Plant Archives Vol. 20, Supplement 2, 2020 pp. 25-36 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

IDENTIFICATION OF *SARCOCYSTIS* SPP. IN IMPORTED BEEF BY TRADITIONAL AND MOLECULAR TECHNIQUE

Jinan Khalid Kamil and Azhar Ali Faraj
Department of Parasitology, College of Veterinary Medicine, University of Baghdad, Iraq

Abstract

These was first research in Iraq conducted for identified *Sarcocystis* spp. in imported beef, by using (100) imported beef samples randomly collected at the start of December 2018 to the end of September 2019. Samples were examined by traditional methods (Trichnoscopy, squeezing, pepsin digestion and blender). In addition, all samples were examined by molecular methods using sequencing and phylogenic to determine the *Sarcocystis* spp. Result by using traditional methods show 64% of samples were positive of *Sarcocystis* infection, additionally found pepsin digestion method was the best technique used for the detection of *Sarcocystis* in imported beef tissues, followed by Blender technique, while trichnoscopy and squeezing technique were less sensitive, also the molecular test registered 69% of positive samples among all 100 samples. Phylogenetic topology of 15 samples bootstraps of our samples registered high sequence resemble to diagnosed species, also BLAST searches revealed that the *18S rRNA* sequences shared 98-100% similar to *S. fusiformis, S. levinei* and *S. buffalonis.* Phylogenetic tree of our study appeared similarities between isolated strain and the distant world.
Keywords: *Sarcocystis* spp., beef, molecular technique

Introduction

Sarcocystosis identified as one of the most commonly protozoan diseases in the world, it have specific life cycle, because it depend on prey – predator relationship. Carnivores acting as definitive, as well as humans, while the intermediate host are mainly livestock animals, which there meat are consumed by the final hosts, include cattle, buffalo, sheep, and goats, as well as pigs (Şaki *et al*., 2010). These animals affected with asexual form of parasite which called (Tissue or muscular cyst), studies registered that frozen buffalo meat has multiple *Sarcocystis* infections (Mohamed *et al*., 2016; El-kady *et al.*, 2018). Typically, the definitive hosts do not show any clinical signs of *Sarcocystosis*, while the disease mainly was asymptomatic in intermediate host when infected. Occasionally, some animals have clear clinical singes according the site of tissue cyst such as mild fever, diarrhea, chills, general weakness, respiratory problems may occur and neurological singes (Faraj and Kawan, 2012; Lau *et al.*, 2014).Traditional methods such as macroscopic and microscopic techniques are mainly used to diagnosis, additionally, molecular assay are a range of DNA based technique for the detection of *Sarcocystis* parasites (Eslami *et al*., 2015; Kawan, 2019).

Materials and Methods

Collection of Samples

One hundred specimens weight 250 gram in labeled clean plastic bags samples of raw imported beef, and carried by cool box to the laboratory of the parasitology (Narges *et al.*, 2013), were collected from different stores, and super markets in diverse area of Baghdad city, at the start of December 2018 to the end of September 2019.

Macroscopic Examination

Gross Examination of fresh muscle were examined by the naked eye to detect macroscopic *Sarcocystis* (Dubey *et al*., 1989).

Microscopic Methods

Squeezing method done by crush the pieces of meat by Garlic press, crush solution drop produced from crushing, transferred to slide then covered with cover slide to examination under microscope, another method was trichnoscopy by cutting fresh muscle to small pieces, then compressed between two cleaned slides, which carry to the light microscope for examination under X10 and X40 magnification to detection the tissue cyst (Claveria *et al*., 2000). The classical microscopic method was pepsin digestion, which done with some modification, by collected 25 gm of fresh muscles from each animal samples, then put it in cleaning flask to digested for 24 h at 25 °C in 100 ml of digestion medium composed of (pepsin 1.3 gm, 2.5 gm NaCl and 3.5 ml HCl all these materials dissolve in 500 ml disinfected distilled water). After digestion, the mixture were filtered by using double layer gauze, after that centrifuged for 3 min at 2500/ rpm (Hamidinejat *et al*., 2015), finally the sediment storage in ependorf tube (1.5)ml under -20C^0 for molecular test. Blender technique was first used in our study to detect the *Sarcocystis* in imported beef, it was done by taken 50 gram of tissue pieces from imported meat, thane cut for small pieces as 2 cm^2 and smallest, and the pieces putted in blender with 100 ml saline for 10-15 second. Smashed materials filtered by using double layer gauze, thane centrifuged for 5 mint at 2800 /rpm (Silva *et al*., 2002). After pouring the supernatant, 10 drops of sediment were used per samples to perpetrated 10 slides by put one drop of sediment on slid and covered by coverslip thane examine by microscopy in 40X. Then the slides of both digestion and blender methods staining by giemsa stain and finally exanimated under microscope 100X for detection the bradyzoites (Tong *et al*., 2018).

DNA Extraction

Genomic DNA of bradyzoites which storage and isolated from imported beef isolate was extracted by using tissue DNA extraction kit Geneaid/ USA and (Bioneer)/Korea, the extraction made by fellow the manufacturer protocol from tissue cells extraction of digestion test. Nano drop spectrophotometer (ActGene/USA) used to measure the DNA purity and concentration.

PCR Amplification

The PCR primer used for *Sarcocystis* spp. detection was based on 18S rRNA, The lyophilized primer was used in this study purchased from Bioneer (Bioneer, Daejeon, South Korea), which almost amplify at 574 bp using the primer BLAST tool on the NCBI of the Genbank. This primer was prepared according to the information of the company Table (1).

Table 1 : Show the primer used in the study according there reference:

Primers	Primer sequence(5- to3-)		Product size bp	Annealing	References
Sar2	F2	AACCGTGGTAATTCTATGGCTAA	574	56	NCBI Primer Blast
	R2	TGCCAGAATTCATCAAGTGC			

For detecting 18S rRNA gene by sar2, the PCR amplification mixture which was used for detection of the gene preparated according to Table.2.

Table 2 : PCR master mix component for 18S rRNA Region reaction by primer Sar2

PCR master mix reaction components		Volume 1Rxn
Master Mix		10 µl
Primers	Primer	1 µl
	Reversed	1 µl
PCR water		3 µl
DNA template		5 µl
Total volume		20 µl

The program of amplification was started with initial denaturation of Sar2 at 94°C. for 5. minute, 30. cycles of denaturation at 94°C for 30. second annealing at 56°C for 30 second and extension at 72°C for 30 second then. ending program by final extension at 72°C. for 5 minuet The amplification reactions were carried out in PCR thermocycler (Bioneer /Korea), electrophoresed on 1.5% agarose gel, stained with ethidium bromide. U.V transilluminator and photographed using to examination.

