Consequently, all further prevalence analyses and phylogenetic characterizations were based on these 12 confirmed *M. bovis* isolates. Analysis of prevalence by product type showed significant variation. Raw milk exhibited the highest rate of contamination, with 7 of the 50 samples (14%) testing positive. Traditional cheese had a lower prevalence, with 4 positive samples out of 50 (8%). The lowest detection rate was in artisanal yogurt, with only 1 of 50 samples (2%) positive for the target sequence (Table 2).

Geographic distribution of positive samples

The geographic distribution of *M. bovis*-positive samples across the 12 surveyed counties of Ardabil province is presented in Table 2. Contamination was not uniformly distributed. Regarding geographical distribution, Khalkhal County exhibited the highest number of positive samples, with 4 positive cases out of 20 samples (20%), indicating contamination across all three dairy product types. Ardabil, Meshkinshahr, and Parsabad each reported two positive samples, whereas Namin, Kowsar, Sarein, Germi, Nir, and Angut showed no positive samples in any of the tested products.

Phylogenetic analysis and genetic characterization

To validate the molecular identification, representative amplicons were sequenced and subjected to phylogenetic analysis. BLASTn analysis confirmed that the *IS6110*, *16 S rRNA*, and *gyrB* sequences obtained in this study shared ≥ 99% nucleotide identity with reference *M. bovis* strains (Fig. 2, 3 and 4).

The phylogenetic trees constructed using the Neighbor-Joining method revealed that the isolates from Ardabil (milk, cheese, and yogurt) formed a distinct, monophyletic cluster closely related to reference *M. bovis* strains (e.g., BCG strains and virulent field isolates) retrieved from GenBank. Specifically, the *gyrB*-based tree (Fig. 4), which offers higher discriminatory power than *16 S rRNA*, demonstrated a robust grouping of the local isolates with global *M. bovis* lineages, supported by high bootstrap values. This topological arrangement confirms the taxonomic classification of the isolates and suggests a close evolutionary relationship with circulating *M. bovis* strains in the region.

Statistical analysis

To investigate the relationship between the type of samples and contamination with the genome of *M. bovis*, Fisher and Chi-square tests of SPSS software version 25 were used. According to Table 3, only a significant relationship was observed between milk samples and contamination with the DNA of the *M. bovis*, ($p = 0.044$). There was no significant relationship between yogurt and cheese samples and contamination.

