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RESEARCH ARTICLE

Molecular diagnostics insights of little leaf and witches' broom disease of sunnhemp in Tamil Nadu

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Abstract

Little leaf and witches' broom disease associated with phytoplasma infection has recently been reported in Tamil Nadu, across all growing seasons. A survey was conducted to assess the incidence of sunnhemp little leaf and witches' broom disease in various sunnhemp growing regions of Tamil Nadu during 2022-2024, across all seasons. The highest incidence of little leaf and witches broom disease was observed during the *Kharif* 2023 in Pudukkottai district with an infection rate of 0.55 % to 88.67 %. Infected plants exhibited characteristic symptoms including phyllody (leaf-like structures replacing flowers), virescence (green pigmentation in floral parts), yellowing, reduced leaf size, stunted growth, witches' broom (proliferation of shoots), leaf curling, bunchy appearance at stem ends, floral gigantism, twig die-back and unseasonal yellowing or reddening of leaves. The disease is caused by *Candidatus* phytoplasma and is transmitted by leafhoppers. However, the phytoplasma cannot be cultured under *in vitro* conditions. Therefore, in this study, nested PCR approach was employed as a rapid and reliable molecular diagnostic technique for detecting phytoplasma. Specific second-round primers were used to confirm the presence of phytoplasma in little leaf and witches' broom infected samples. The infected plant material was analyzed using a 16S rRNA region-specific primer to identify the pathogen. Sequence analysis revealed that the phytoplasma infecting sunnhemp had over 99 % nucleotide similarity with other isolates of *Candidatus: Phytoplasma aurantifolia* from the NCBI database, confirming the association of *Candidatus: Phytoplasma aurantifolia* (16SrII group) with sunnhemp little leaf and witches' broom disease.

Keywords: 16S rRNA gene; little leaf; nested PCR; phytoplasma; sunnhemp; witches' broom

Introduction

Sunnhemp (*Crotalaria juncea* L.) is a versatile annual legume widely cultivated for various purposes. It is considered as the oldest bast crop grown in tropical regions for its fiber (1). The plant was introduced into Europe in 1791 where it has since been cultivated as an alternative green manure crop (2). Studies have shown that *Crotalaria* can produce ample dry matter which helps protect the soil from erosion while also supplying nitrogen that benefits subsequent crops in diversified rotations (3). *C. juncea* is native to India and has been cultivated there since ancient times. Sunnhemp is cultivated in nearly all Indian states; however, the leading producers include Uttar Pradesh, Madhya Pradesh, Odisha, Bihar, Maharashtra, Rajasthan, Jharkhand, Punjab, Haryana and West Bengal. These states are prominent in the production of both fiber and green manure. It was first mentioned in Sanskrit literature around 400 BCE (4). Commonly referred to as brown hemp, Indian hemp, Madras hemp or sunnhemp (5), *C. juncea* is a tropical Asian plant in the legume family (Fabaceae). It is generally considered to have originated in India. In India, the crop is known by various regional names, including "*sonai*" or "*san*" (Hindi), "*sanpat*" (Bengali and Oriya), "*tag*" (Marathi), "*janumu*" (Telugu), "*sanabu*" (Kannada), "*sanappai*" (Tamil Nadu) and "*vakku*" (Malayalam).

Many pathogens including fungi, bacteria, viruses, phytoplasma and nematodes have been found to pass on a disease to sunnhemp. Among these, phytoplasmas are wall-less prokaryotes that do something as obligate parasites, multiplying in isotonic niches within plant phloem tissues and insect hemolymph. They are little and in the middle of the simplest self-replicating organisms. Phytoplasmas are connected with over 600 diverse plant diseases worldwide, primarily transmitted by phloem-feeding insects, particularly leafhoppers and planthoppers and psyllids. They are known to infect more than 700 plant species belonging to different families worldwide, displaying a wide array of symptoms in the infected plants (6, 7). For several decades, the lack of effective methods to identify and characterize phytoplasmas made it impossible to determine whether the same bacterium was responsible for disease exhibiting similar symptoms across different host plants and locations. Phytoplasma infections in plants trigger various symptoms including witches' broom, phyllody, yellowing, virescence (greenish-white discoloration), little leaf, stunted growth, flower sterility and purple tops (8).