Sequencing and genotyping of the Sarcocystis isolate

The sequencing according manuals instruction of the company (Macrogen Inc. Geumchen, Seoul), PCR amplicons were determined commercially sequenced from termini, forward and reverse, following by further analyzed of sequence file with Applied. Biosystem. extension (ABI), obtain that the entourage and divergence not on account of artifacts of PCR or sequencing. Moreover, confront DNA sequences of local samples result from this study, with the regain DNA sequencing of *Sarcocystis spp.*, in order to identified. PCR products sequencing results of multiple isolate were edited, aligned, and resolve as long as with the certain sequences in the reference database using Bio.Edit.Sequence. Alignment Editor Software Version 7.1 .

Results and Discussion

Macroscopically Examination

Imported beef with macroscopic cyst of *Sarcocystis* parasite collected, same morphological characteristic found in study done on frozen beef collected from Alexandria markets, Egypt, were creamy white in color, with different shapes as spindle, fusiform and globular (Mohamed *et al.*, 2016) also, with different sizes ranged from 2.0 - 3.0 mm x 1.0 -5.0 mm (Fig. 1).

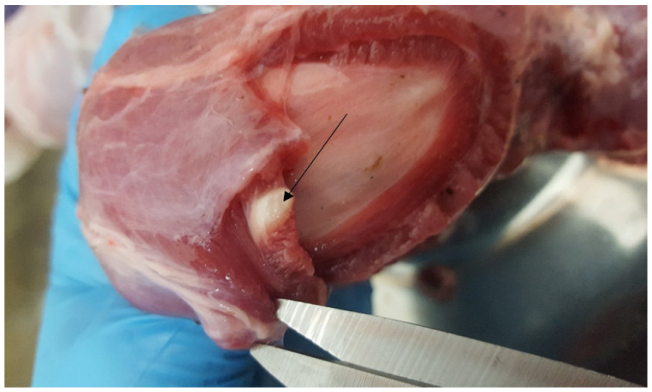

Fig. 1 : Photograph of imported beef showing macroscopic cyst of *Sarcocystis* spp.

Microscopic Examination

Sarcocystis cyst in Fig (2) by using trichnoscopy technique and examination by light microscope, look as oval, elliptical and conical form, divided into compartments were numerous intercostal with various size from (166 × 52.2) µm (40X and 100X)

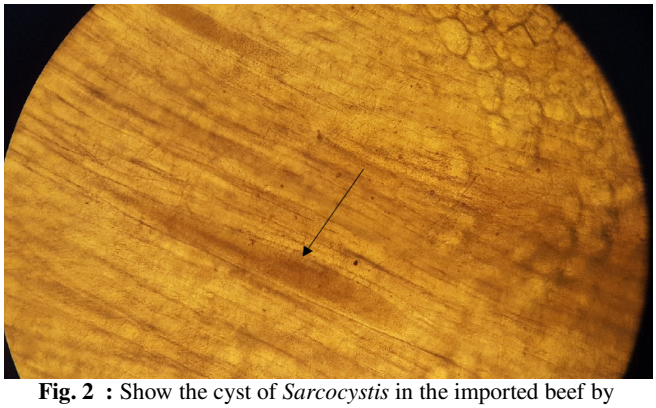

Fig. 2 : Show the cyst of *Sarcocystis* in the imported beef by Trichnoscopy method.

Morphology of bradyzoites

Using peptic digestion which are golden method for detection the bradyzoites, because its lead to liberated it after analyses the tissue cyst (Ferreira *et al*., 2018). In this methods the bradyzoites were seen by examining one drop of the sediment of the digested muscle fluid (Fig. 4). Bradyzoites appeared as banana form, with a spiked end of front and rounded rear end, nucleus seem as slightly clear lying near the rear end, measurements 13.2× 2.8 µm (100X).

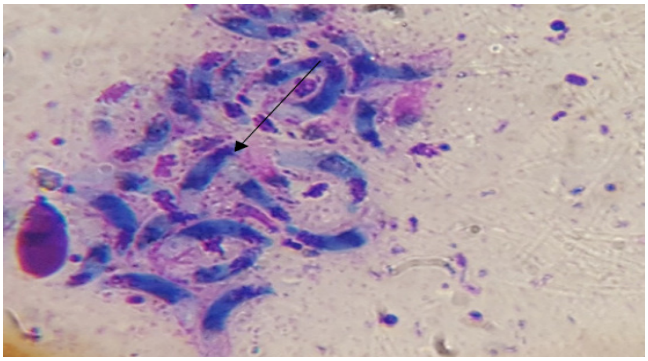

Fig. 4 : Bradyzoites in imported beef stained by Giemsa (100X)

Infection rate of macrocyst and microcyst (cystisoites) in all samples

The study show highly significant differences (P<0.01) table (2) between macrocytic and microcytic infection of imported beef these were dealing with many study which found the microscopically infection was highest detection rate of *Sarcocystis* and thus may be due to infected of the animal tissue or condemnation of carcass (Mohamed *et al*., 2016; Imer *et al*., 2019).

Table 2 : Show the infection rate of macrocyst and microcyst in imported beef samples by traditional technique

Samples	No .of meat samples examined	No .of meat Infected	Macrocyst Positive	%	Cystisoites positive	%	Chi-Square (χ^2)
Imported meat	100	64 (64%)	13	13%	51	51%	9.962 **

** (P<0.01).

***Sarcocystis* infection rate according the different traditional test**

Table (3) Show highly significant differences (P <0.01) registered between three traditional test in imported beef. Pepsin digestion was high percentage in diagnosis 100% followed by the blender method at 61% while, the squeezing and trichnoscopy methods were less sensitive in diagnosis at 37% and 34% respectively. The explanation for the elevated of Sarcocystosis prevalence according bad management system practiced by cattle owners where they allowed to roam about and scavenge for food and water, in this process they pick up the sporocysts of *Sarcocystis* shed by different definitive hosts such as dog, cat, humans and non-human primates thus resulting to *Sarcocystis* formation in the muscle of the cattle (Dubey *et al*., 1989; More *et al*., 2011). Also the external factors such as high temperature, freezing, moisture and multiple disinfectant, could be affecting on sporocysts by increased its resist and retain their infectivity in the environment for a long period (Duby *et al*., 2016).

Table 3 : The infection rate of *Sarcocystis* in imported beef according to different traditional techniques.