Discussion

This cross- sectional study provides the first systematic molecular evidence of *Mycobacterium bovis* DNA contamination in bulk unpasteurized dairy products within Ardabil Province, a significant livestock farming region in northwestern Iran. Our findings reveal an overall prevalence of 8%, demonstrating that these products serve as a tangible vehicle for potential zoonotic transmission. Crucially, we identified raw bulk milk as the highest-risk product, with a contamination rate of 14% and only a significant relationship was observed between milk samples and contamination with the DNA of the *M. bovis* ($p = 0.044$). Specific geographic hotspots, such as Khalkhal County (2.6% prevalence), where the risk to public health may be most acute. The stark variation in *M. bovis* prevalence across different dairy products is a key finding with direct implications for consumer risk assessment. The 14% contamination rate in raw milk is alarming and aligns with its established role as a primary vector for bovine tuberculosis. This is biologically plausible, as milk can be directly contaminated via shedding from an infected udder or indirectly during the milking process. In contrast, the lower prevalence in cheese (6%) and especially yogurt (2%) suggests that traditional processing methods, even if uncontrolled, may have a partial protective effect. The fermentation process, characterized by a drop in pH and the production of antimicrobial compounds by starter cultures, likely creates an environment that is less hospitable to the survival and proliferation of *M. bovis*. The significantly lower prevalence in yogurt (2%) compared to raw milk (14%) supports the hypothesis that the acidic environment (low pH) and the presence of inhibitory metabolites produced during fermentation may compromise the survival of *M. bovis*. However, as molecular detection does not differentiate between viable and non-viable DNA, this protective effect requires further validation through survival assays. Our findings contribute critical regional data to the broader epidemiological landscape of bovine tuberculosis in the Middle East. The 14% prevalence in raw milk from Ardabil is similar to the 26.9% reported by Zahrakar et al[21]. in the neighboring Lorestan Province of Iran, suggesting that high levels of contamination may be a widespread issue in traditional Iranian dairy production systems. This consistency reinforces the urgent need for national-level surveillance and control measures. However, our results stand in stark contrast to data from other parts of the country and the wider region. For instance, Solghani et al[22]. detected no *M. bovis* in milk or cheese from Tehran Province. This discrepancy is likely multifactorial, attributable not only to different molecular methods but also to fundamental differences in agro-climatic conditions. The extensive pastoral farming systems in Ardabil, with its favorable climate and abundant pastures, stand in contrast to the more intensive, less pasture-based dairy farming prevalent around Tehran, potentially leading to different transmission dynamics. Similarly, the prevalence in Ardabil milk is significantly higher than the 10.3% reported in Baghdad, Iraq[22], and the remarkably low 1.4% found in the Anatolian region of Turkey[13]. While methodological differences exist, these variations likely reflect underlying differences in cattle density, herd management, and the effectiveness of national bovine TB control programs. The very low prevalence in Turkey, a country with a developed livestock industry, suggests that effective veterinary oversight and control strategies can dramatically reduce the public health risk, offering a potential roadmap for intervention in high-prevalence areas like Ardabil[19]. In a comprehensive study conducted across 14 provinces of Iran, *M. bovis* was examined in 213 cattle samples using the RFLP technique. Among these, 56 samples tested positive for bovine tuberculosis. Ardabil Province was included as one of the study regions. The researchers identified seven distinct genotypes, one of which was found to be unique to Ardabil, indicating a possible region-specific strain of *M. bovis*[13]. Consistent with our findings, a study conducted by Haghi et al. in Zanjan Province reported a 13.3% contamination rate of *M. bovis* in raw milk samples. This prevalence is closely aligned with the 14% reported in our study. The similarity in results may be attributed to the use of comparable PCR methodologies and the resemblance in climatic conditions and livestock management practices between Ardabil and Zanjan provinces[16]. In contrast, a study by Solghani et

