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ORIGINAL ARTICLE

Detection of SYT-SSX mutant transcripts in formalin-fixed paraffin-embedded sarcoma tissues using one-step reverse transcriptase real-time PCR

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Abstract

Background: Synovial sarcoma (SS) is a rare cancer and accounts for 5-10% of adult soft tissue sarcomas. Making an accurate diagnosis is difficult due to the overlapping histological features of SS with other types of sarcomas and the non-specific immunohistochemistry profile findings. Molecular testing is thus considered necessary to confirm the diagnosis since more than 90% of SS cases carry the transcript of t(X;18)(p11.2;q11.2). The purpose of this study is to diagnose SS at molecular level by testing for t(X;18) fusion-transcript expression through One-step reverse transcriptase real-time Polymerase Chain Reaction (PCR). *Method:* Formalin-fixed paraffin-embedded tissue blocks of 23 cases of soft tissue sarcomas, which included 5 and 8 cases reported as SS as the primary diagnosis and differential diagnosis respectively, were retrieved from the Department of Pathology, Tengku Ampuan Afzan Hospital, Kuantan, Pahang. RNA was purified from the tissue block sections and then subjected to One-step reverse transcriptase real-time PCR using sequence specific hydrolysis probes for simultaneous detection of either SYT-SSX1 or SYT-SSX2 fusion transcript. *Results:* Of the 23 cases, 4 cases were found to be positive for SYT-SSX fusion transcript in which 2 were diagnosed as SS whereas in the 2 other cases, SS was the differential diagnosis. Three cases were excluded due to failure of both amplification assays SYT-SSX and control β-2-microglobulin. The remaining 16 cases were negative for the fusion transcript. *Conclusion*: This study has shown that the application of One-Step reverse transcriptase real time PCR for the detection SYT-SSX transcript is feasible as an aid in confirming the diagnosis of synovial sarcoma.

Keywords: synovial sarcoma, SYT-SSX mutant transcript, real-time PCR

INTRODUCTION

Soft tissue sarcomas (STS) are heterogenous groups of tumours of mesoderm origin and make up 1% and 15% of adult and paediatric malignancies respectively.[1] In Malaysia, STS are the seventh most common paediatric tumours with an incidence rate of 3.7 cases per million population.[2] Although rare, STS are associated with a high mortality rate.[3] Confirming the diagnosis of the various types of STS through the conventional histopathological method can be difficult for the pathologists as haematoxylin and eosin stained-sections of different types of sarcomas are known to have overlapping microscopical features. Although the use of immunohistochemical stains assists in the diagnosis, they are not without limitation when it comes to the confirmation of diagnosis. About 25% to 40% of STS cases have not been diagnosed correctly by histology and immunohistochemical results alone.[4] Fortunately advances have been made in the field of genetics that allow for identification of distinctive molecular markers in certain types of STS and hence improvement in the accuracy of diagnosis.[5]

Synovial sarcoma (SS) is one of the STS

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that has been identified to carry characteristic genetic changes and comprises 5-10% of all STS.[6,7] It is a rare malignant tumour and occurs predominantly in young adults. It can arise from any part of the appendicular skeleton,[8] and typically presents as a mass lesion usually on the extremities. SS constitutes two major histological subtypes, the monophasic and the biphasic variants. The monophasic variant is composed entirely of spindle-shaped cells while biphasic SS has both the spindle cell and epithelial component with glandular differentiation.[9]

Cytogenetically, SS is characterised by the specific chromosomal translocation t(X;18) (p11.2;q11.2) which is found in more than 90% of the cases.[10,11] The translocation fuses the SYT [SS18 (MIM 600192)] gene from chromosome 18 to one of the two most common homologous genes, SSX1 or SSX2 on the X chromosome resulting in the production of SYT-SSX fusion transcript.[12] As this translocation is unique to SS, its detection provides a definitive diagnosis, even in cases with unusual clinical or histological features.[13] In addition to SYT/SSX1 and SYT/SSX2 fusion genes which are identified in the majority of SS cases, there are a few case reports of SYT/SSX4[14] and SYT/SSX4v[15] fusion genes. In about 3% of cases, however, despite exhibiting the characteristic histomorphology features of SS, SYT/SSX fusions are not detected.[16] Cytogenetics, interphase fluorescence *in-situ* hybridization, conventional reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR have all been used to diagnose SS.[17] Hence SYT-SSX fusion gene is considered pathognomonic of SS. Also targeted therapies are being explored based on the possible mechanism of actions of the SYT-SSX fusion protein in relation to tumour growth.[18] There are, however, controversies surrounding the role of SYT-SSX as a prognostic marker of SS.[19]