Molecular diagnostics is essential for the reliable detection of phytoplasmas due to their high sensitivity. PCR-based amplification of the 16S rRNA gene has significantly advanced the documentation and description of previously unknown phytoplasmas (9). This method is more sensitive than microscopic, serological and hybridization assays. Therefore, investigations were conducted to detect a potential association of phytoplasmas causing little leaf and witches' broom diseases in sunnhemp.

Materials and Methods

Survey

A roving survey was conducted in sunnhemp-growing districts of Tamil Nadu during the 2022-2023 *Kharif* (June to September), *Rabi* (October to January) and Summer (February to May) and 2024 Summer (February to May), *Kharif* (June to September) seasons to assess the incidence of little leaf and witches' broom disease. During these periods, a vegetative disorder was observed on sunnhemp (*C. juncea*) in the surveyed areas. The disease incidence was determined using the following formula:

Disease Incidence (%) = (Number of symptomatic plants / Total number of plants observed) × 100

Healthy and infected leaf samples from sunnhemp were collected from affected locations and used for DNA extraction. The modified CTAB method was employed to extract total DNA from both healthy and infected leaf samples for phytoplasma detection (10). Additionally, a well-documented case of phytoplasma infection causing little leaf disease in brinjal in India was used as a positive control in the PCR study.

The infected sample (0.4 g) was incubated for 10 min in 2 mL of phytoplasma grinding buffer (PGB: 100 mM K_2HPO_4, 30 mM KH_2PO_4, 10 % sucrose, 0.15 % bovine serum albumin fraction V, 2 % polyvinylpyrrolidone, 25 mM ascorbic acid) in a mortar kept on ice. The sample was then finely crushed using a pestle & mortar and an additional 1.25 mL of PGB was added. The homogenate was centrifuged at 2500 rpm for 5 min. The resulting supernatant was transferred to clean tubes and centrifuged again at 15000 rpm for 25 min. The pellet obtained was dissolved in 1 mL of CTAB buffer (2 % CTAB, 100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA). After 1 hr incubation at 65 °C, nucleic acids were purified using a chloroform-isoamyl alcohol (24:1) extraction. Ice-cold isopropanol, in an equal volume was added to the aqueous phase and the mixture was incubated on ice for 1 hr. After centrifuging at 10000 rpm for 10 min, the pellet was dissolved in 400 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Subsequently, 40 µL of 3 M sodium acetate and 0.9 mL of 95 % ethanol were added. The mixture was incubated at -20 °C for 45 min and centrifuged at 10000 rpm for 10 min. The supernatant was discarded and the tubes were washed with 80 % ethanol. Finally, the pellet containing DNA was dissolved in 35 µL of sterile water and the extracted DNA was analyzed using 0.8 % agarose gel electrophoresis.

The nested PCR approach was utilized to detect phytoplasma in sunnhemp infected plant samples (11). Two pairs of primers, p1 and p7 (12) and R16f2n and R16R2 (13) were employed to specifically amplify the 16S rRNA gene of phytoplasma (Table 1).

PCR reactions were performed with a uniform concentration of ingredients in a total volume of 20 µL, prepared in 0.2 mL PCR tubes. Each reaction mixture contained 25 ng/µL DNA, 2.5 mM dNTPs, 0.5 µM of each forward and reverse primer, 10 × assay buffer, 3 units/µL Taq polymerase, 1.5 mM magnesium chloride and sterile distilled water. The PCR assays were conducted using a thermal cycler (Eppendorf Gradient Master Cycler, Germany).

Table 1. Primer sequences used in this study

Primer name	Sequences	Nested PCR
p1	AAGAGTTTGATCCTGGCTCAGGATT	First round primer
p7	CGTCCTTCATCGGCTCTT	
R16f2n	GAAACGACTGCTAAGACTGG	Second round primer
R16R2	TGACGGGCGGTGTGTACAAACCCCG	

The PCR conditions for the first-round amplification with the primer pair p1/p7 involved an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. The reaction concluded with a final extension at 72 °C for 10 min. The first-round PCR product was then used as a template for the second-round PCR. The second-round amplification performed with the primer pair R16f2n and R16R2 began with an initial denaturation at 92 °C for 75 sec followed by 35 cycles of denaturation at 92 °C for 30 sec, annealing at 62 °C for 30 sec and extension at 72 °C for 60 sec with a final extension at 72 °C for 10 min.