Samples	No.of meat samples examined	Total infection	Pepsin digestion %	Squeezing %	Trichnoscopy %	Blender %	Chi-Square (χ^2)
Imported meat	100	64(64%)	64(100%)	37(37%)	34(34%)	61(61%)	9.80 **

** (P<0.01).

PCR analyses:

The positive bands of DNA were shown in 574 bp, 579bp and 591bp, Fig.(5, 6 and 7). When PCR was analyzed by an agarose gel electrophoresis (1.5%), ethidium bromide used as stain with voltage at 100 volts for 1.5 hour

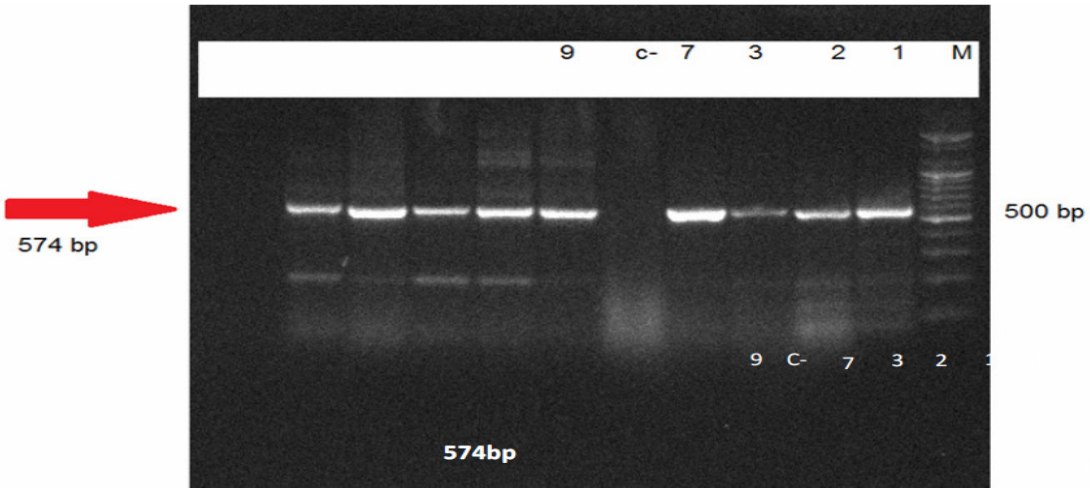

Fig. 5 : Agarose gel electrophoresis. M: molecular marker 100bp,C-: Control negative. Showed the PCR product analysis of 18S rRNA gene in *Sarcocystis spp*. Positive samples, line (Sar2),1.2,3,7,9at 574 bp with 1.5% agarose gel contain ethidium bromide, 60 volt for 1.5 hours.

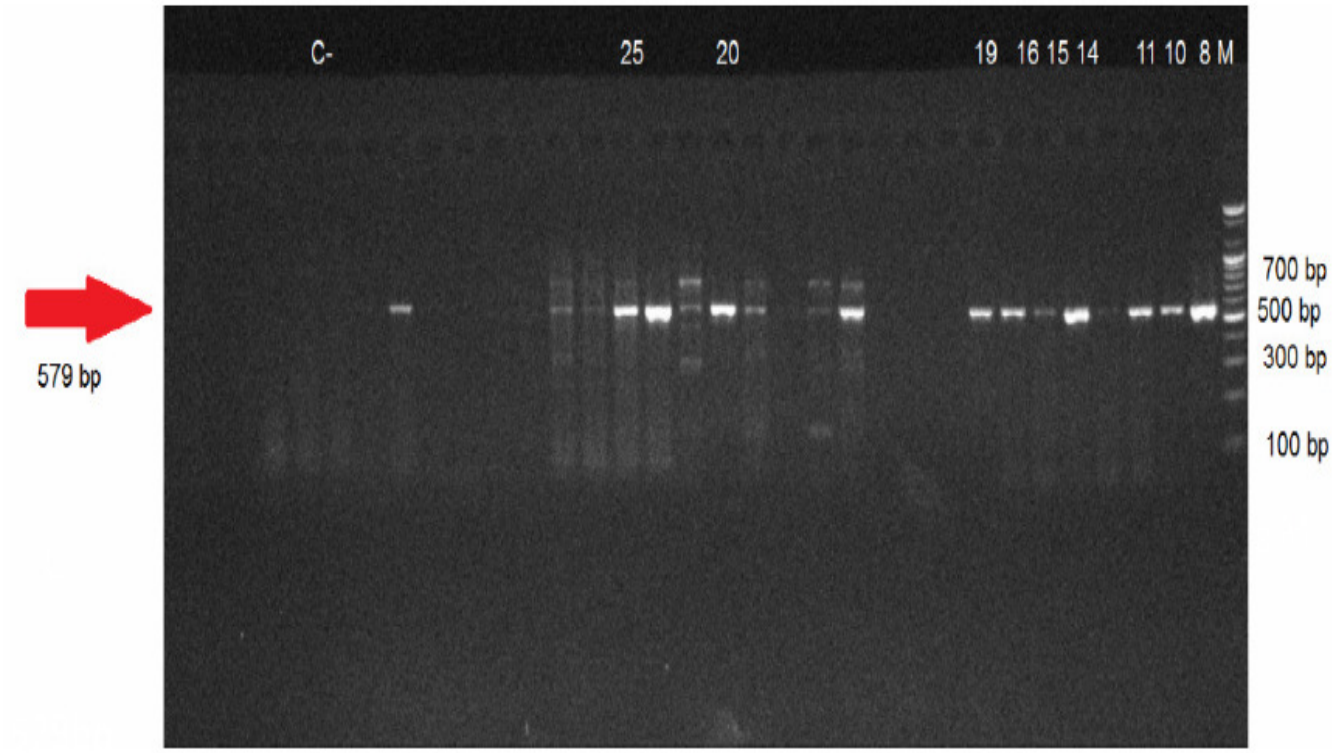

Fig. 6 : Agarose Gel electrophoresis, M: molecular marker 100bp, C-: Control negative. Showed the PCR product based on of 18S rRNA gene in *Sarcocystosiss* .Positive isolate, line (Sar2), 7.8,9,10,11,14,15,16,20,25 at 579 bp in 1.5% agarose gel with ethidium bromide, 60 volt and 1.5 hours.

