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Current Situation of Environmental Mycobacteria in Raw Milk in Some Egyptian Governorates

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Abstract

IN EGYPT, raw milk is widely consumed and can serve as a vector for transmitting certain zoonotic diseases. *Mycobacterium tuberculosis complex* (*MTBC)* is the most extensively studied group of mycobacterial agents in veterinary microbiology, known for causing tuberculosis in both man and animals, other *non-tuberculous mycobacteria* (*NTM*) or environmental mycobacteria are also significant animal pathogens. Our study is directed to detect *NTM* in raw milk in several Egyptian governorates. Three hundred milk samples were collected from El-Sharqiya, El-Qalyubia, and El-Daqahlia Governorates to examine the presence of *NTM*. Twenty-two milk samples exhibited bacterial growth on Lowenstein-Jensen and Middlebrook agar media enriched with sodium pyruvate and glycerol. Molecular identification of the bacterial isolates through 16S ribosomal RNA gene PCR confirmed their positivity. We identified five various species of *NTM*: *M. fortuitum*, *M. kansasii*, *M. scrofulaceum*, *M. chelonae*, and *M. smegmatis*, with isolation counts of 27, 18, 12, 9, and 15, respectively. This study highlights that raw milk poses a prospective *NTM* infection source for animals and humans.

Keywords: Raw milk, *Non-Tuberculous Mycobacteria*, Molecular characterization, public health significance.

Introduction

A wide range of organisms is included in the category of *Mycobacterium*, such as: (i) obligate pathogens from the *M. tuberculosis complex*, which cause serious disease to animal and human(ii) opportunistic pathogens, which are mostly represented by the *M. avium complex*; and (iii) saprophytic species [1].

M. tuberculosis and *M. bovis* are the main pathogenic mycobacteria causing serious illnesses in animals and humans [2]. However, *non-tuberculous mycobacteria* (*NTM*) are now recognized as significant causes of diseases in humans and animals, leading to both pulmonary and extrapulmonary infections in these populations [3]. It is commonly known that milk may transmit *Mycobacterium spp*., particularly *M. bovis* and *NTM*, to humans and animals [4].

Although the single intradermal cervical tuberculin test is the main diagnostic method for bovine TB, non-tuberculous mycobacteria may impede the existing diagnostic procedures. Few studies have looked at finding environmental mycobacteria in milk [5,6,7].

NTMs can cause skin, soft tissue, and respiratory tract infections in healthy individuals, especially those with immune system deficiencies [8]. Over 286 Mycobacterium species have been identified, with most belonging to the *NTM* group according to the List of (LPSN), Prokaryotic Names withstanding in Nomenclature [9].

NTMs can cause various diseases, including mastitis in cows and cutaneous granulomas in cats and dogs, associated with species such as *Mycobacterium chelonae*, *M. thermoresistibile*, *M. fortuitum*, *M. phlei* and *M. smegmatis* [10, 11]. The rise in *NTM* infections is primarily due to an increase in immunocompromised individuals, including those with AIDS, cancer, organ transplant recipients, and those using immunosuppressive drugs [12].

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Nontuberculous mycobacteria (*NTM*) infections are often misdiagnosed, especially in regions where tuberculosis is widespread. This is due to the resistance of *NTMs* to standard anti-TB medications, which can lead to them being mistakenly identified as *Mycobacterium tuberculosis* (MTB) [13]. Therefore, accurate identification of *NTM* species in associated infections is crucial for determining the most suitable treatment.

The target of the study is to detect atypical mycobacteria in unpasteurised milk from several farms in the El-Sharqyia, El-Qaliubia, and El-Daqahlia governorates. This was accomplished through bacterial culture and Ziehl-Neelsen (ZN) staining. Additionally, molecular-based speciation was performed to further analyse the isolates through 16S rRNA gene sequence analysis.

Material and Methods

Sampling

Three hundred samples of raw milk were randomly collected from various dairy farms in El-Sharqyia, El-Qaliubia, and El-Daqahlia Governorates, between January 2023 and January 2024. Samples were transported to the laboratory in an ice box for processing and bacteriological culture.

Sample preparation

A 50 mL aliquot of each milk sample was centrifuged at 3000 rpm for 15 minutes. Post centrifugation, the supernatant liquid was carefully extracted following the separation of the cream. The sediment was then subjected to a decontamination procedure as distinct samples [14].