In Malaysia publications related to molecular analysis of SS are few in number. Most such publications are limited to isolated case reports. Thus the purpose of this study is to identify the mRNA fusion of the t(X;18) (p11.2;q11.2) using a real-time PCR with sequence specific probe method in cases diagnosed as SS by the conventional histopathological techniques.

METHODS

Selections of cases

All records of cases of sarcomas diagnosed in the Department of Pathology, Tengku Ampuan Afzan Hospital, Kuantan, Pahang from January, 2005 to January 2012 were reviewed. Formalin-fixed paraffin-embedded (FFPE) tissue blocks of cases where SS was the final diagnosis or SS was one of the differential diagnoses were all retrieved. Cases diagnosed as other types of STS but shared the clinical features of SS, namely tumour location in the extremities or morphologically exhibiting both epithelial and spindle cell components were also included in the study.

Sample preparation and RNA purification

Four 5-µm thick sections were cut from the tissue blocks and placed in a 2 mL microcentrifuge tube. The tubes were kept at 4°C prior to RNA purification. RNA was subsequently extracted from the tissue sections using the QIAGEN® RNeasy FFPE purification kit. The purity and concentration of the RNA was determined using BioPhotometer Plus ™ (Eppendorf, USA) based on the ratio of absorbance at 260 ηm and 280 ηm.

Determination of positive and negative controls

Uterine leiomyoma tissue was used as negative control. For the positive control, 'N1' a tumour tissue diagnosed as poorly differentiated SS based on the conventional method was selected. The immunohistochemical profile indicated positivity with vimentin, cytokeratin, S-100 and myoglobin. Both positive and negative control samples were subjected to similar method of RNA purification as described above. Conventional RT-PCR as described in our previous study was carried on the control samples.[20] Direct nucleotide sequencing was subsequently performed on the positive control. The procedure involved purification of PCR products after it has been separated by 1.5% agarose gel electrophoresis. The purified product was cycle sequenced with ABI BigDye Terminator Kit (Applied Biosystem, US) with the forward primer, purified with ethanol precipitation technique and sequenced using the ABI3130 Genetic Sequencer (Applied Biosystem, US).

One-step reverse transcriptase real time-PCR detection of SYT-SSX using sequence-specific probes

Primer and probe sequences for the SYT-SSX and β-2-microglobulin(β-2M) was chosen based on the study by Bijwaard *et al.*[21] The details of the probes and primers used are as shown in Table 1. The assay utilized multiplex real-time hydrolysis probes for detection of either

TABLE 1: Primers and probes sequence used for SYT-SSX detection

Target	Primer Names	Sequence (5'-3')
t(X;18)/*SYT-SSX*	*SYT*	AGA GGC CTT ATG GAT ATG ACCAGA
	SSX	C(A/G)T TTT GTGGGCCAG ATGCC
	SSX1 probe	FAM- TCCCTT CGA ATCATT TTCGTCCTC TGC T -TAMRA
	SSX2 probe	VIC- TCT GGCACT TCCTCCGAATCA TTTCCT T -TAMRA
β2M	β2M-246F	TGACTT TGTCACAGCCCAAGATA
	β2M-330R	AATCCAAATGCGGCATCTTC
	β2M-275R	VIC-TGATGCTGC TTACATGTCTCGATCCCA -TAMRA

