Journal University of Kerbala , Vol. 15 No.2 Scientific . 2017

Molecular identification of some causes of ringworm isolated from cattle in Al-Diwaniya region

التعرف الجزيئي على بعض مسببات مرض القوباء الحلقية المعزولة من الابقار في مدينة الديوانية

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Abstract :

The ringworm is fungal disease results in high economic losses particularly in the fields that used for leather production due to the down grading of healthy skin; a decrease in meat and milk production. this disease caused by several fungi genera like microsporium, epidermophytone, and trichophyton.

Trichophyton spp is one of fungi most common zoophilic and dermatophyte fungi that parasitizes and keratinized tissues in human and animal.

Our study results showed that percentage of total isolated fungi from infected cattle are (34.2%) by using classical media culture, while it was (100%) using molecular methods (PCR). in this study isolated two type of fungi are *Trichophyton verrucosum and Trichophyton rubrum.*

Percentage of *Trichophyton rubrum* that diagnosed in cattle in our study are (33.3%); while was (66.6%) in *Trichophyton verrucusum.*

phylogenetic analysis tree of Trichophyton spp was done by using a primer designed for specific rRNA gene for detection of *Trichophyton rubrum and Trichophyton verrucusum.*

whenever ,*Trichophyton rubrum* showed high likely similarity with *Trichophyton raubitschekii* (99%), *Trichophyton kanei* (99%) and *Trichophyton megninii* (98%).

while *Trichophyton verrucusum* show similarity with *Trichophyton megninii* (93%), *Trichophyton circonvolutum* (92%) and finally similarity with *Trichophyton interdigital* (93%).

Percentage of similarity of the isolates of this study with phylogenic tree analysis is (100%) by using rRNA gene.

Keyword: Ring worm, Trichophyton spp, PCR, phylogenic.

الخلاصة :

مرض القوباء الحلقية مرض فطري يسبب خسائر اقتصادية كبيرة خصوصا في الحقول التي تستخدم لانتاج الجلود الطبيعية نتيجة انخفاض في انتاج الجلود السليمة ، ونقصان في انتاج الحليب واللحم ، هذا المرض يتسبب بواسطة عدة اجناس من الفطريات هي الميكروسبوريوم والابديرموفايتون والترايكوفايتون. الترايكوفايتون واحدة من الفطريات الاكثر شيوعا والمحبة للحيوانات والتي تستعمر على الانسجة المتقرنة للانسان والحيوان .نتائج دراستنا اظهرت بان نسبة العزلات الكلية الفطرية المعزولة من الابقار كانت 34.2% بواسطة استخدام الزراعة على الاوساط الزرعية، بينما كانت نسبتها 100% بواسطة استخدام الطرق الجزيئية (تفاعل السلسلة المتعدد). في هذه الدراسة تم عزل نوعين من الفطريات هم الترايكوفايتون فيريوكوسم وفرطيات الترايكوفايتون ربرم.كانت نسبة الترايكوفايتون ربرم 33.3% بينما كانت نسبة الترايكوفايتون فيريومكوسم كانت 66.6% تحليل الشجرة الوراثية لانواع الترايكوفايتون بواسطة استخدام بادئ صمم لهذا الغرض لجين (ار ار ان اي) لتشخيص فطريات الترايكوفايتون فيروكوسوم والترايكوفايتون ربرم .

على كل ، فان فطريات التترايكوفايتون ربرم اظهرت تشابه كبير مع الراتيكوفايتون راوبيتشكي بنسبة 99%

والترايكوفايتون كاني بنسبة 99% وترايكوفايتون مكنيني بنسبة 98% .
بينما الترايكوفايتون فيريوكوسوم اظهرت تشابه كبير مع الترايكوفايتون مكنيني بنسبة 93% والترايكوفايوتون بنسبة سيركونفيليتوم بنسبة 92% واخيرا تسابه مع الترايكوفايتون انتريديجتال بنسبة 93% .
نسبة التشابه ما بين العزلات المعزولة في هذه الدراسة مع تحليل الشجرة الوراثية كانت بنسبة 100% بواسطة استخدم مورثة (اراران اي).
الكلمات الافتتاحية : القوباء الحلقية ، انواع الترايكوفايتون ، تفاعل السلسلة المتعدد ، الشجرة الوراثية .