Sample cultures

A volume of 5 mL of milk samples were centrifuged for 10 minutes at 4°C, at 3000 rpm, following an equal volume treatment with a 5% oxalic acid decontamination procedure. A 200µL aliquot from the decontaminated sample was inoculated into Lowenstein-Jensen with sodium pyruvate and glycerol added and Middlebrook 7H10 agar media enriched with OADC growth supplement (Himedia®). Then, incubated in duplicate at 37°C, with and without 5-10% CO2, for. Daily checks were conducted to monitor the growth of mycobacteria [14].

Biochemical identification of mycobacterial species:

To identify the *Mycobacterial* species, several phenotypic tests were conducted in the lab following the observation of growth. These tests included the following: temperature preference (°C), growth rate at 42°C and 52°C, pigment production under both dark and light conditions, growth on PNB (Peptone Nutrient Broth) and MacConkey agar, catalase activity, and nitrate reduction test. In addition, growth in the presence of 5% NaCl, tween 80 hydrolysis, pyrazinamidase activity, aryl sulfatase activity after three days, then after 14 days, utilization of citrate, urea hydrolysis, iron uptake, niacin testing, mannitol, tellurite reduction, and inositol were estimated [14].

Molecular identification of mycobacterial species

To confirm the isolates' positivity, molecular identification of the bacteria that targeted the 16S ribosomal RNA gene was employed.

DNA extraction procedures

The Qiagen® QIAamp DNA Kit was used for DNA extraction, with modifications to the manufacturer's instructions. Specifically, 200 µl suspension of the sample was treated with 200 µl of lysis buffer and 10 µl of proteinase K, followed by incubation for ten minutes at 56°C, additional 200 µl of 100% ethanol to the lysate. The sample was eluted using 100 µl of the elution buffer provided in the kit.

PCR Amplification

Table 1 shows the oligonucleotide primers used. Primers at a concentration of 20 pmol were employed in a 25µl reaction that included EmeraldAmp Max PCR Master Mix (12.5µl), Takara® (Japan), 4.5µl water, 1µl of primer, and 6µl of DNA template. The reaction was carried out using an Applied Biosystems 2720 thermocycler.

Analysis of PCR Products

PCR products were subjected to electrophoresis at a gradient of 5V/cm on a 1.5% agarose gel (Applichem, Germany, GmbH) in a 1x TBE buffer at ambient temperature. For gel analysis, 15µl of each PCR product was loaded into the gel slots. Fragment sizes were determined using the Generuler 100bp ladder from Fermentas, Thermo. The gel was captured using a gel documentation system (Alpha Innotech, Biometra), and the resulting data was analysed using Gelanalyzer software.

Discussion

Nontuberculous mycobacteria (*NTM*) species are increasingly documented as sources of human and animals' diseases globally [16]. They are known to be the primary pathogens responsible for both pulmonary and extrapulmonary infections [17]. *NTM* can be found in various environments on dairy farms. They can be isolated from soil, manure, organic waste, plants, and water [18, 19]. The consumption of tainted food and water is the main way that cattle contract *NTM* [20]. Infections can also occur through the intramammary route [21]. The existence of *NTM* in raw milk and dairy products has been documented in several investigations carried out globally.

Turkey released a report on the discovery of *NTM* species in dairy cows by Konuk et al. [22] and Aydin et al. [23], in Pakistan by Qamar et al. [24], and also in Tanzania by Kazwala et al. [25]. Several reports

detected *NTM* in heat-treated milk by Leite et al. [26], Sgarioni et al. [27], and Bolanos et al. [28] in Brazil.

Out of 300 raw milk samples, 81 were tested for bacterial growth as shown in Table 2.

According to the OIE (2024) [29], microbiological culture is still the most reliable technique for isolating *Mycobacterium species*. Sgarioni et al. [27] observed similar results in Brazil and detected *Mycobacterium chelonae*, *Mycobacterium kansasii, Mycobacterium smegmatis*, and *Mycobacterium fortuitum*, with an overall detection rate of 28.8%. In contrast, a study conducted in Iran [30] found *Mycobacterium fortuitum* in all examined raw milk samples, indicating a higher prevalence.

Biochemical assays have successfully identified *NTM* over the last fifty years. However, certain species remain difficult to identify due to their limitations [31].