SYT-SSX1 or SYT-SSX2 transcripts whilst β-2M served as control. The whole procedure of One-step reverse transcriptase real-time PCR was carried out using the Qiagen's QuantiTect™ probe Real-time-PCR kit. The Real-Time-PCR reaction consists of purified RNA, 2x Quantifast probes Real-time PCR master mix, primers solution; probe solution and RNase-free water. The process of cDNA synthesis is incorporated into the PCR kit. The mixture of QuantiTect Probe Real-time-PCR Master Mix together with QuantiTect RT mix allowed for both reverse transcription and PCR to take place in a single tube. For each of the samples, the assay was repeated at least once and yielded similar results. The real-time assay was performed on Bio-Rad CFX-96 real-time system, and analysed using the endpoint analysis of CFX96 Manager Software, Bio-Rad, US. The software also determined the cut of value of Relative Fluorescence Unit (RFU). The Receiver Operating Characteristic (ROC) values were analysed using the MedCalc statistical software Version 16.1 (https://www.medcalc.org). The RFU values of the end point analyses were used as the measurement of interest for the synovial sarcoma (as primary or differential diagnosis) and the control (other soft tissue sarcomas) groups.

RESULTS

Demographic and histopathological features

In all there were 127 cases of sarcomas during the study period. 23 cases were included in the study. In 5 of the 23 cases SS was considered as the primary diagnosis with the patients' mean age being 27.2 years (SD 12.6). In 8 cases SS was a differential diagnosis. The remaining 10 cases were included based on tumour location (in the extremities) and morphology (histologically, they exhibited both epithelial and spindle cell components). The demographic and histopathological data of the cases are as shown in Table 2.

Positive control result

The positive control (N1) was confirmed by sequencing (Figure 1) to carry SYT-SSX1 fusion transcript upon BLAST alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with Homo sapiens Synovial Sarcoma X Breakpoint 1(SSX1) transcript [NCBI Reference Sequence: NG_012528.1]. The nucleotide sequences were also aligned with Homo sapiens chromosome 18, GRCh37.p5 Primary Assembly [NCBI Reference Sequence: NC_000018.9] for the SYT region.

Detection of SYT-SSX Fusion Transcripts

Of the 23 selected cases that were tested for SYT-SSX fusion transcripts, 3 cases namely, N5, N6 and N20 were excluded from analysis as the samples showed repeated negative amplification plot for both SYT-SSX and control β-2M. The above failure of PCR amplification was most probably due to impurity or insufficiency of the starting RNA materials. N5, a case of SS and N6 diagnosed as sarcoma were both biopsy samples of less than 10mm tissue obtained in 2012 and 2008 respectively whilst N20 a sample obtained in 2010 was diagnosed as poorly differentiated sarcoma and exhibited significant areas of necrosis. The ratio of 260 ηm and 280 ηm absorbances for the three samples were only about 1.00 with total RNA concentration of only slightly above 30 ng/μL.

Four cases, N1 (which was also the positive control), N2, N8 and N13 showed positive amplification for the SYT-SSX. The amplification plots for SYT-SSX and control β-2M are as shown in Figure 2(A) and Figure 2(B) respectively. N1 and N2 cases were histologically diagnosed as poorly-differentiated SS and biphasic SS respectively while N8 and

TABLE 2: Demographic and histopathological profile of the selected cases

Sample	Age	Gender	Tumour Site	Immunohistochemistry profile (Positive)	Primary Diagnosis
Synovial Sarcoma					
*N1	17	F	Right iliac fossa	Vimentin, CK, S-100, Myoglobin	Synovial sarcoma
N2	26	F	Right thigh	Vimentin, CK 7, EMA, S100	Biphasic synovial sarcoma
N3	22	M	Left frontal sinus	Vimentin, S100, CD 99, BCL 2	Synovial sarcoma
N4	22	M	Left maxillary sinus	Vimentin, SMA, CD99, S100 NSE	Synovial sarcoma
N5	49	M	Right anterior chest	CK AE1/AE3, EMA, Vimentin, CK7, p63, S100	Synovial sarcoma
Synovial Sarcoma as a differential diagnosis					
N6	38	M	Psoas	CD 117	Sarcoma
N7	37	F	Left abdominopelvic	CK-MNF 116, CK AE1/AE3, Vimentin, CD 99, EMA	Malignant soft tissue sarcoma.
N8	21	F	Middle lung lobe	Vimentin, SMA, EMA. CK7	Spindle cell tumour
N9	47	M	Right forearm	Vimentin, CK AE1/AE3, EMA, CD 99, CD 34	Epitheloid sarcoma
N10	59	M	Proximal left thigh	Vimentin, EMA, CD 99, SMA,CD 34, S100, NSE	Spindle cell sarcoma
N11	33	M	Left forearm	Vimentin, CD 99, BCL 2	Spindle cell sarcoma
N12	34	F	Right thigh	Vimentin, BCL 2	Malignant mesenchymal tumour
N13	34	F	Proximal right thigh	Vimentin, S100	Spindle cell mesenchymal tumour.
Other soft tissue sarcomas					
N14	17	M	Right leg	Not Done	High grade sarcoma
N15	72	M	Right chest wall	Vimentin, SMA	High grade sarcoma
N16	53	m	Right thigh	Not Done	Benign fibrous tumour
N17	53	F	Left gluteal	CD34	Dermatofibrosarcoma protuberance
N18	35	M	Anterior abdominal wall	Vimentin, Desmin, CK	Epitheloid sarcoma
N19	15	F	Left wrist	Vimentin, CD 34	Dermatofibrosarcoma protuberance
N20	51	M	Pelvic	Vimentin, CK AE1/AE3	Poorly differentiated sarcoma
N21	25	F	Right shoulder	Vimentin, P53, NSE, SMA	High grade spindle cell sarcoma
N22	59	M	Left thigh	Vimentin, EMA, SMA, CD99, CD68	Malignant fibrous histiocytoma
N23	54	F	Left gluteal	Not done	Myxoid liposarcoma