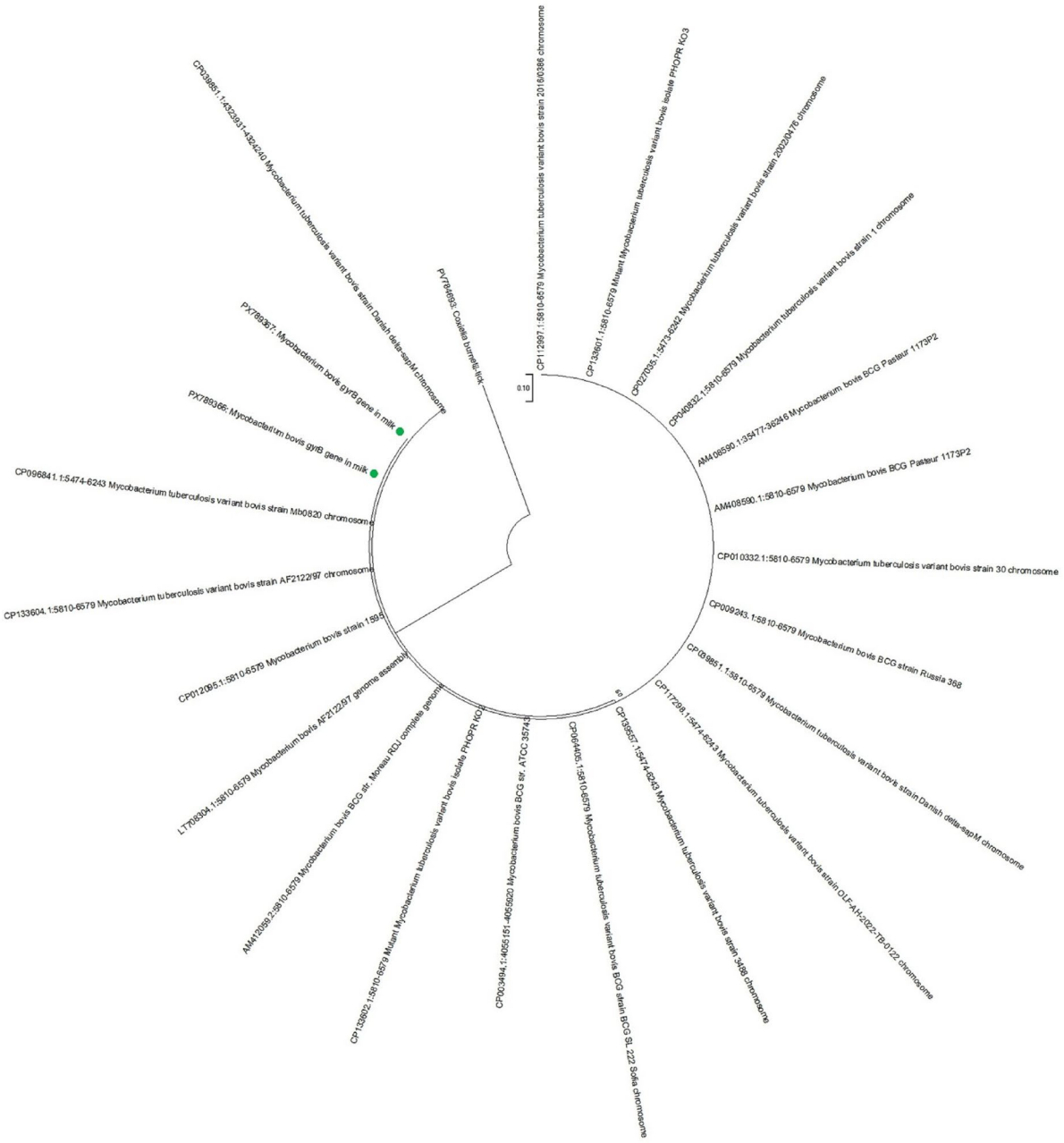

Fig. 4. Evolutionary relationships of *M. bovis* isolates based on partial *gyrB* gene sequences. The tree was generated using the Neighbor-Joining method. Bootstrap values (> 50%) from 1000 replicates are displayed at the nodes. The scale bar represents nucleotide substitutions per site. Isolates from milk samples are indicated by green circles (Accession Nos. PX789366 and PX789367). The distinct clustering of these isolates with reference to *M. bovis* strains confirms species-level identification. *Coxiella burnetii* (PV784693) served as the outgroup.

Sample type	Milk	Cheese	Yogurt	Total
Contamination	4 of 50	3 of 50	1 of 50	8 of 150
p-value	0.044	0.083	0.322	0.385

Table 3. Statistical analysis.

al. in Tehran Province, which employed PCR targeting the *16 S rRNA* and *hsp65* genes, along with culture on Löwenstein–Jensen medium, revealed significantly lower contamination rates. Among 175 samples of milk and cheese, only 4 milk samples were found to be positive for mycobacteria, and none of the isolates were identified as *M. bovis*. Additionally, no cheese samples were contaminated. This stark difference may be explained by less favorable environmental conditions for livestock farming in Tehran, including limited pasture availability and climatic differences compared to Ardabil[22]. Results of studies suggest that both geographic and methodological factors play essential roles in the detection rates of *M. bovis* in food products. Environmental conditions, farming practices, sample types, and diagnostic techniques all contribute to variations in prevalence across different regions. Preliminary molecular evidence suggests the presence of *Mycobacterium bovis* in unpasteurized dairy, but culture-based studies are needed to clarify the true risk and guide One Health interventions.

Conclusion

This study provides the first molecular evidence of *M. bovis* contamination in the traditional dairy supply chain of Ardabil Province, with a concerning prevalence of 8%, particularly in raw milk. While the use of PCR confirms the presence of the pathogen's genome, the lack of culture-based isolation limits definitive conclusions regarding infectivity. Nevertheless, these findings highlight a tangible One Health risk. Immediate interventions, including stricter veterinary surveillance, public education on the risks of raw dairy consumption, and the implementation of pasteurization protocols in traditional processing units, are imperative to mitigate the threat of zoonotic tuberculosis in this region.

Data availability

The datasets generated and analyzed during the current study are available in the NCBI GenBank repository, under the accession numbers: PV948194, PV948195, PV948196, PX789259, PX789893, PX789366, and PX789367.

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Author contributions
FMB: Methodology, Investigation, and Formal analysis, HRA: Methodology, Investigation, and Formal analysis, MRV: Software, and FK: Conceptualization, Supervision, Project administration, and Original draft preparation.

Declarations

Competing interests
The authors declare no competing interests.

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