Introduction:

Dermatophytes cause surface infections of the skin, characterized by the non-inflammatory lesions due to *Trichophyton spp* as the most common [1].

Superficial infection in human and animals caused by dermatophytes are spread all the animals and all the age . The diseases have a zoonotic importance because the infection transmitted between the animals and human by contact [2].

In dairy beef production and also in public health, dermatophytosis may be of economic importance due to costs of treatment, decreased skin value and weight [3-4] and its incremented for high economic losses in cattle due to damages and loss of the good leather [5].

Trichophyton verrucosum is a zoophilic fungus and is a cause factor of disease of the ringworm in human and domestic animals e.g a camel and cow [6]. Direct contact is common methods to spread the disease; also *T. verrucosum* also doing financial loos in field animals [7]. It is transmitted to human through direct contact with contaminated cattle or its products to result inflammatory lesion in the head, face etc[8].

Trichophyton rubrum, is the spreadable and common factor of mycosis over the worldwide, basically infect all the creatures [9]. This fungus commonly causing cutaneous diseases and considered the discovery of its genomic structure may reduce the health costs of those who affected with different forms of fungal disease caused by *T. rubrum* [10].

T. rubrum causes cutaneous infection and deformity of the shape [10]. Our informations are very little about the mechanism or pathogenesis of this fungus [11]. It is usually not life-threat and don't cause acute infection, but usually are chronic infections, reinfection, but it's difficult to heal. The fungal pathogen’s can formed and secrete specific enzymes considered as pathogenic and virulence factor [12]. *T. rubrum* transmitted to the animals and the human skin via contact by keratin layers [10،12] .

Trichophyton verrucosum is the animals' fungi causing in cow dermatophytosis in humidity environments of our plant and the goat and sheep but less percentage [13,14].

The Ringworm disease occurs in calves more prevalent than adult age(cow) and is speedily spreading between the animals of the one herd by infected flockes, like hyphae, and fungal spores called arthrospores. The disease occurs in all worldwide [15,16].

Furthermore, this disease in cattle also has been reported and confirmed in ruminants as examples goat and sheep [13,14].

PCR technique is one of the unique and very sensitive ways which used for detection the dermatophytosis disease, such as tinea unguium disease and tinea pedis disease [17,18].

(19)uses real-time PCR to identification of the organisms a are assay with universal primer kit and its probe for whole the fungi, also the specific primer used to detect *T. rubrum* as accuracy [19].

Our study has been designed to diagnose and confirm the molecular Identification types of Trichophyton spp which collected from the skin of cow by some media culture like SDA agar and PCR then tested by phylogeny analysis tree to confirm the final diagnosis these fungi.

Materials and Methods:

Sample collection and culture:

The study was conducted by collection samples from cattle from different regions of Al-Diwaniya, the sample collected was done by using scraping by sterile scalpel , keep the samples in a petri dish, sent to the lab.

Samples were treated with several drops from solution KOH (10%) were added to each sample to dissolve the keratin for (2) hours for complete dissolving and separating the colony. tocopherol cotton blue was used to staining the fungi and examined under the microscope at 40X, then grew on SDA media where kept at 25°C - 10 days [20, 21].

Bacterial genomic DNA extraction:

Genomic pellet DNA was extracted from fungal samples by using (specific kit called EZ-10 Spin Column).

Fungal cell pellets were collected from the 1ml fungal culture by centrifugation then ground in liquid nitrogen using a pestle and transferred to a clean 1.5 ml small centrifuge tubes. Extraction of fungal DNA was done according to company instructions. After that, the extracted fungal genomic was tested by Nanodrop spectrophotometer apparatus, after store in -30C at freezing for PCR assay time.

Polymerase chain reaction methods:

PCR technique has done for diagnosis dermatophytes fungi which called Trichophyton spp. depended on small subunit ribosomal RNA gene by using specific primers that designed in this study by using NCBI-GenBank and primer3 plus site design online. These primers were prepared and made by (Bioneer com. Korea) as following table (1).

Table(1): The primer used in this study with its sequence, and it's pb (amplicon).