**N1 used as a positive control. EMA: epithelial membrane antigen. CK: Cytokeratin SMA: Smooth Muscle Actin NSE: Neuron Specific Enolase.*

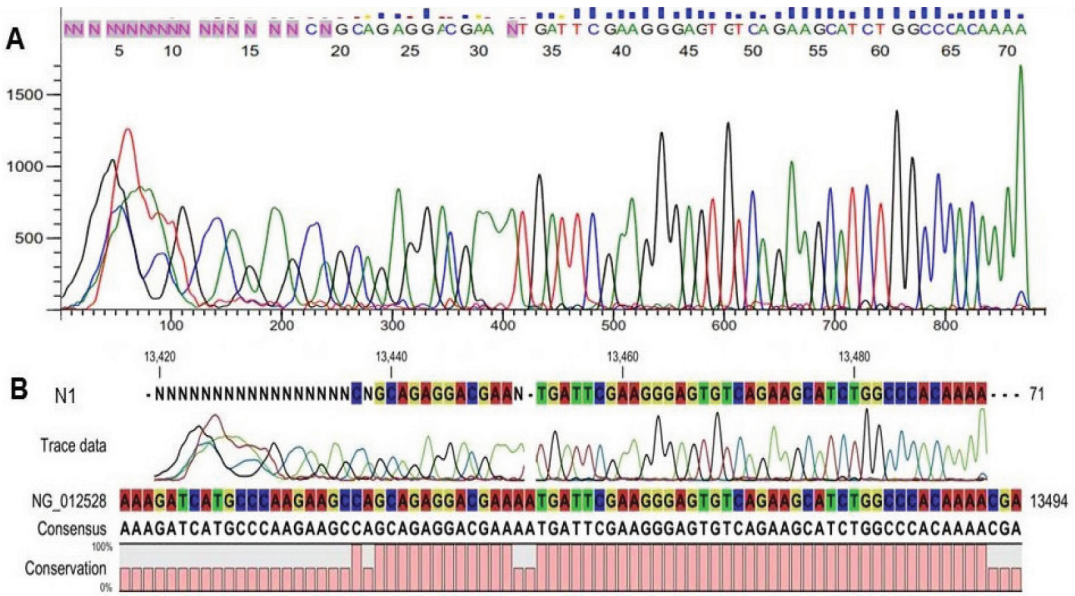

FIG. 1: **(A)** Direct sequencing result of SYT-SSX amplification products for sample N1. **(B)** The sequence is in alignment with Homo sapiens Synovial Sarcoma, X breakpoint 1 (SSX1), (NCBI Reference Sequence: NG_012528.1).

