Materials and Methods:

Sampling

A total of 225 samples were collected from animal and human surroundings, including 75 Soil samples, 75 Water and 75 fecal samples. Soil samples included (sandy soil, clay soil, gerby soil, stony soil, agriculture soil, agriculture drainage soil), water samples included (artois water, agriculture water, Nile water), feces samples included (avian feces, bovine feces, rabbit feces). These samples were collected from Feb.2017 till Feb. 2018, for isolation and identification of NTM.

1. Traditional techniques

1.1. Culture technique:

A) Sample preparation:

1- Fecal samples:
It was prepared by method described by [11] diced separately with sterile disposable surgical blades (Swann-Morton) and were homogenized using a sterile porcelain mortar and pestle (Cole-Parmer). The contents were then suspended in 10 ml of phosphate-buffered saline (PBS) (Sigma-Aldrich).

2- Soil samples:
It was prepared using a modified version of the method described by [12]. Approximately 5-g soil samples were suspended in 30 ml of PBS in sterile 50-ml Falcon tubes (BD Biosciences). Samples were shaken vigorously for 1 min and then centrifuged at 600 g for 5 min at 4°C, to pellet the soil particles. The turbid supernatants (15 ml) were transferred into new sterile 50-ml Falcon tubes (BD Biosciences) and centrifuged at 7,000 g for 10 min at 4°C, and the pellets were suspended in 10 ml of PBS.

3- Water samples:
It was processed according to [12]. It was vortex-mixed to homogeneity and centrifuged at 1,700 g for 30 min, to sediment all suspended bacteria the supernatant was decanted and the resulting pellet was suspended in 10 ml of PBS (SigmaAldrich). Prepared sample suspensions were kept at 4°C.

A) Sample decontamination:

According to [13]
Wet soil or feces samples of approximately 5 gm were collected from a depth of 3 cm, and 50ml water samples were collected from different sources. Soil or fecal sample was suspended in 20 ml of sterile double-distilled water (D/W) in polycarbonate centrifuge tubes. After being shaken manually for 60 s, the suspension was centrifuged at 600 xg for 5 min at 4°C to pellet the soil particles. The turbid supernatant (10 ml) was transferred into other sterile centrifuge tubes and centrifuged at 8,000 x g for 15 min at 4°C. Water samples were centrifuged at 8,000 x g for 15 min at 4°C.
Pellets from the soil, water and feces samples were re-suspended in 20 ml of treatment solution (3% sodium dodecyl sulfate [SDS] plus 4% NaOH) and then divided into two parts: A and B. Part A was incubated at room temperature (RT) for 15 min to obtain the growth of rapid growers, and part B was incubated at RT for 30 min to obtain the growth of slow growers. After incubation, both the suspensions were centrifuged at 8,000 x g for 15 min at 4°C, and then the supernatants were decanted. Sediments were processed for cetrimide treatment. The pellets were resuspended in 20 ml of 2% cetrimide. Part A was incubated at RT for 5 min to obtain the growth of rapid growers, and part B was incubated at RT for 15 min to obtain the growth of slow-growing mycobacteria, following which the suspensions were centrifuged at 8,000 x g for 15 min at 4°C. Subsequently the pellets were washed twice with 20 ml of D/W and finally resuspended in 0.5 ml of D/W. A 0.1-ml sample of the suspension was inoculated on Lowenstein-Jensen (L-J) slants in duplicate and incubated at 30 and 37°C.

1.2. In vitro test for some clinically significant Mycobacterium According to [14]:

Once the growth was observed, some phenotypic tests were performed in the laboratory for identification - temperature preference (ºC), growth at both 42ºC, 52ºC growth rate, pigment production in dark and on exposur to light, growth on PNB, growth on MacConkey, growth in the presence of 5% NaCl, niacin, semi-quantitative catalase (mm), heat stable catalase, nitrate reduction, tellurite reduction, tween 80 hydrolysis, aryl sulphatase three days, aryl sulphatase 14 days, urea hydrolysis, utilization of citrate, mannitol, and inositol, iron uptake and pyrazinamidase .

2. ***Molecular technique:***Five isolates were selected for molecular identification of isolated NTM from all types of samples including two from water samples, two from soil samples and one from fecal samples as shown in fig (1).