An aliquot of 20 µL of the PCR products was analyzed by horizontal electrophoresis using a 1.5 % agarose gel containing ethidium bromide (0.5 µg/mL). The electrophoresis was carried out in TAE buffer consisting of Tris, EDTA, glacial acetic acid and water. Prior to loading, the sample was mixed with 2 µL of gel loading buffer (0.25 % bromophenol blue and 40 % sucrose in water) and carefully loaded into the gel slots. The gel was visualized under a UV trans-illuminator and photographed using a gel documentation system (Alpha Innotech Corporation, California, USA).

The nested PCR-amplified product from the phytoplasma-infected sunnhemp sample was cloned. The cloned product was sequenced using the Sanger sequencing method. The sequence was then deposited in GenBank and aligned with 16S rRNA sequences of other *Candidatus Phytoplasma* species using BLASTN (14). Multiple sequence alignment was performed using Clustal W (15), with consistent 16S rRNA sequences derived from phytoplasma -associated diseases or various groups of phytoplasma strains (Table 2). A phylogenetic tree was constructed using the neighbor-joining method (16) based on the distance matrix, generated using NGPhylogeny.fr and was visualized with PRESTO, (Phylogenetic tReE visualization) (17).

Results and Discussion

Throughout the cropping seasons from 2022 to 2023 and during the Summer and *Kharif* seasons of 2024, the incidence of little leaf and witches' broom disease in sunnhemp ranged from 0.55 % to 18.89 %. In 2022, the highest incidence was recorded in the *Kharif* season at 4.58 % while the lowest was 0.55 % in the *Rabi* season. In 2023, the incidence peaked at 18.89 % during *Kharif* and dropped to 1.56 % in *Rabi*. For 2024, the highest incidence was 5.78 % in *Kharif* with the lowest at 2.16 % in Summer. Across all three years, the maximum disease incidence was consistently observed during the *Kharif* season (Fig. 1). During the surveyed areas, the following typical symptoms including phyllody (leaf-like structures replacing flowers), virescence (green pigmentation in floral parts) (18), yellowing, reduced leaf size, stunted growth, witches' broom (proliferation of

Table 2. Name, group and GenBank accession numbers of phytoplasma 16S rRNA sequences used in this study

Phytoplasma associated disease/strains	Designation	Phytoplasma group	GenBank accession number
Sunnhemp phytoplasma	SP	This study	PQ623358
Litle leaf disease and witches' broom of sunnhemp	LL &WB S	16S rRNA-I	OL891522
Withania somnifera phytoplasma	WSP	16S rRNA-I	DQ151998
Tomato big bud	TBB	16Sr I	L33760
Lethal yellowing phytoplasma	LY	16S rRNA-IV	EF186822
Peanut witches' broom	WB	16S rRNA-II	L33765
Western X disease	WX	16S rRNA-III	L04682
Coconut lethal yellowing	CLY	16S rRNA-IV	U18747
Elm yellows	EY	16S rRNA-V	AF189214
Brinjal little leaf disease	BLL	16S rRNA-VI	X83431
Ash yellows	AY	16S rRNA-VII	AF189215
Loofah witches' broom	LWB	16S rRNA-III	AF248956
Pigeon pea witches' broom	PPWB	16S rRNA-IX	AF248957
Apple proliferation	AP	16S rRNA-X	AF248958
Rice yellow dwarf	RYD	16S rRNA-XI	D12581
Australian grapevine yellows	AGY	16S rRNA-XII	L76865
Mexican periwinkle virescence phytoplasma	MPVP	16S rRNA-XIII	AF248960
Bermuda grass white leaf phytoplasma	BGWL	16S rRNA-XIV	AJ550984
Hibiscus witches' broom phytoplasma	HWB	16S rRNA-XVI	AF147708
Candidatus Phytoplasma caricae	PC	16S rRNA-XVII	AY725234
Acholeplasma laidlawii	AL	16S rRNA	NR074448