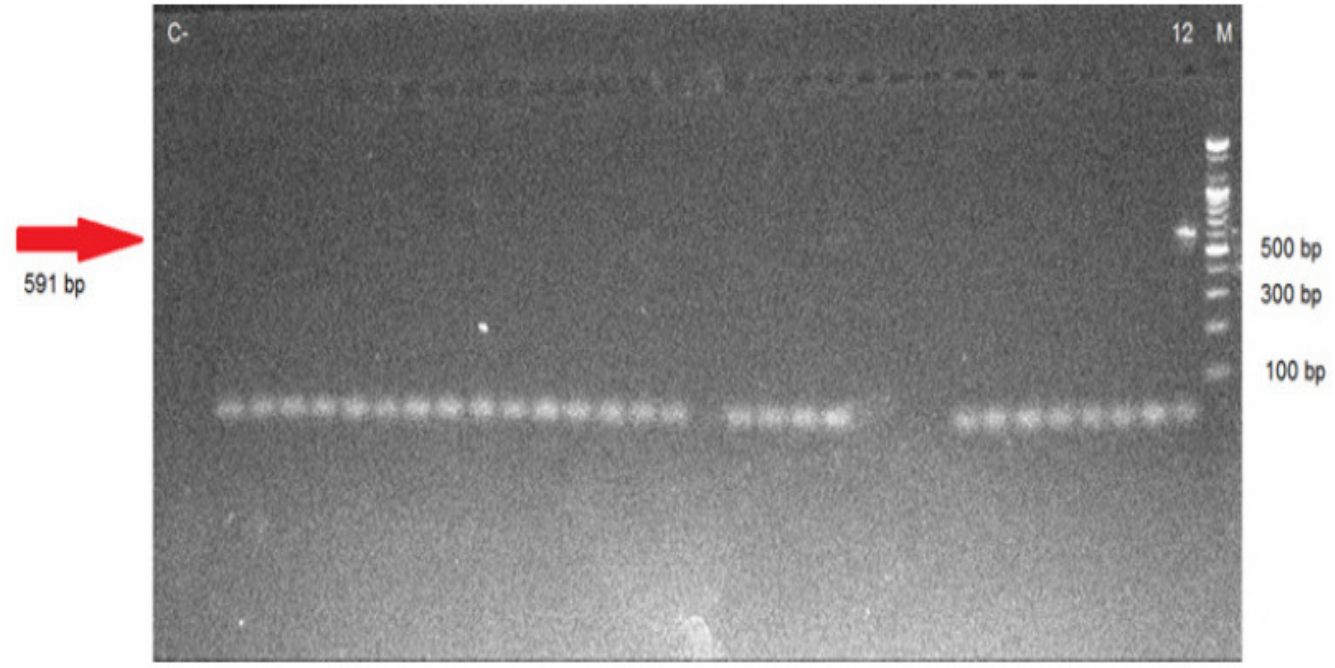

Fig. 7 : Agarose gel electrophoresis, M: molecular marker 100 bp. Showed the PCR product analysis of 18S rRNA gene in *Sarcocystis spp.* Positive samples, line (Sar2),12 at 591 bp in 1.5% agarose gel with ethidium bromide, 60 volt and 1.5 hours.

Sequence analysis

Extension is concerned with the DNA peaks, green, black, blue, and red for T, G, C, and T respectively). For ensuring that the annotation and. variations are not due to PCR. or sequencing artifacts, sequence files will be further analyzed. All these done by comparing the observed DNA sequences of local isolate with the retrieved DNA sequences of *Sarcocystis spp.*, the virtual positions and other details of the regain PCR fragments were identified in (Table 4)

Table 4 : Homology Sequence identity (%) between Iraqi *Sarcocystis* spp. of imported beef isolates and NCBI-BLAST submitted *Sarcocystis* spp. isolates, the specific primers' pairs selected to amplify 18S rRNA locus within *Sarcocystis spp.* genomic DNA sequences. The symbols (+) and (-) refer to the orientation of the positive and negative strand, respectively. The bold letters refer to the start and end of the amplicon fragment

No.	*Sarcocystis spp*. isolate	Genbank accession No.	Amplicon size	NCBI-BLAST Homology sequence identity (%)		
				Identical *Sarcocystis Spp*.	Genbank Accession No.	Identity (%)
1	*Sarcocystis* spp. Imported beef isolate No.1	MN096328	574 bp	*Sarcocystis fusiformis*	KR186121.1 (150 – 172) + (704 – 723) -	100
2	*Sarcocystis* spp.Imported beef isolate No.2	MN096329	574 bp	*Sarcocystis fusiformis*	KR186121.1 (150 – 172) + (704 – 723) -	100
3	*Sarcocystis* spp. Imported beef isolate No.3	MN096330	574 bp	*Sarcocystis fusiformis*	KR186121.1 (150 – 172) + (704 – 723) -	100
4	*Sarcocystis* spp. Imported beef isolate No.7	MN096334	574 bp	*Sarcocystis fusiformis*	KR186121.1 (150 – 172) + (704 – 723) -	100
5	*Sarcocystis* spp. Imported beef isolate No.9	MN096336	574 bp	*Sarcocystis fusiformis*	KR186121.1 (150 – 172) + (704 – 723) -	100
6	*Sarcocystis* spp. Imported beef isolate No.8	MN096335	579 bp	*Sarcocystis levinei*	MG957194.1 (5– 27) + (564– 583) -	100
7	*Sarcocystis* spp. Imported beef isolate No.10	MN096337	579 bp	*Sarcocystis levinei*	MG957194.1 (5 – 27) + (564– 583) -	100
8	*Sarcocystis spp*. Imported beef isolate No.11	MN096338	579 bp	*Sarcocystis levinei*	MG957194.1 (5 – 27) + (564– 583) -	100
9	*Sarcocystis spp*. Imported beef isolate No.14	MN096341	579 bp	*Sarcocystis levinei*	MG957194.1 (5 – 27) + (564– 583) -	100
10	*Sarcocystis spp*. Imported beef isolate No.15	MN096342	579 bp	*Sarcocystis levinei*	MG957194.1 (5 – 27) + (564– 583) -	100
11	*Sarcocystis spp.* Imported beef isolate No.16	MN096343	579 bp	*Sarcocystis levinei*	MG957194.1 (5 – 27) +(564 – 583) -	100
12	*Sarcocystis spp*. Imported beef isolate No.19	MN096346	579 bp	*Sarcocystis levinei*	MG957194.1 (5 – 27) + (564– 583) -	100
13	*Sarcocystis spp*. Imported beef isolate No.20	MN096347	579 bp	*Sarcocystis levinei*	MG957194.1 (5 – 27) + (564– 583) -	100
14	*Sarcocystis spp*. Imported beef isolate No.25	MN197852	579 bp	*Sarcocystis levinei*	MG957194.1 (5 – 27) + (564– 583) -	100
15	*Sarcocystis spp*. Imported beef isolate No.12	MN096339	591 bp	*Sarcocystis buffalonis*	MF595843.1 (5 – 27) + (576 - 595) -	100

Sequencing of the *18S rRNA* gene for samples S1, S2, S3, S7, S9

The sequencing reactions indicated the exact positions after performing NCBI blast n for these PCR amplicons (Zhang *et al*., 2000). NCBI BLASTn engine has indicated the presence of remarkable of homology with the expected target that covered a portion of the *18S rRNA* within *Sarcocystis fusiformis* genomic DNA sequences, it done when comparing local isolate with the (**GenBank acc. KR186121.1**), observed DNA sequences (Fig. 8).