Primer	Sequence	Amplicon	GenBank
small subunit ribosomal RNA gene	GATCAGCGTTCCATCAGGGG	540bp	AF168126.1
	GAGAGATTTGGGGGAAGGCC		

The PCR solution mix was prepared by the company called (Bioneer). The PCR solution kept at small tube containing freeze-dried pellet of DNA and the PCR master mixture reaction was done according to kit instructions, then filled to the top by the PCR premix tube by deionized water into 20µl and used vortex centrifuge (Bioneer. Korea) for good mixing. The reaction was done in a thermocycler system (MygeneBioneer) by orders we can sit it on the control panel the following thermocycler conditions; The products were examined by electrophoresis on a 1% agarose gel under UV light.

DNA sequencing method:

genetic sequencing of subunit ribosomal RNA gene by using analysis of phylogenetic relationship and study level of alignment by mega multiple sequence software alignment programs. At 540bp product was purified from the gel by using (Gel Extraction Kit). The purified rRNA gene PCR product was sent to DNA sequence Company in Korea for doing the DNA sequencing using 18-rRNA primer designing by (ABDNA sequencing system).

Results :

In this study used classical methods and molecular methods to diagnosis of fungi , used SDA agar for culture that keep at 25°C for [10] days as figure (1), however the tested (35) isolates by culture methods produce the positive is (12) (34.2%) while these(12) isolates used molecular detection methods for confirm these (12)(100%) isolates see the table (2) **.**

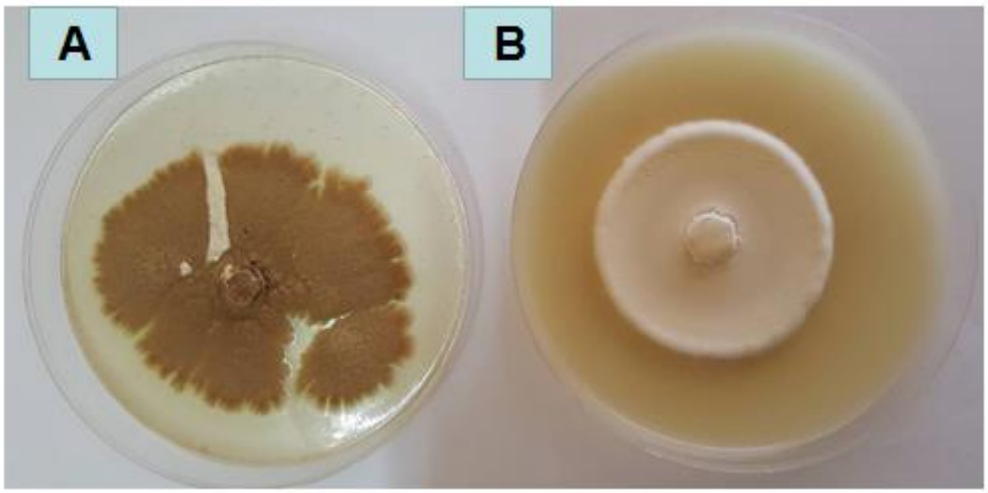

Figure (1): Dermatophytes fungal growth cultures on (SDA) that incubated at agar. Where A: Dermatophyte *Trichophyton rubrum* and B: *Trichophyton verrucosum* according to PCR technique and phylogenetic tree analysis results.

Table (2): Prevalence of dermatophytes fungi that detected by two methods shows its number and percentage.

Method	No .of tested isolates	Positive	Prevalence (%)
Culture method	35	12	34.2%
Dermatophytes fungi based PCR	12	12	100%

Trichophyton rubrum and *Trichophyton verrucosum* were (2) isolate out of (35) isolate By use PCR methods.

PCR used to determination species of fungi, *trichophyton rubrum* is (4) out from (12)(33.3%) tested isolates, while *Trichophyton verrucosum* is (8) out from (12) (66.6%) tested isolates the table (3). it has been read on electrophoresis also Figure (2)**.**

Table(3): The prevalence of dermatophytes fungi species that detected by phylogenetic tree analysis:

Fungal isolates species	No .of tested isolates	Positive	Prevalence (%)
Trichophyton rubrum	12	4	33.3%
Trichophyton verrucosum	12	8	66.6%

After making DNA extraction, PCR, and finally used electrophoresis to read the results of agarose gel electrophoresis see figure (2).