- **DNA extraction.** DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min.
  After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then

washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

- Oligonucleotide Primer

TABLE (1): SHOWING PRIMERS WHICH WERE SUPPLIED FROM METABION

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

- **PCR amplification.** Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.
- **Analysis of the PCR Products.** The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. Generuler 100 bp (Fermentas, Thermo) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Results

Out of 225 environmental samples (75 of water samples, 75 of soil samples and 75 of fecal samples), 41 NTM isolates were obtained. Of the 41isolates, 13 were isolated from water, 18 were isolated from soil and 10 were isolated from feces.

TABLE (2): ISOLATION RATE OF NTM FROM ENVIRONMENT

Type of samples	No. of samples	Isolation rate
Water	75	13(17.33%)
Soil	75	18(24%)
Feces	75	10(13.33%)
Total	225	41(18.22)

***the percentage of isolation was calculated according to each type of examined samples.**

TABLE (3): TYPE OF NTM ISOLATES ACCORDING TO IN VITRO TEST

Pathogenicity	Type of NTM	Number of isolates	Total
Pathogenic strains	**M. avium**	**8 (19.5%)**	**31(75.6)**
	M. fortuitum	**5(12.2%)**	
	M.chelonae	**2(4.9%)**	
	M. marinum	**5(12.2%)**	
	M. ulcerans	**9(22%)**	
	M. Kansasii	**2(4.9%)**	
Non-pathogenic strains	**M. flavescens**	**4(9.8%)**	**10(24.4)**
	M. smegmatis	**6(14.6%)**	
Total	**41**		

TABLE (4): BIOCHEMICAL IDENTIFICATION OF THE ISOLATED NTM

Type of	Type of Mycobacterial isolate							
	Fortuitum	**Marinum**	***Avium***	**Chelonae**	**Kansasii**	**Smegmatis**	**Flavescence**	**Ulcerans**
Niacine production	-	-	-	-	-	Nd	-	-
Aryl sulfatase						Nd		-
3 Days	+	-	-	+	-		-	Nd
2 Weeks	+	-	+	+	+		+	
Urease production	+	+	+	+	+	Nd	+	-
Nitrate reduction	+	-	-	-	+	+	+	-
Tween hydrolysis						Nd		-
+10 Days	-/+	+	-	+/-	+		+	Nd
(1 Week)	+		-		-		+	
Catalase						Nd		-
Semi Quantative	+	-	-	+	+		+	+
Heat stable	+	-	+	+/-	+		+	Nd
Iron uptake	+	-	-	-				
Growth in Macconkey agar with crystal violet	+	-	-	+	-	-	-	Nd
Carbon source					Nd	Nd	Nd	Nd
Sodium citrate	-			+				Nd
Inositol	-			-				Nd
Mannitol	-			-				

***Nd: non available data**

Table (5): Relationship between isolated NTM species and type of samples

		Type and number of isolated NT							
Type of sample	**Total Examined Isolates**	M. avium	M. fortuitum	M. chelonae	M. marinum	M. smegmatis	M. kansasii	M. ulcerans	M. flavescence
Water	**13**	1	2	1	3	0	0	6	0
Soil	**18**	2	2	1	2	4	2	3	2
Feces	**10**	5	1	0	0	2	0	0	2
Total	**41**	8	5	2	5	6	2	9	4