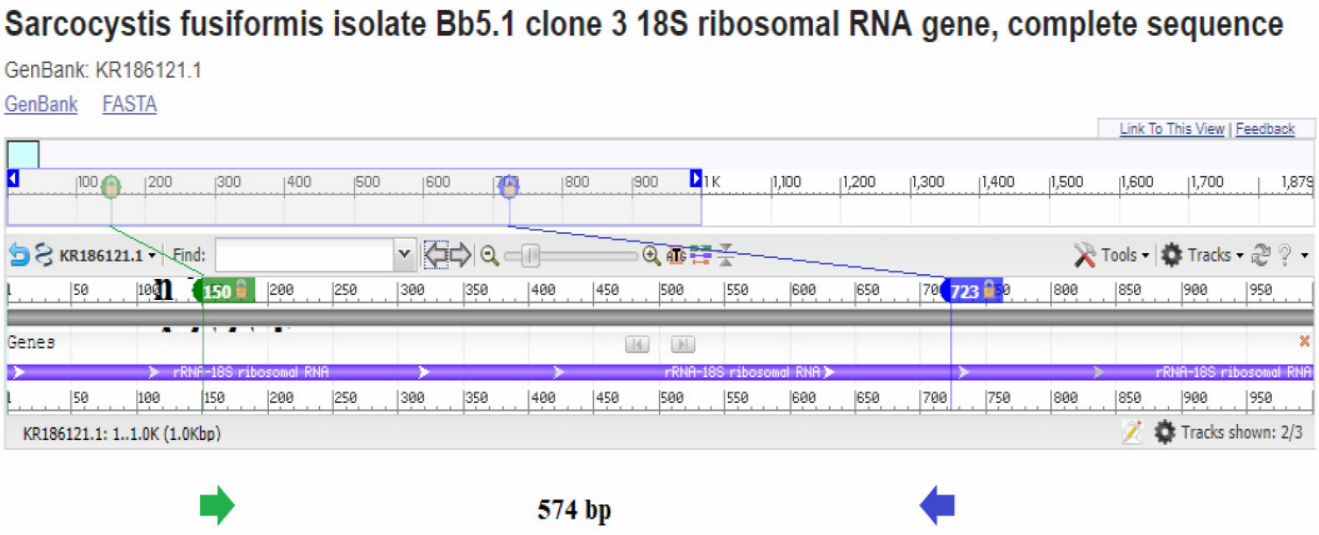

Fig. 8 : The exact position of the retrieved 574 bp amplicon that entirely covered a portion of the *18S rRNA* locus within the *Sarcocystis fusiformis* DNA genomic sequences (acc no. KR186121.1). The green arrow refers to the starting point of this amplicon while the cyan arrow refers to its end point.

The alignment show tow mutations in the local isolate of the 574 bp (Fig. 9). These mutations were taken a different distribution in the analyzed samples. S3 was shown one substitution mutation of C516A.

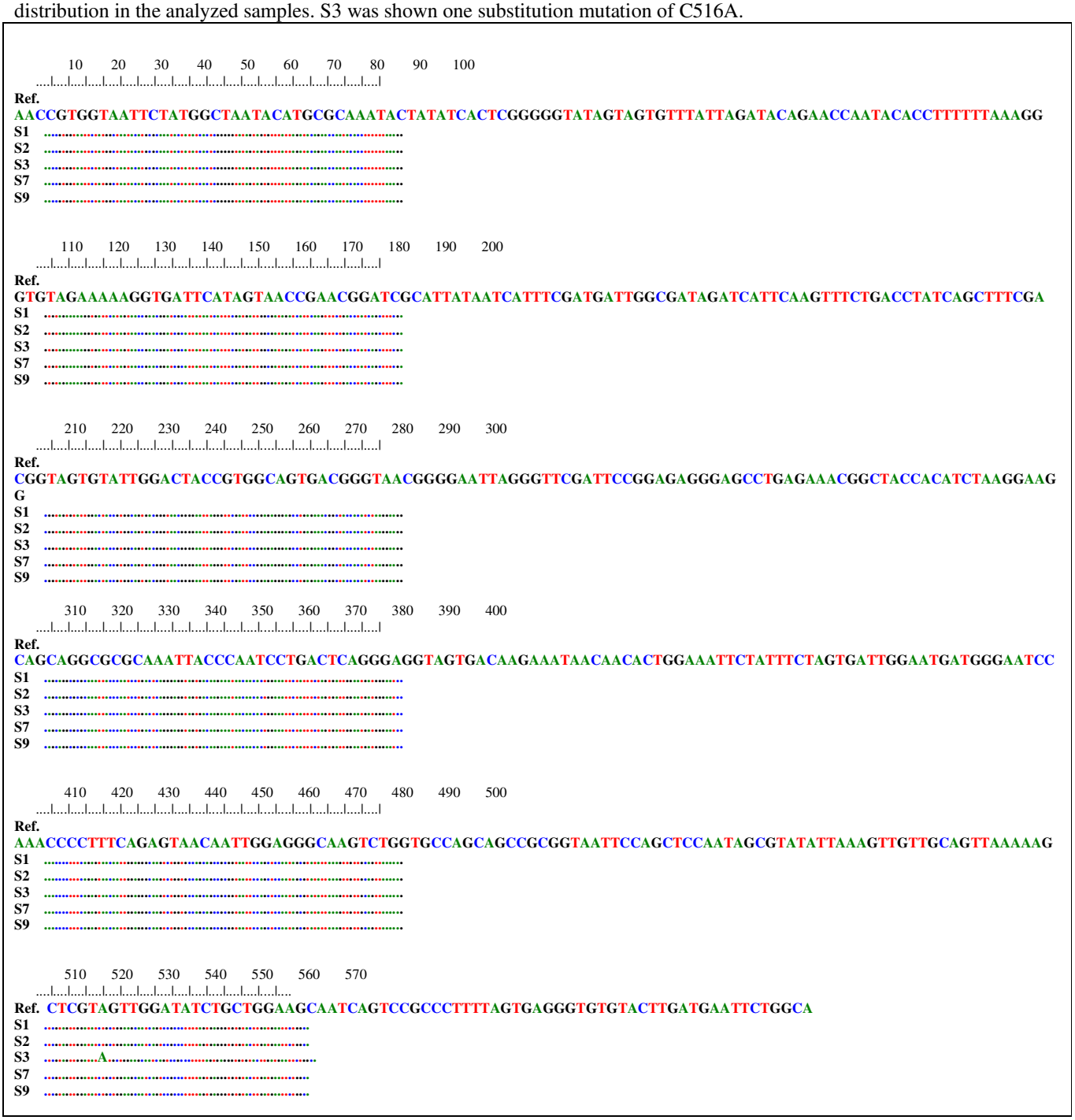

Fig. 9 : DNA sequences alignment of 5 local protozan isolate with their 574 bp amplicons of the 18S rRNA locus, corresponding reference sequences within the genomic DNA sequences of *Sarcocystis fusiformis*. The symbol "ref" refers to the NCBI reference sequences, while S1, S2, S3, S7 and S9, refer to the local isolate No. 1, 2, 3, 7 and 9 respectively.