Results in Table 3 were similar to Eman Mahrous et al. [19], who noted that out of 225 environmental samples, 41 sample tested positive for *mycobacteria*. Among these positive samples, 75.6% were pathogenic, including *M. avium, M. fortuitum, M. chelonae, M. marinum, M. kansasii and M. ulcerans*, *as* (19.5%), (12.2%), (4.9%), (12.2%), (4.9%), and (22%); respectively. The remaining 25.4% comprised non-pathogenic strains of nontuberculous mycobacteria, such as *M. smegmatis* (14%) and *M. flavescens* (10%).

Additionally, Ali et al. [35] noted that *M. fortuitum* dominant *NTM* isolates from tank milk samples; the most often discovered isolate from milk filters was *M. chelonae/abscessus*.

Other studies also recovered *Mycobacterium scrofulaceum* and *M. chelonae* from pasteurized and raw milk [27]. Other NTM group members, including *Mycobacterium terrae*, *M. kansasii*, *Mycobacterium haemophilum*, and *Mycobacterium agri*, were detected in raw milk in Turkey. [22].

Mycobacterium fortuitum, one of the rapidly growing mycobacteria (RGM), is associated with various health issues, including joint infections, osteomyelitis, pneumonia, hospital-acquired infections, and local skin diseases [30].

Additionally, *Mycobacterium kansasii* is often detected in the lymph nodes and tissue lesions of cattle, and it can cause lung diseases and disseminated infections in humans that are comparable to conventional tuberculosis [30].

A useful surrogate for mycobacterial research is Mycobacterium smegmatis, a fast-growing, nonpathogenic species of the Mycobacterium genus. Due to its conserved gene orthologs, similar cell architecture, and physiological characteristics.

The discovery in 1990 of a mutant strain known as mc2155 facilitated the identification of physiological targets for tuberculosis (TB) medications and provided insights into its slow-growing relatives. Consequently, *M. smegmatis* continues to advance microbiology and mycobacterial studies [32].

Another species of *NTM* that is frequently identified as a human pathogen is *Mycobacterium chelonae*. Although *M. chelonae* is associated with lung infections and commonly causes localized and disseminated infections of the skin and soft tissues [33].

This study is the first documented record of Nontuberculous Mycobacteria (NTM) in raw milk in Egypt. Based on extensive research and data collection, it provides new insights into the microbiological quality of raw milk within the Egyptian context.

This discovery is significant for public health, as it highlights potential risks associated with the consumption of raw milk. This study aims to contribute to the existing body of knowledge and to pave the way for future research in this area, emphasizing the need for stringent monitoring and regulation of raw milk to ensure consumer safety.

Conclusion

The identification of opportunistic human pathogenic species of Mycobacterium highlights a significant health risk associated with raw milk. This underscores the importance of processing milk hygienically before it is consumed to ensure safety.

Results confirmed by 16S rRNA gene detection and showed a higher percentage of similarity with the isolation and identification by traditional techniques.

Recommendations

- Implementing measures to avoid contamination of milk with non-tuberculous mycobacteria (*NTM*) during and after milking to prevent illness.
- Further research using guinea pig and cattle models is essential to determine whether specific non-tuberculous mycobacteria (NTM) strains trigger non-specific immune reactions in cattle that are not infected with bovine tuberculosis (bTB). Additionally, investigating various antigens may help differentiate immune responses observed in skin tests versus interferon-gamma assays. These insights could enhance the accuracy of diagnostic tools, refine risk management strategies, and support more effective disease eradication efforts.
- Mitigating the risk of consuming raw milk contaminated with non-tuberculous mycobacteria (NTM) requires a combination of strict control measures. Here are some key strategies: such as:

a) Improved Hygienic Practices: Ensure cleanliness in milking equipment, storage containers, and the milking environment.

b) Training farm workers on proper handling practices and sanitation procedures.

c) Regular Screening and Testing: Conducting routine microbiological tests to identify *NTMs* in raw milk.

-Screening cattle for *NTM* infection and isolating infected animals.

d) Enhanced Pasteurization Techniques: Implementation of adequate thermal processing methods tailored to effectively eliminate *NTMs*.

e) Water Quality Management: Testing and treating water sources used in farms to avoid introducing *NTMs* during milking or cleaning processes.

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Conflicts of interest

According to the authors, there isn't a conflict of interest.

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TABLE 1. Oligonucleotide primers targeting 16S ribosomal RNA for NTM molecular identification.