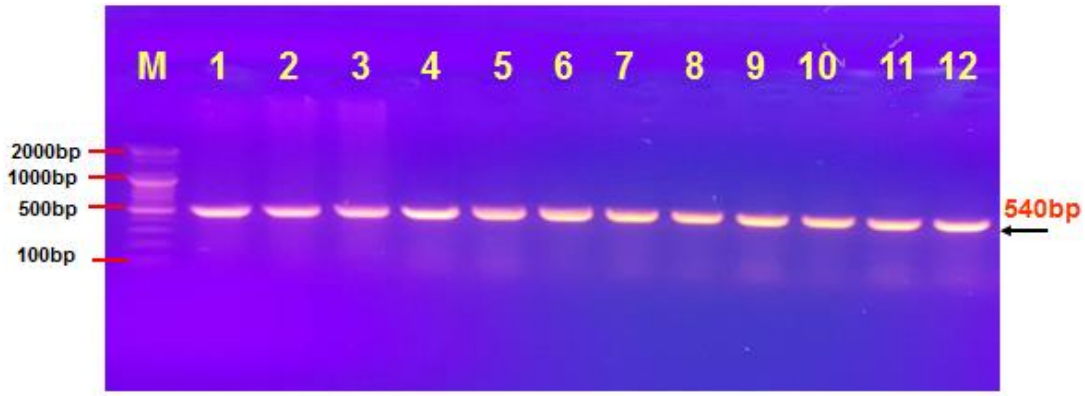

Figure (2): this picture show under UV light by use Agarose gel in electrophoresis analysis apparatus that shows the PCR product analysis of small subunit ribosomal RNA gene in Trichophyton spp. isolates. M: marker (range between 100 to 2000bp), lane (1-12) some of positive Trichophyton spp. in (540bp) PCR product size.

phylogenic of *trichophyton verrucosum* has been making Homology sequence identity by use rRNA gene according to NCBI-BLAST site show table (4) highly sensitivity to accuracy molecular detection.

Table (4): NCBI-Blast Homology sequence identity for rRNA gene in Trichophyton sp. isolate-1 with NCBI-BLAST Trichophyton spp.:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download Graphics Distance tree of results

	Max score	Total score	Query cover	E value	Ident	Accession
Trichophyton verrucosum genomic rRNA gene (LN614528.1)	975	1603	100%	0.0	100%	Query_183680
Trichophyton megninii genomic rRNA gene (AF170464.1)	769	1683	96%	0.0	93%	Query_183686
Trichophyton circonvolutum genomic rRNA gene (AJ270791.1)	758	1520	96%	0.0	92%	Query_183684
Trichophyton interdigitale genomic rRNA gene (AJ270790.1)	751	1501	98%	0.0	93%	Query_183683
Trichophyton violaceum genomic rRNA gene (AJ270796.1)	749	1471	96%	0.0	92%	Query_183682
Trichophyton rubrum genomic rRNA gene (AJ270806.1)	749	1528	96%	0.0	92%	Query_183681
Trichophyton raubitschekii genomic rRNA gene (AF170468.1)	747	1635	96%	0.0	92%	Query_183687
Trichophyton kanei genomic rRNA gene (AF170460.1)	747	1593	96%	0.0	92%	Query_183685

