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COMBATING THE EMERGING DRUG-RESISTANT *PSEUDOMONAS AERUGINOSA* BY AN ANTIBIOTIC PURIFIED FROM THE NOVEL *STREPTOMYCES VIOLASCENS* STRAIN *VS*

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Keywords:

Antibacterial activity, Antibiotic production, 16S rDNA sequencing, *Streptomyces violascens* strain VS, purification of antibiotics, Antimicrobial assay

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ABSTRACT: Aim: The emergence of drug-resistant pathogens enforces the isolation of potent and novel antibiotic-producing *Streptomyces* sp., from the undisclosed ecological niches. **Methodology:** Unique *Streptomyces violascens* strain *VS* isolated by serial dilution agar plate method and strain confirmed by 16S rDNA sequencing. Antibacterial assay against multidrug resistant *Pseudomonas aeruginosa* isolated from bronchoalveolar lavage fluid was documented by primary (cross streak method) and secondary screening (agar well diffusion assay). Antibiotics are purified by organic solvent extraction, column chromatography, and thin-layer chromatography. The MIC and MBC value of antibiotics is determined against the pathogen by tube dilution method. **Results:** Fourteen *Streptomyces* species were isolated from Erode district, Tamil Nadu, with six different soil samples. Among the isolated *Streptomyces* sp., AC01 exhibited stable antibacterial activity against the MDR strain both in primary and secondary screening. It produced an opulent growth of powdery white colonies with yellow colour spores arranged in chains. Based on 16S rDNA sequencing, the isolated identified as *Streptomyces violascens* strain VS. Crude antibiotic was extracted from ISP-2 broth by ethyl acetate extraction and further purified by column chromatography with chloroform: methanol as solvent. Collected 3rd fraction, validated for maximum antibacterial activity by bioautography. MIC concentrations of antibiotics are determined as 1250 µg/ml and MBC as 2500 µg/ml. **Interpretation:** The novel antibiotic produced by the *S. violascens* strain vs would be a promising drug to combat multidrug-resistant *P. aeruginosa*.

INTRODUCTION: *Pseudomonas aeruginosa* is a ubiquitous, versatile, and opportunistic pathogen spread through immunologically weak persons. Pseudomonas infections range from uncomplicated to most complicated infections [1].

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Complicated infections of healthcare-associated infections include pneumonia, bloodstream infections, urinary tract infections and surgical site/wound infections.

It is transmitted at a faster rate, acquiring drug resistance to most of the currently available antibiotics, including highly effective carbapenem and third-generation cephalosporins [2, 3]. In 2019, Antibiotic Resistance (AR) threats reported that multidrug-resistant (MDR) *P. aeruginosa* was listed as a serious threat causing severe and deadly infections in hospitalized patients.

P. aeruginosa was considered as the second critical threat by the World Health Organization (WHO) in 2017, causing severe to deadly infections in hospitalized patients resulting in around 44.6% of mortality, which requires an urgent need to develop new antibiotics [4]. The improper usage of antibiotics results in a catastrophic event called antimicrobial resistance (AMR) of these pathogens [5]. A high rate of multidrug resistance, particularly in clinical pathogens like *P. aeruginosa*, could result in a lack of treatment options in the future. The centers for disease control and prevention also reported in its newsletter that existing drugs are becoming impotent to treat multidrug-resistant infections because these antibiotics are unable to treat drug-resistant [6]. So, there is a dire need to develop new antimicrobial agents from natural sources, and WHO urges for an immediate solution to control the vehemently increasing antimicrobial resistance threats. In 2021, WHO declared that new antibiotics must be one of the right solutions to address the growing problem and provide drugs effectively [7].