To summarize all the get results from the sequencing of 574 bp fragments, lay of the observed variations were described in the NCBI reference sequences (Table 5).

Table 5 : The pattern of the observed SNP in the 574 bp amplicons in comparison with the NCBI referring sequences (Gen Bank access no. KR186121.1). the symbol "S" refers to the "sample" code.

Sample No.	Native	Allele	Position in the PCR fragment	Position in the reference genome	Variant summary
S3	C	A	516	665	KR186121.1;g.5116C>A

Sequencing the *18S rRNA* gene for samples S8, S10, S11, S14, S15, S16, S19, 20 and S25

NCBI BLASTn engine has indicated the presence of remarkable of homology with the expected target that covered a portion of the *18S rRNA* within *Sarcocystis levinei* genomic DNA sequences. All above isolate comparing with the retrieved DNA sequences (GenBank acc. MG957194.1), additionally all details of the retrieved PCR fragment were clarified in (Fig. 10).

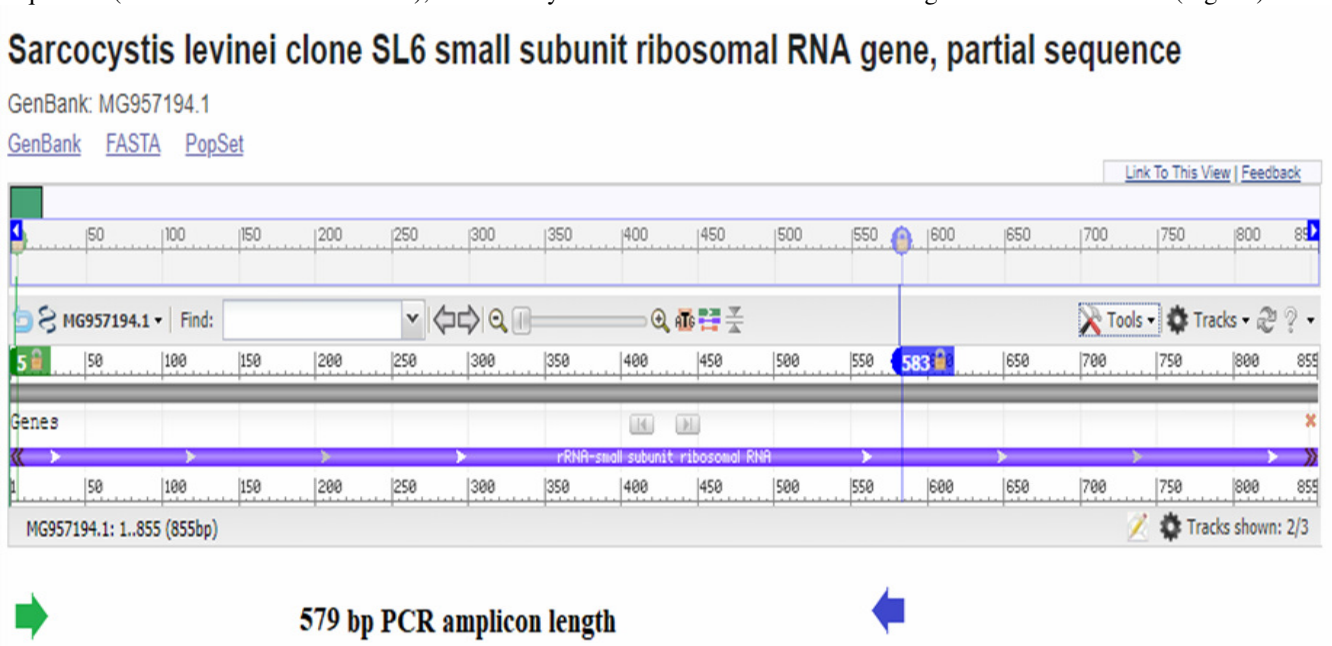

Fig. 10 : The exact position of the retrieved 579 bp amplicon that entirely covered a portion of the *18S rRNA* locus within the *Sarcocystis levinei* DNA genomic sequences (access no. MG957194.1). The green arrow refers to the starting point of this amplicon while the cyan arrow refers to its end point.

Result of alignment on 579 bp, revealed the presence of two mutations in some of the isolate when comparison with the *18S rRNA* genetic sequences referral. These 2 mutations were taken a different distribution in the analyzed isolate. **S8, S10, S11,** and **S20** were shown one substitution mutation of C78A, while **S10, S11**, and **S14** were shown one substitution mutation of C86A (Fig. 11).

10 20 30 40 50 60 70 80 90 100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Ref.
CCGTGGTAATTCTATGGCTAATACATGCGCAAATATCCTTTTTCGCAAGAAAGAGGATAGTGTTTATTAGAT ACAGAACCAATACACCATCTGTTTATCA
S8 .....C.....
S10 .....C.....C.....
S11 .....C.....C.....
S14 .....C.....
S15 .....
S16 .....
S19 .....
S20 .....C.....

S25 .....
110 120 130 140 150 160 170 180 190 200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Ref.
GGTGGTGTAAAAAAGGTGATTCATAGTAACCGAACGGATCGCATTATGGTCATTTTATATGGCTGGCGATA GATCATTCAAGTTTCTGACCTATCAGCTT
S8 .....
S10 .....
S11 .....
S14 .....
S15 .....
S16 .....
S19 .....
S20 .....
S25 .....

210 220 230 240 250 260 270 280 290 300
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ref.
TCGACGGTAGTGTATTGGACTACCGTGGCAGTGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGG GAGCCTGAGAAACGGCTACCACATCTAAGG
S8 ....
S10 ....
S11 ....
S14 ....
S15 ....
S16 ....
S19 ....
S20 ....

S25 ....
310 320 330 340 350 360 370 380 390 400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ref.
AAGGCAGCAGGCGCGCAAATTACCCAATCCTGACTCAGGGAGGTAGTGACAAGAAATAACAACACTGGAAA TTTTATTTCTAGTGATTGGAATGATGGGA
S8 ....
S10 ....
S11 ....
S14 ....
S15 ....
S16 ....
S19 ....
S20 ....