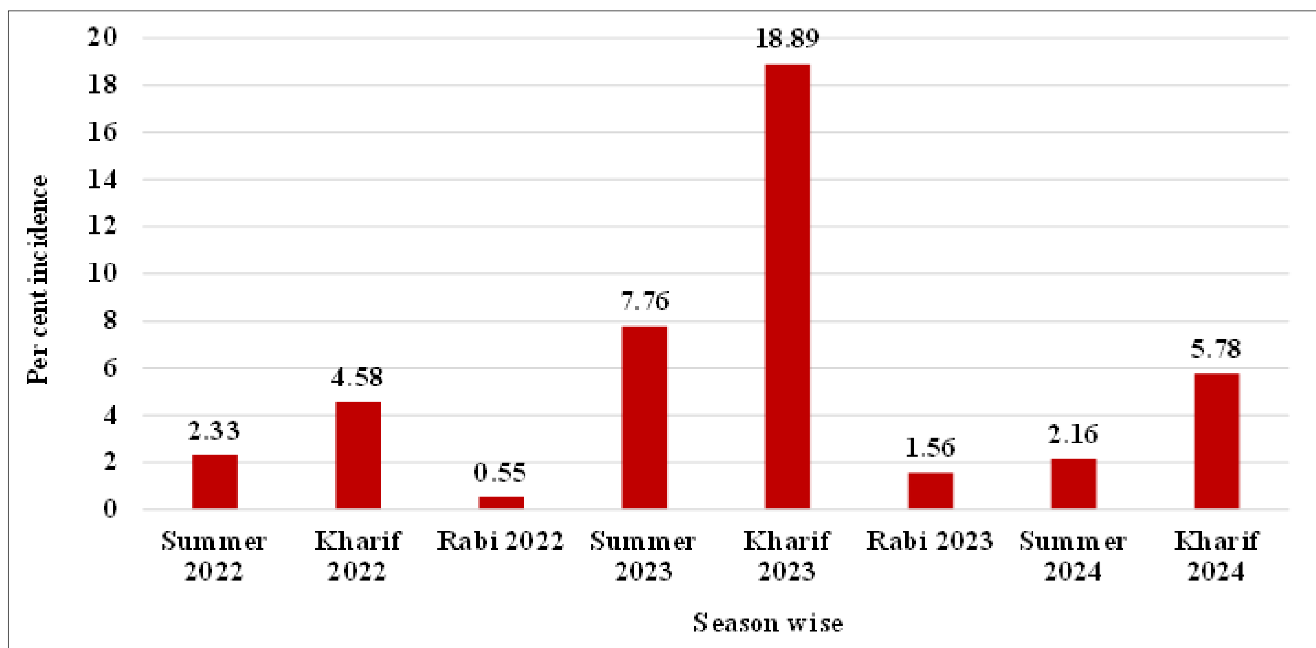

Fig. 1. Season wise incidence of sunnhemp little leaf and witches' broom disease in Tamil Nadu.

Kharif (June to September), *Rabi* (October to January), Summer *(*February to May).

shoots) (19), leaf curling, reduction of the internode length, a bunchy appearance at stem ends, floral gigantism, twig die-back and unseasonal yellowing or reddening of leaves general stunting, yellowing and proliferation of shoots (Fig. 2) was observed on sunnhemp in all sunnhemp growing districts of Tamil Nadu, India. Normally the above-mentioned symptoms were appeared on at the time of flowering to pod formation stage.

DNA extracted from malformed bud tips was used as a template for PCR. In the first round of PCR, the primers p1/p7 failed to produce any noticeable amplified DNA fragments from both infected and healthy sunnhemp tissues. This may have been due to the DNA concentration being below the detection limit in the ethidium bromide-stained agarose gel. When the first-round PCR products were re-amplified using nested PCR with primers R16f2n/R16R2, a 1.2 kb DNA fragment was obtained from both the diseased sunnhemp samples and a known phytoplasma-positive sample (brinjal little leaf) (20), but not from asymptomatic plants (Fig. 3). The amplified PCR products from the phytoplasma-infected sunnhemp leaves were cloned into the T/A vector of pGEMT® and transformed into *Escherichia coli* strain DH5α. The nucleotide sequence of the clones was then determined. The sequenced region, consisting of 1186 bases was compared with the corresponding regions of phytoplasma isolates from different hosts and regions. The sequence was deposited in GenBank and its accession number is listed in Table 2. Comparative sequence analysis showed that the sunnhemp phytoplasma shared the highest sequence identity (99.47 %) with a phytoplasma isolate belonging to the *Candidatus: Phytoplasma aurantifolia* group (16SrII).