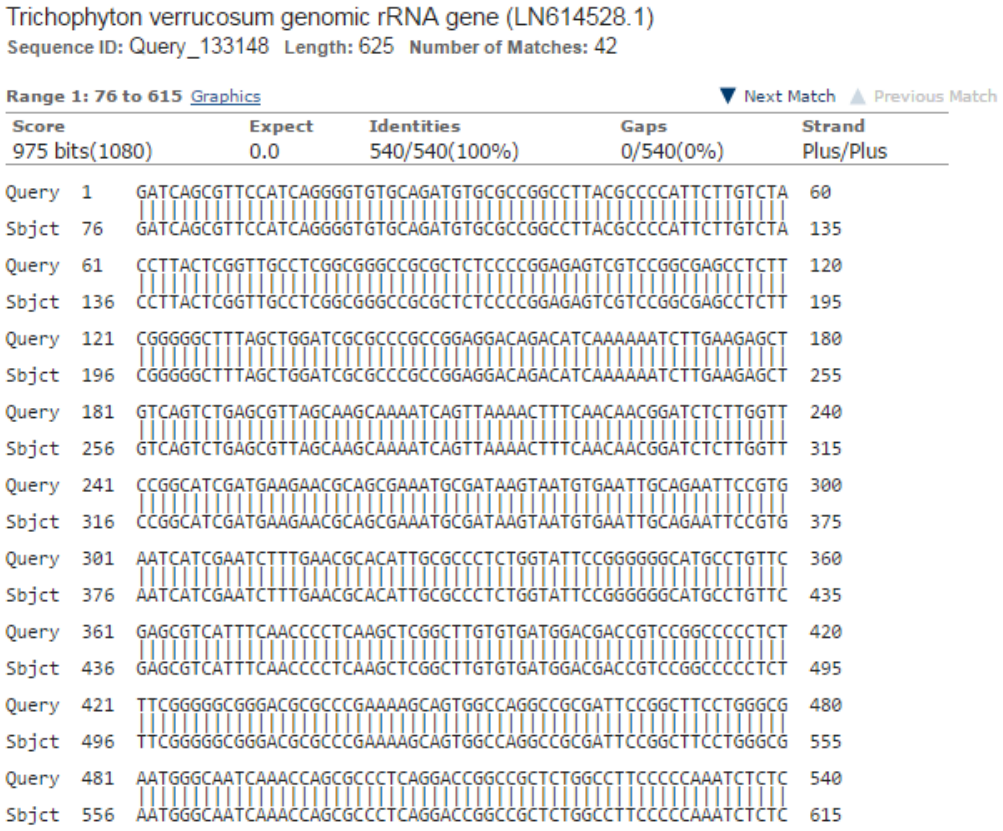

Figure (2): Basic local sequence alignment analysis of local Trichophyton sp. isolate-1with NCBI-BLAST *Trichophyton verrucosum* at 100% identity.

phylogenic of *trichophyton rubrum done in the* NCBI-BLAST site *are Homology sequence identity by use* rRNA gene highly sensitivity to accuracy molecular detection see table (5).

Table (5): NCBI-Blast Homology sequence identity for rRNA gene in Trichophyton sp. isolate-2 with NCBI-BLAST Trichophyton spp.:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download Graphics Distance tree of results

	Max score	Total score	Query cover	E value	Ident	Accession
Trichophyton rubrum genomic rRNA gene (AJ270806.1)	976	1823	100%	0.0	100%	Query_183681
Trichophyton raubitschekii genomic rRNA gene (AF170468.1)	964	1933	100%	0.0	99%	Query_183687
Trichophyton kanei genomic rRNA gene (AF170460.1)	964	1861	100%	0.0	99%	Query_183685
Trichophyton megninii genomic rRNA gene (AF170464.1)	926	1879	100%	0.0	98%	Query_183686
Trichophyton circonvolutum genomic rRNA gene (AJ270791.1)	922	1717	100%	0.0	98%	Query_183684
Trichophyton violaceum genomic rRNA gene (AJ270796.1)	910	1687	100%	0.0	97%	Query_183682
Trichophyton verrucosum genomic rRNA gene (LN614528.1)	686	1358	91%	0.0	92%	Query_183680
Trichophyton interdigitale genomic rRNA gene (AJ270790.1)	677	1409	100%	0.0	88%	Query_183683