S25 ....
410 420 430 440 450 460 470 480 490 500
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ref.
ATTTAAACCCCTTTCAGAGTAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA ATAGCGTATATTAAAGTTGTTGCAGTTAA
S8 ....
S10 ....
S11 ....
S14 ....
S15 ....
S16 ....
S19 ....
S20 ....
S25 ....A..
510 520 530 540 550 560 570
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
Ref.
AAAAAGCTCGTAGTTGGATGTCTGCTGGAAGCAATCAGTCCGCCCTATTTGTAGGGTGTGCACTTGATGAA TTCTGGCA
S25 ....A..

510
....|....|..
Ref. AAAGCTCGTAGT
S8 ....
S10 ....
S11 ....
S14 ....
S15 ....
S16 ....
S19 ....
S20 ....

Fig. 11 : DNA sequences alignment of 8 local protozan isolate of the 579 bp amplicons, the DNA sequences of *Sarcocystis levinei*. The symbol "ref" refers to the NCBI reference sequences, while S8, S10, S11, S14, S15, S16, S19,20 and S25 refer to the isolate No. 8, 10, 11, 14, 15, 16, 19,20 and 25 respectively.

To summarize all the results obtained from the sequencing 579 bp fragments, at the target positions of the observed variations were described in the NCBI reference sequences (Table.6).

Table 6 : The pattern of the observed SNP in the 579 bp amplicons in comparison with the NCBI referring sequences **(Gen Bank access no. MG957194.1).** The symbol "S" refers to the "sample" code.

Sample No.	Native	Allele	Position in the PCR fragment	Position in the reference genome	Variant summary
S8, S10, S11, S20	A	C	78	84	MG957194.1;g.A>C84
S10, S11, S14	A	C	86	92	MG957194.1;g.A>C92
S25	G	A	498	502	MG957194.1;g.498G>A
S25	G	A	531	535	MG957194.1;g.531G>A

Sequencing of the *18S rRNA* gene for samples S12

NCBI BLAST n engine has indicated the presence of remarkable of homology with the expected target that covered a portion of the *18S rRNA* within ***Sarcocystis buffalonis*** genomic DNA sequences when comparing with the retrieved DNA sequences (**GenBank acc. MF595843.1**), all details were identified in (Fig. 12).

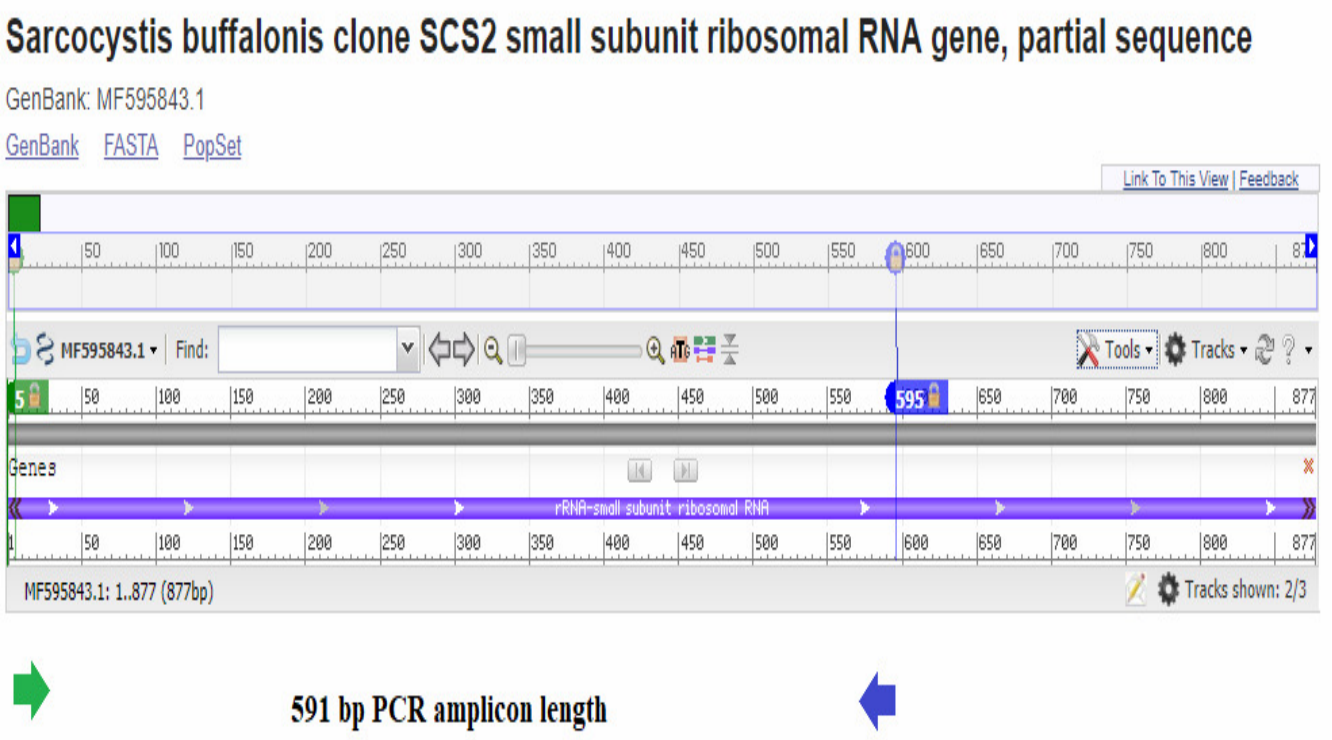

Fig. 12 : The exact position of the retrieved 591 bp amplicon that entirely covered a portion of the *18S rRNA* locus within the *Sarcocystis buffalonis* DNA genomic sequences (access no. MF595843.1). The green arrow refers to the starting point of this amplicon while the cyan arrow refers to its end point.

The alignment results of the 591 bp sample in the **S12** sample revealed the presence of two mutations in comparison with the referring *18S rRNA* genetic sequences, namely A27T and C74T (Fig.13).

10 20 30 40 50 60 70 80 90 100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Ref.
GCGCAAATACTATATTATCTCACTTTACGGTGGATGATATAGTAGTGTTTATTAGATACAGAACCAATACAC CCGTAACAAGGTGTAGAAAAAGGTGATT
S12 .....T.....T.....