FIG. (1): RESULTS OF TRADITIONAL PCR ON SOME SELECTED ISOLATED NTM

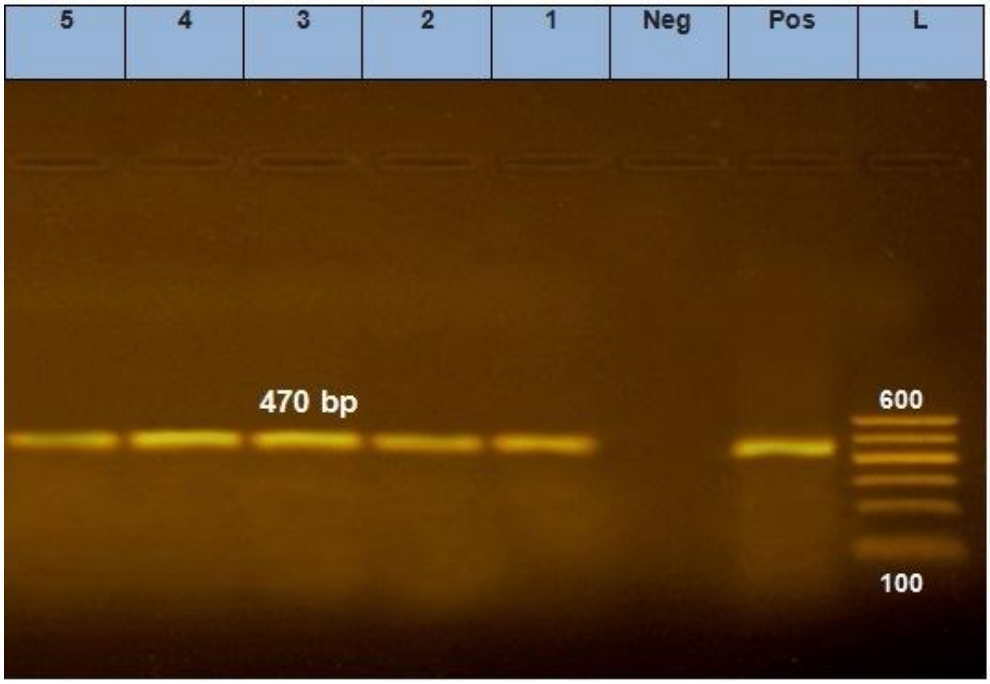

Fig (1): Agarose gel electrophoresis showing:
Lane L: 100 bp ladder.
Lane 1,2,3,4 and 5 showing amplification of 470 bp fragment (Positive NTM).
Pos.: positive control.
Neg.: Negative control.

Discussion:

In Tb endemic countries, due to heavy burden of disease caused by M. tuberculosis complex, NTM diseases have been considered less important. The objective of our study was to through light on public health significance of NTM and try to isolate it from different environmental samples, using the best of the evaluate method to isolate and to profile NTM in environmental samples.

In our study 75.7% of environmental isolates included potentially pathogenic mycobacterial spp. Such as M. fortuitum, M. ulcerans ,M. chelonae, M. avium, M. kansasii and M. marinum.

Most types of NTM present in environment may interfere the protective efficacy of BCG vaccination [16]. Table (2) revealed that, the isolation rate of NTM was 13% from water, 18% from soil and 10% from feces. Environmental Mycobacteria have an extraordinary starvation survival [17] persisting despite low nutrient levels in water, furthermore, tolerance of temperature extremes, adaptation to acid and microaerophilic conditions which aid in the virulence of intracellular pathogens.

The greatest number of Mycobacterial spp. Were isolated from soil, that's due to the abundance of organic matter serve as a nutrient source for NTM in the environment since most of them are saprophytic in nature, moreover, the hydrophobic nature of NTM affect their ability to attach to the soil surface and survive in the environment [18].

Identification of environmental Mycobacteria by biochemical tests has been with considerable success during the last 50 years, but it has some limitations in identifying some environmental species [19].

Based on biochemical characters, our study indicated that, out of 225 examined environmental samples (75 water, 75 soil samples and 75 fecal samples), there were 41 positive samples. Out of 41 environmental isolates, 75.6% of them were pathogenic including; M. avium (19.5%), M. fortuitum (12.2 %), M. chelonae (4.9%), M. marinum (12.2%), M. kansasii (4.9%) and M. ulcerans (22%). The remaining (25.4%) were nonpathogenic strains of NTM including M. smegmatis (14%) and M. flavesence (10%).

Our results were approximately similar to [20] who have higher isolation rate from soil with a percentage of (33%).

Isolation of M. chelonae and M. fortuitum were higher from water than soil, which is similar to [12], on the other hand, [21] isolated M. chelonae and M. fortuitum higher from soil.

The steady increase of mycobacteria species, the use of time-consuming techniques and the lack of standardized identification methods makes the NTM diagnosis is challenging. Additionally, inaccurate diagnosis can lead to in accurate therapeutic approaches [22], so the use of PCR becomes a must to reach to an accurate diagnosis.

Conclusion

There are many health disorders caused by several species of NTM such as; increasing the prevalence of autoimmune disorders, cervical lymphadenitis in children. Moreover, it is associated with chronic bowel disease, allergies, strong dysregulation of pulmonary immunity and blocking the replication of BCG which prevents the protection against M. tuberculosis so we have to pay attention to environmental mycobacteria . Moreover, it is important to realize that, there is no "stand alone" assay for the identification of NTM. Many new species may not be recognized using single assay. Water, soil and animal feces revealed to be a significant health hazard for both animals and humans in their surroundings.

Recommendations:

Agriculture farmers, lifeguards, veterinarian during their field work and children particularly during play time, are constantly exposed to soil and vegetation in the environment; therefore, it is important for them to wear protective clothing, which has been shown to offer some form of protection for NTM.

It is important to pay attention to health education and self-cleansing.

Application of strict hygienic measures in farms and sanitation.

Hygienic disposal of animal manure.

Strict application of quality control measures on water sources.

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