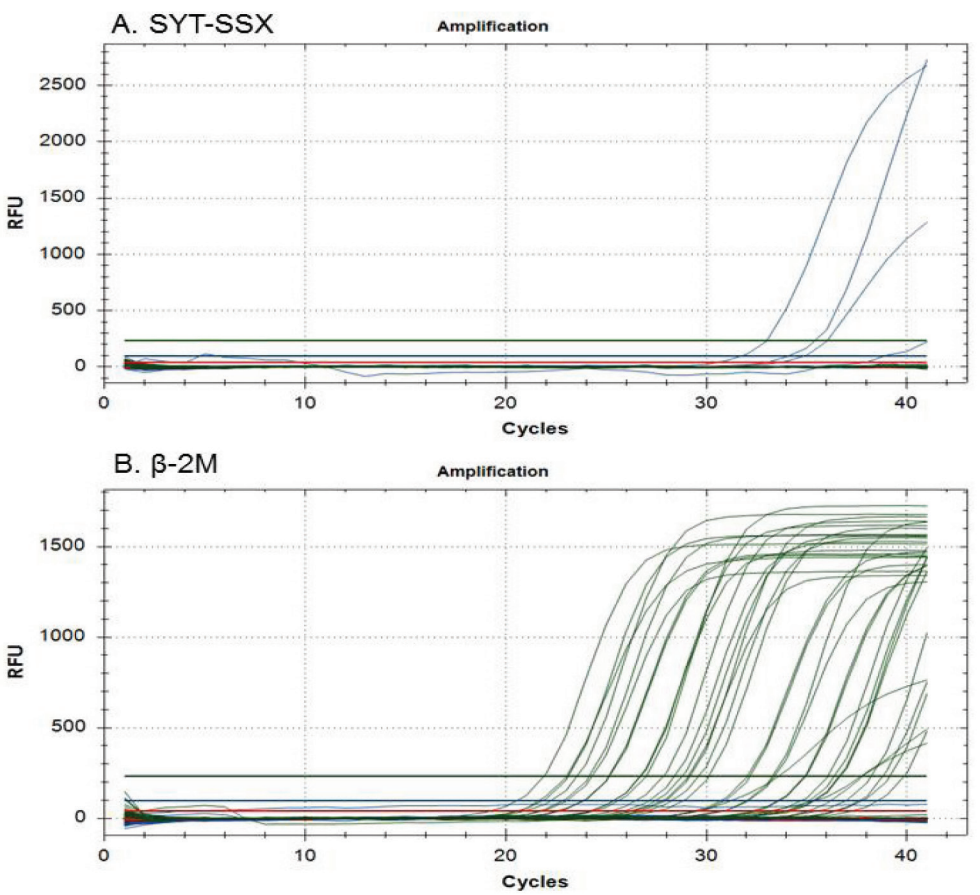

FIG. 2: Amplification plots for A: SYT-SSX. B: β-2 Microglobulin.

N13 were diagnosed as spindle cell tumour with SS as the differential diagnosis. None of the additional 10 cases of STS selected revealed SYT-SSX fusion transcripts. Overall, of the 5 cases with primary diagnosis of SS (N1 – N5), 1 was excluded due to amplification failure (N5), 2 (N1 and N2) of the remaining 4 cases (50%) were positive for the fusion transcript.

Receiver Operating Characteristic (ROC) values
The lowest and highest RFU values of the SYT-SSX assay were -5.73 and 2328 respectively. The cut off value was 229. ROC curve analysis found that the specificity and sensitivity of the assay were both 100% for a criterion fixed at >0.557 with area under the ROC curve = 1.000 (Figure 3).

DISCUSSION

Of the STS studied, 23 cases were selected for molecular analysis for SYT-SSX1 and SYT-SSX2 fusion transcripts. In these cases, SS was either the primary diagnosis or the differential diagnosis in 13 cases. Although SS has been reported to be commonly found in the extremities, only about 50% of the 13 cases presented with tumour in the extremities and only 1 case exhibited biphasic variant morphologically.[13,22] The immunohistochemistry profiles exhibited by the 13 cases were variable as also noted by other researchers[17,23,24] who showed that immunohistochemistry profile is not a reliable marker in distinguishing SS from other types of sarcoma.