Fig. 2. Little leaf and witches' broom symptoms in sunnhemp (*Crotalaria juncea*). (a, b, c) Infected plants; (d) healthy plant.

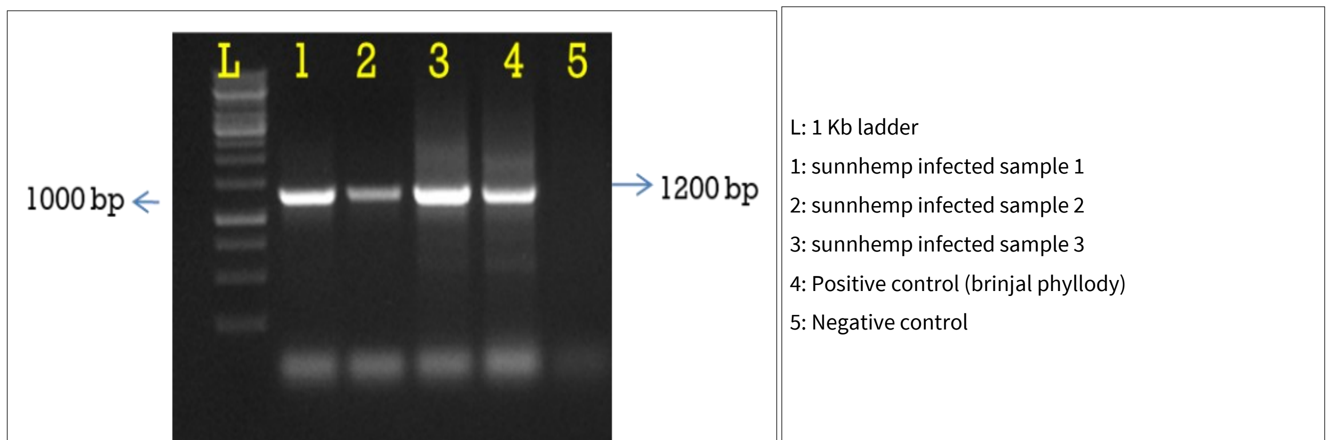

Fig. 3. PCR amplification of 16S rRNA gene from sunnhemp little leaf and witches' broom phytoplasma using of nested primer (R16F2n/ R16R12).

The 16S rRNA sequence identities are used as a key criterion for identifying and grouping phytoplasma isolates. In sunnhemp, sequence similarities between two distinct 16S rRNA groups of phytoplasma were observed, ranging from 88 % to 94 % in sunnhemp phytoplasma (21, 22). Since the 16S rRNA gene sequence similarity of the sunnhemp phytoplasma with members of the *Candidatus: Phytoplasma aurantifolia* group (16SrII) exceeds the 99 % threshold, it is proposed that the sunnhemp phytoplasma be classified as a member of the *Candidatus: Phytoplasma aurantifolia* group (16SrII).

Phytoplasmas belonging to the 16SrII-D subgroups have been widely distributed across various plant crops in India (23, 24). Previous studies have reported the association of *Candidatus: Phytoplasma asteris* and *Candidatus: Phytoplasma aurantifolia* with sunnhemp in South India (25, 26). Additionally, the 16SrII-A and 16SrII-C subgroups have been identified in sunnhemp in Myanmar (18) and Brazil (26), respectively.

The partial 16S rRNA gene sequences of sunnhemp were aligned with those of other phytoplasmas from NCBI GenBank using CLUSTALW software. Phylogenetic analysis confirmed that the sunnhemp phytoplasma is closely related to members of the peanut witches' broom group (Fig. 4). Based on the phylogenetic tree, the 16S rRNA gene sequence of the sunnhemp phytoplasma (PQ623358) is closely aligned with the peanut witches' broom group within the 16S rRNA II group (Fig. 4) with *Acholeplasma laidlawii* as the outgroup. The sunnhemp phytoplasma (PQ623358) shares 100 % sequence similarity with the Praxelis witches' broom phytoplasma (MK967469) and 99.91 % similarity with the Longbean phyllody phytoplasma (AB690306), Goat-weed witches' broom phytoplasma (AB741637), peanut witches' broom phytoplasma (JX871467), Cassava witches' broom phytoplasma (897457) and sunnhemp witches' broom phytoplasma (AB558143).