110 120 130 140 150 160 170 180 190 200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

Ref.
CATAGTAACCGAACGGATCGCATATATTATAATCATTTCGGTGATTATTGGCGATAGATCATTCAAGTTTCT GACCTATCAGCTTTCGACGGTAGTGTAT
S12 .....

```
 210 220 230 240 250 260 270 280 290 300
 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ref.
TGGACTACCGTGGCAGTGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGG
CTACCACATCTAAGGAAGGCAGCAGGCGCG
S12 ..........................................................................................

 310 320 330 340 350 360 370 380 390 400
 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ref.
CAAATTACCCAATCCTGACTCAGGGAGGTAGTGACAAGAAATAACAACACTGGAAATTCAATTTCTAGTGAT
TGGAATGATGGGAATCCAAACCCCTTTC
S12 ..........................................................................................

 410 420 430 440 450 460 470 480 490 500
 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ref.
AGAGTAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAA
GTTGTTGCAGTTAAAAAGCTCGTAGTTGG
S12 ..........................................................................................

 510 520
 ....|....|....|....|...
Ref. ATATCTGCTGGAAGCAATCAGTC
S12 ........................
```

Fig. 13 : DNA sequences alignment of one imported beef isolate with its same reference sequences of the 591 bp amplicons of the *18S rRNA* position within the genomic DNA sequences of *Sarcocystis buffalonis*. The symbol "ref" refers to the NCBI reference sequences, while S12 refer to the sample No. 12.

To summarize the variant in S12 show in (Table.7).

Table 7 : The pattern of the observed SNP in the 591 bp amplicons in comparison with the NCBI referring sequences (Gen Bank access no. MF595843.1). The symbol "S" refers to the "sample" code.

Sample No.	Native	Allele	Position in the PCR fragment	Position in the reference genome	Variant summary
S12	A	T	27	59	MF595843.1;g.A>T59
S12	C	T	74	106	MF595843.1;g.C>T106

The Phylogenetic tree analysis of samples (S1,2,3,7,9,8,10,11,12,14,15,16,19 and20)

Four samples of *Sarcocystis*, including S1, S2, S3, S7 and S9, were found to be positioned within the *S. fusiformis* phylogenetic area. With regard to S1, a close positioning was found with access no AF176927.1, which belongs to a Chinese strain of *S. fusiformis*. However, S2, S3, such positioning was relatively found in the vicinity of the Indian strain SFS10 of the same species (acc. no. MF595830.1). Simultaneously, another nine samples of the investigated *Sarcocystis* were found to belong to *S. levinei*, including S8, S10, S11, S14, S15, S16, S19,S20 and S25. Considering they were clustered in the vicinity to a Norway Bb20.2 isolate (access no. KU247922.1) and to an Indian SL5 clone (access no. MG957193.1), respectively. The investigated samples was found in S12, which localized within *S. buffalonis*. However, S12 was suited in a relatively distinctive position compared with other related *S. buffalonis* strains beside a Norway strain of the same describe species (access no. KU247909.1). This is because S12 had exerted two novel substitutions of A27T and C74T (Fig. 14)

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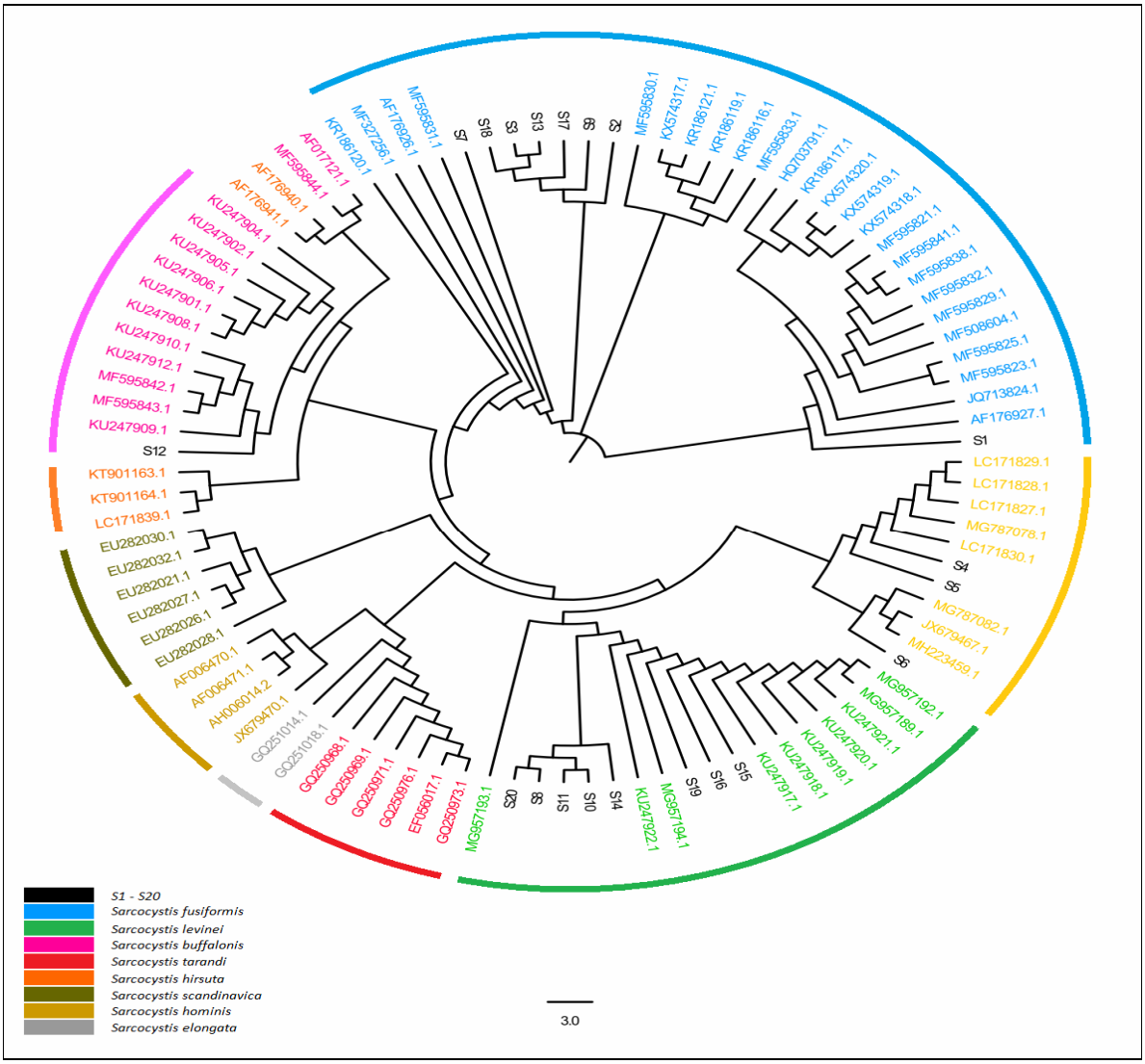

Fig. 14 : The comprehensive phylogenetic tree of genetic variants of *18S rRNA* gene fragment of *Sarcocystis* local isolate. The black color refers to the sequenced *S1 – S20* variants, while the other colors refer to the related referring NCBI *Sarcocystis* deposited species. All the mentioned numbers referred to Genbank acc. no. of each referring species. The number "3.0" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

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