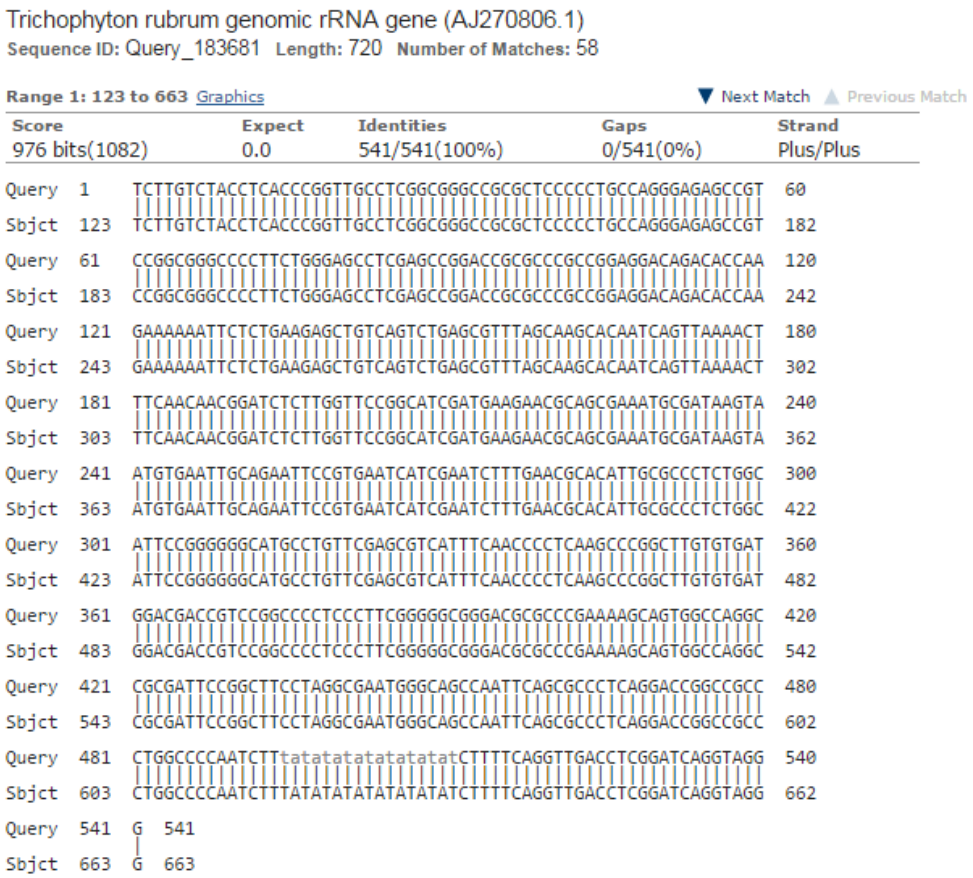

Figure (4): Basic local sequence alignment analysis of local Trichophyton sp. isolate-2 with NCBI-BLAST *Trichophyton rubrum* at 100% identity.

Phylogenetic analysis

Analysis of Phylogenetic tree has done depend on the clone rRNA, a that used for final detection of *trichophyton* spp. draw a tree for trichophyton by Phylogenetic analysis of rRNA gene sequences has become the principal method for knowing prokaryotic phylogeny**.** Our result show the phylogenetic tree has done according to these isolates see figure (3) and Table (6).

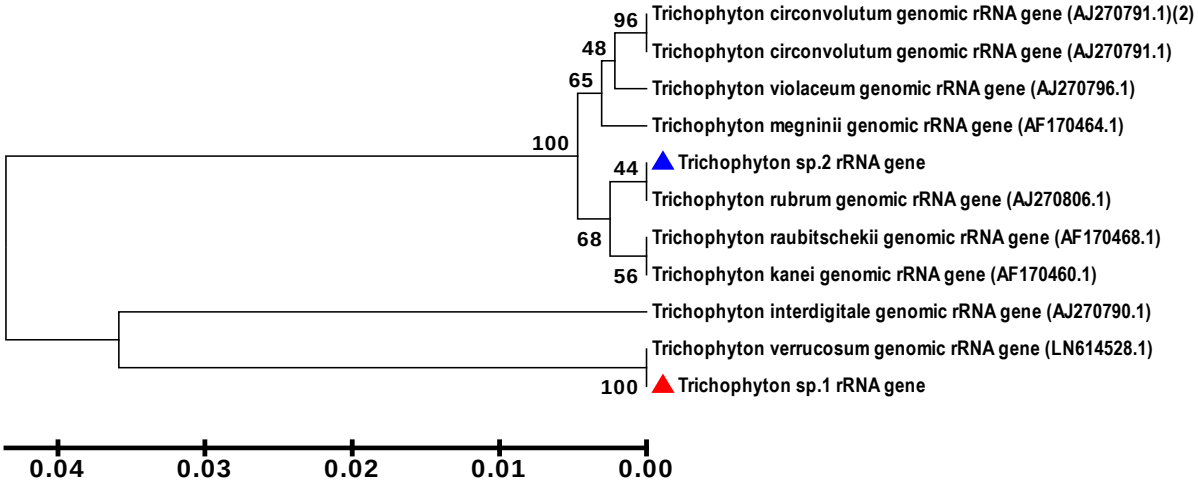

Figure (3): Trichophyton spp typing family analysis of Phylogenetic tree was created by using method named (UPG) Unweighted Pair Group with in (MEGA software 6.0 version).