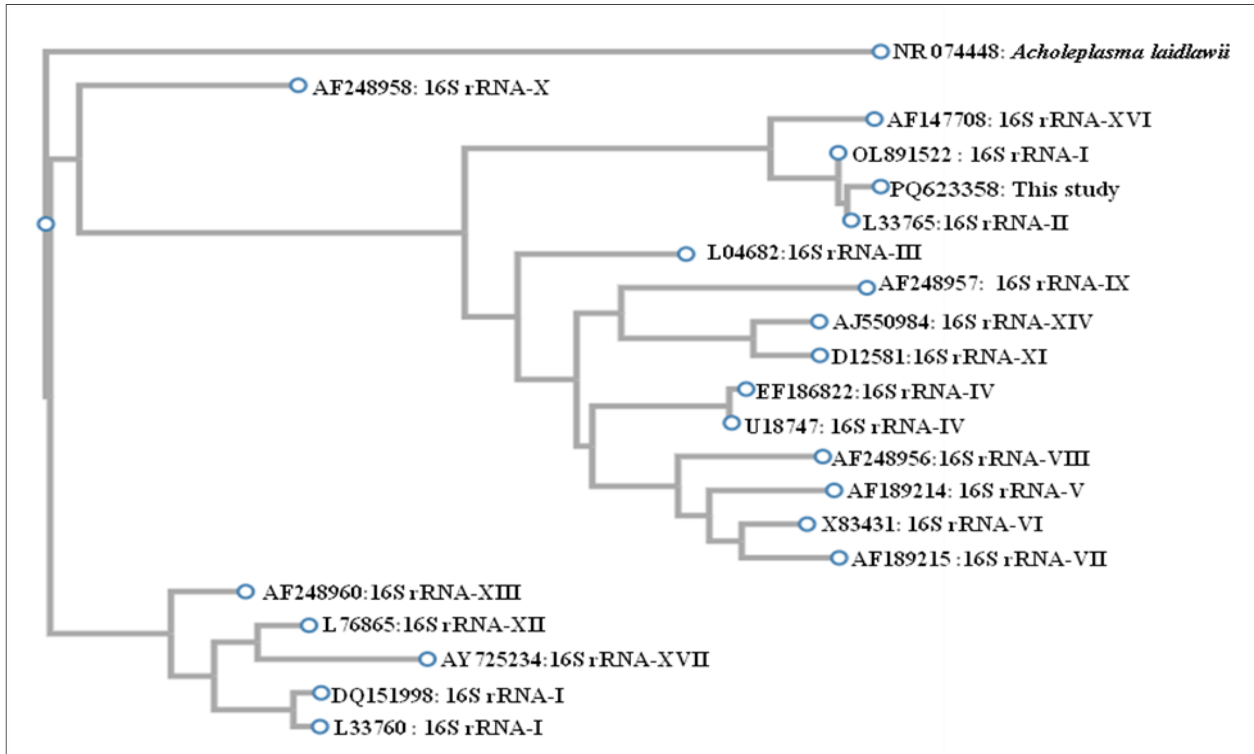

Fig. 4. Phylogenetic tree of 16S rRNA sequences from phytoplasma associated disease /different groups and sunnhemp little leaf and witches' broom phytoplasma (PQ623358) employing *Acholeplasma laidlawii* as the out group constructed by neighbor joining method.

Conclusion

To the best of our knowledge, the present study reports the association of a *Candidatus: Phytoplasma aurantifolia* (16SrII group)-related strain infecting sunnhemp in Tamil Nadu, India. The increasing incidence of this spread strongly emphasizes the need to investigate the sources and other epidemiological factors contributing to the dynamic spread of the disease, in order to develop an effective management strategy.

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Authors' contributions

RR[1] contributed to the original draft writing, formal analysis and data curation. LK was responsible for conceptualization, methodology design and investigation. RR[2] contributed to the review of the manuscript and validation of the content. MP, MD and KT were involved in the investigation process. IJ contributed to validation and manuscript editing. AY provided overall supervision of the work [RR[1] stands for R Ramjegathesh and RR[2] for R Ramesh].

Compliance with ethical standards

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical issues: None

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