In essence, SS is difficult to diagnose accurately by histology and immunohistochemical results alone, even by the most experienced sarcoma pathologists. Diagnosis based on histomorphology may lead to a wrong diagnosis due to the overlapping morphology with different types of sarcoma and also between carcinoma and sarcoma.[25] Small biopsies of tumours arising outside the usual age-range and anatomical locations are often even more problematic.[17]

Hence, since almost all cases of SS (>90%) contain the characteristic translocation t(X;18) (p11.2;q11.2), molecular testing is considered helpful or even necessary. This is particularly so when the diagnosis of SS is only possible with the need to consider other differential diagnoses such as other spindle sarcomas, round cell sarcomas, carcinomas, myoepitheliomas and epithelioid fibrosarcomas.[26] If however the clinical presentations, morphology and immunohistochemistry profile findings are consistent with SS, molecular testing is generally

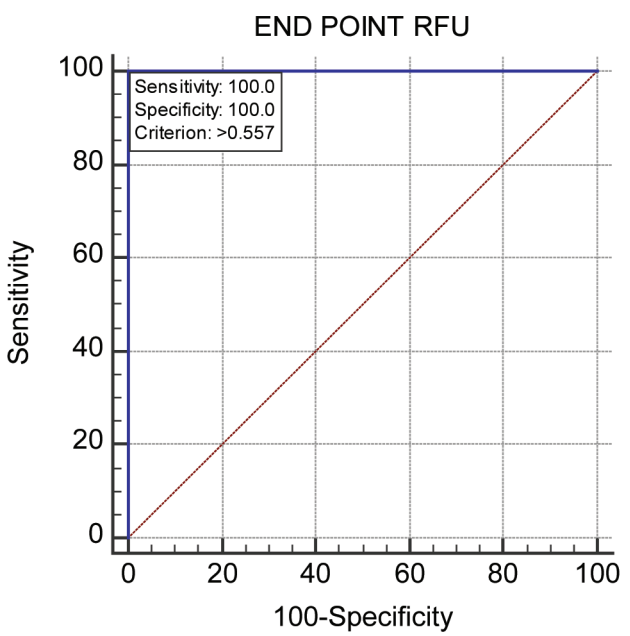

FIG.3: ROC curve analysis on 23 samples [13 positive group (56.5%) and 10 Negative group (43.5%)]. Confidence interval (CI) is set at 95%. Area under the ROC curve = 1 [CI (0.852-1.000)]; p<0.0001. Criterion >0.557; Sensitivity 100% [CI75.3-100)], Specificity 100% [CI(69.2-100)].

considered non-contributory or necessary.[26] Our finding indicated that 50% of the cases (2 of 4) with primary diagnosis of SS analysed for SYT-SSX fusion transcripts were positive for the transcript. This result is comparatively lower than other published studies, which range from 75% to > 90%.[10,11,22] The additional 2 cases that were positive for the fusion transcript only had SS as a differntial diagnosis. These findings further reiterate the fact that synovial sarcoma is diagnostically challenging histologically in addition to the non-availability of specific immunohistochemical markers.

In this study we utilized real time PCR analysis for the detection of t(X;18) fusion transcripts SYT-SSX1 and SYT-SSX2. As opposed to the conventional cytogenetic testing, in addition to utilizing small tissue sample, this technique does not require fresh tissue since RNA can be extracted from FFPE tissue. It is however well documented that the integrity and yield of total RNA purified from FFPE tissue are often compromised. The application of real time detection is plausible since fluorescent detection at the exponential phase of the PCR amplification would require only very minute amount of starting cDNA. In addition, it is quick and simple[21] with rapid turnaround time and it is relatively cheaper, making it suitable to be used in a diagnostic laboratory. The test is further shortened and simplified with the integration of cDNA synthesis steps during the amplification processes (One-step reverse transcriptase real-time PCR). The primers and probes utilized in this study allow for detection of either SYT-SSX1 or SYT-SSX2 transcripts,[21] the two most common transcripts of SS.[27] Our study has also shown that the detection of SYT-SSX fusion transcript in SS via One-step reverse transcriptase real-time-PCR from FFPE tissues is comparatively less laborious than the conventional RT-PCR with flexible annealing temperature. Kinetic real time PCR technique is known to be highly sensitive and reliable.[28,29] The test is capable of discriminating SS from other types of sarcoma.[6,24,30]

In conclusion, molecular testing for the detection of SYT-SSX fusion transcript through One-step reverse transcriptase real-time-PCR technique employed greatly assists in confirming the diagnosis of SS.

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