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)				Final extension	Reference
			Primary Denaturation	Secondary denaturation	Annealing	Extension		
Nontuberculosis Mycobacterium 16S rRNA	ATGCACCACCTGCACACAG G GGTGGTTTGTCGCGTTGTTC	470	94 5 min	94 30 sec	55 40 sec	72 45 sec	72 10 min	[15]

TABLE 2. Occurrence of non-tuberculous mycobacteria in milk samples.

Samples	Positive		Negative	
	No.	%	No.	%
Raw milk (N=300)	(81/300)	27%	(219/300)	73%

TABLE 3. Type of NTM isolates according to Biochemical tests.

Pathogenicity	NTM isolates (N=81)	
	Type	No.
Pathogenic strains	*M. fortuitum*	27
	M. kansasii	18
	M. scrofulaceum	12
	M. chelonae	9
Nonpathogenic strains	*M. smegmatis*	15

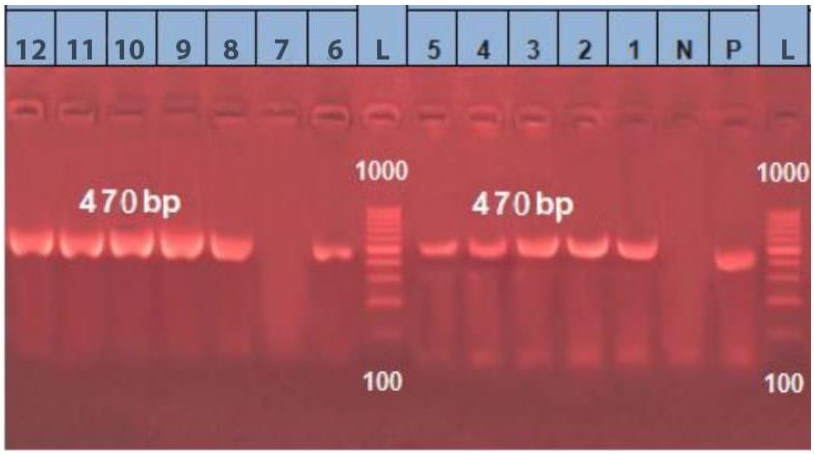

Fig. 1. PCR product amplification of *NTM* at 470 bp is demonstrated by agarose gel electrophoresis. Controls (positive and negative), MWM-molecular weight marker (100–1000bp DNA ladder). Eleven isolates were confirmed positive at 470 bp.

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الوضع الحالي لمتطفرة السل البيئية في الحليب الخام في بعض المحافظات المصرية

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الملخص

يُستهلك الحليب في مصر على نطاق واسع ويمكن أن يكون ناقلاً لبعض الأمراض الحيوانية المنشأ .بينما تعتبر مجموعة المتطفرة السلية المجموعة الأكثر دراسة من العوامل الممرضة في علم الأحياء الدقيقة البيطرية، المعروف بتسببه في مرض السل لدى البشر والحيوانات، فإن المتفطرات غير السلية (NTM) أو المتفطرات البيئية تُعد أيضًا مسببات أمراض هامة للحيوانات والإنسان على حد سواء .هدفت دراستنا إلى عزل وتحديد البكتيريا غير السلية من الحليب الخام في عدة محافظات مصرية .

تم تجميع 300 عينة من الحليب من ثلاث محافظات مصرية وهم محافظة الشرقية والقليوبية والدقهلية لفحص وجود متطفرة السل البيئية. أظهرت اثنان وعشرون عينة من الحليب نموًا بكتيريًا على أوساط أجار لوينشتاين-جينسن المدعمة بالصوديوم بيوفوسفات والجلسيرول وأجار ميدلبروك، التعرف الجزيئي على العزلات البكتيرية من خلال تفاعل البوليميراز المتسلسل لجين RNA الريبوسومي16 S أكد إيجابيتها.

تم تحديد خمس سلالات مختلفة من متفطرات السل البيئية *M. fortuitum*، *M. kansasii*، *M. scrofulaceum*، *M. chelonae*، و*M. smegmatis*، بعدد عزل بلغ 27، 18، 12، 9، و15 على التوالي .تسلط هذه الدراسة الضوء على أن الحليب الخام يشكل مصدرًا محتملاً لعدوى السل البيئية لكل من الحيوانات والبشر.

الكلمات المفتاحية: الحليب الخام، المتفطرات غير السلية، التوصيف الجزيئي، الأهمية للصحة العامة.