Table(6): Show names of strains of isolates that sent to NCBI for sequencing and its codes (Accession number) of two isolates that selected in this work.

Fungal isolate		Accession number
Trichophton verrucosum	1- BankIt1987271 Seq1	KY549929
Trichophyton rubrum	2- BankIt1987271 Seq2	KY549930

Discussion :

The ringworm is a cutaneous disease caused by several genera of fungi including microsporium,epidermophyton, and trichophyton that can live on keratinized tissues in the skin are affected by this invasion [25,26].

The result of study in the table (2) revealed that Percentage of total isolated trichophyton spp are (34.2%) by use classical media culture, including isolation, staining, and culture ; that was near to results of [27] where was (31%).

In our report too the percentage of trichophyton spp (100%) when it used molecular methods (polymerase chain reaction) was (100%). while prevalence in [27] was less than our results where it was 17(42.5%)[28].

[29] study Molecular aspects (PCR) of total of trichophyton spp it was (96%) was same to our report results [30].

Percentage of *Trichophyton rubrum* that diagnosed in our study are (33.3%) , that compatibility with [29] that show it was (32.5%) as percentage (29) look at table (3).

Percentage of *Trichophyton verrocosum* that tested in our study is (66.6%) ;That different from [31] that found *T. verrucosum* as percentage (99%) that conserded more than our values [31].

However, Percentage of *Trichophyton verrocosum* was close to our results where was (71.3%) as in [32,33].

[34] found *T. verrucosum* precentage for the ringworm was (85%) as percentage in calves; its considered more little from ours.

Different percentage, Incidence and prevalence range are contract depend on age of young animals particularly the calves susceptible for the ringworm more than adults ; and this disease may be accurate due to a poor immune system and the high pH of the skin in young animals [35,36].

The humid and warm weather of environment and climate could have preferred to the growth and development of fungal spores there by help to replication and infection the fungus in the animals and produce the outbreaks in the population[37][(38].

Finally, The host species; the geographical factors and environmental conditions are main causes to formed contract values phenomones [39,40].

T. verrucosum has been slow growing, white color, cottony consistency, and its yellow pigment. These signs are consistent with the clinical findings of [41] and [42]. In our research, so the microscopic examination revealed hyphae thread and small conidia that were adhesion with the hyphae, that same with the study of [31].

Percentage of infection in mature cattle was less than the calves and that like to our results [35] [43], and [44] .

Genomic sequence identification showed that the isolates and nucleotide of *Trichophyton rubrum* show similarity with *Trichophyton raubitschekii* (99%), *Trichophyton kanei* (99%) and *Trichophyton megninii* (98%). while *Trichophyton verrucusum* show likely similarity with *Trichophyton megninii* (93%), *Trichophyton circonvolutum* (92%) and finally likely with *Trichophyton interdigital* (93%).

[45] reported for the first time *T. rubrum var. raubitschekii* isolated from the toe nail of a hypertensive from old women living in Algeria presenting. The molecular investigation from the isolate led to (99%) of homology with both *T. raubitschekii* JX827168.1 and *T. rubrum* FM178326.1. As previously reported, showed that *T. rubrum* was genetically identical same to *T. rubrum*. [45].

While [49] found the tree of genetic family analysis of *T. rubrum* by use (CHS1) gene that extracted from the isolates detect more than (99%) genome high likely between human and canine isolates of *T. rubrum* then the tests mention that isolates are same sources [46].

Percentage of similarity between our isolates of this study with phylogenic tree analysis database is (100%) by use mega software by use rRNA gene.

[47] found The symmetrical percentage sequencing was more than (97%). according to sources that sequences of new strains, sequencing supply a very sensitive and accurate and he conceded very good method for the knowing of dermatophytes [47].

Using pairwise nucleotide comparisons, a mean match of 81% was noticed among [29] dermatophyte species, with inter-species variety range from 0 to 200 nucleotides [48] ,while our sequencing was same at 100% with the sequence in data base.

This study suggested phylogenetic tree analysis based on rRNA gene, by use primer can be used for confirmative detection of *trichophyton verrucosum* and *trichophyton rubrum* strains and determine the close relationship between These results with informations of data base and showed highlight the importance of detection of trichophyton and it relationship with the cattle that effected with the ringworm disease bu phlogenic analysis tree.

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