Streptomyces sp. is a common inhabitant of soil, carries out nutrient cycles synergistically. These organisms adopt various mechanisms to grasp nutrients available in the soil effectively. Day-to-day, added novel anthropogenic materials effectively degrade and release simple end products or by-products, which play several important roles in the environment [8]. Of them, only 17% were chemically characterized and explored to have functions such as microbial growth regulators, siderophores, antagonistic agents, immuno-modulators, pesticides, herbicides, enzymes, and specific enzyme inhibitors [9]. In the soil environment, one of the secondary metabolites, antibiotics, displays antibiosis, a well-known phenomenon among the *Streptomyces* sp. Though clinical pathogens are highly resistant to antibiotics, their control measures need to be explored. Species of *Streptomyces* found in extreme environments and unexplored areas would also contribute to the production of unique and new classes of antibiotics to control this MDR strains [10]. Most of the naturally occurring and semi-synthetic antibiotics are derived from *Streptomyces* [10, 11]. Soil serves as an excellent habitat for antibiotic-producing bacteria, which verges on the development of new drugs. Today, soil *Streptomyces* serves as the only species producing approximately 80% of the antibiotics [12, 13]. The application of selective isolation and characterization methods reinforces the discovery of novel antibiotics from rare *Streptomyces* [14]. Over the decades, for exploring new *Streptomyces*, the understudied or undisturbed environment has been investigated [15]. Based on this objective, the current study investigates the isolation of *Streptomyces* from unexplored soil samples located around Erode districts of Tamilnadu, India. This study focuses on the isolation, screening, and purification of antibiotic compounds produced by *Streptomyces violascens* strain VS. In addition to this, the antibacterial activity of the isolated genus *Streptomyces* is illustrated in this article.

MATERIALS AND METHODS:

Isolation of *Streptomyces* sp.: Five different soil types were selected for the isolation of *Streptomyces* sp., from unexplored locations in Erode district of Tamilnadu, India. About 100g of soil sample was collected from each location and plated by serial dilution and agar plate technique. The physicochemical properties of the soil, such as pH, colour, and texture, were recorded [16]. Soil samples were subjected to physical heat treatment followed by chemical pretreatment [17, 18] and enrichment culture technique to facilitate the growth of *Streptomyces* [19, 20].

Selection of Clinical Pathogen: The test organism *P. aeruginosa* was procured from Bioline Laboratory, Coimbatore, isolated from a bronchoalveolar lavage fluid sample. The primary selective media such as pseudomonas isolation agar and cetrimide agar were used for cultural characterization.

The typical colony morphology and pigment production were observed after incubation for 24h at 37°C. For the presumptive identification of *P. aeruginosa*, the parameters such as colony morphology, Gram staining, oxidase activity, and Glucose non-fermentation was carried out [21].

Primary Screening by Cross Streak Method: The antibacterial activities of the isolates were determined on yeast extract malt agar (YEMA) medium by perpendicular streaking technique.

The same medium streaked pathogen only and without *Streptomyces* sp., used as a positive and negative control [22].

Secondary Screening: Agar well diffusion method is widely used to evaluate the antimicrobial activity of culture filtrate from *Streptomyces* sp., using the Muller Hinton agar (MHA) [23, 24].

Identification of *Streptomyces* sp.: Based on the morphometric analysis, the representative *streptomyces* colonies were selected and inoculated into ISP-2 medium with nystatin (50µg/ml) and amphotericin B (5µg/ml) and incubated for 14 days at 28 °C [25]. Further, conventional tests like biochemical and cover-slip techniques were carried out to study the physiological and microscopic characteristics of the organisms.

Antibiotic producing potent *Streptomyces* sp. was mass cultured in 1000 ml ISP-2 media, the cell was harvested by centrifugation and DNA extracted by organic solvent extraction method, DNA amplified using actinobacteria specific forward primer: 5'CGCGGCCTATCAGCTTGTTG3 and reversed primer: 5'CCGTACTCCCCAGGCGGGG3' using the Bio-radthermo cycler and sequenced using ABI-3500 DNA Analyzer. Obtained data compared with Ribosomal Database Project (RDP) and a phylogenetic tree constructed by Neighbour-Joining method [2 6, 27].

Partial Purification of Antibiotics: The maximum antimicrobial activity, showing isolate AC01, was sub-cultured separately in 150ml ISP-2 broth and incubated for 14 days at 30℃ in a shaker. After incubation, culture broth was centrifuged at 10,000 rpm for 15 min, the supernatant was collected and mixed with an equal volume of ethyl acetate (1:1 (v/v)) and continuously mixed in a rotatory shaker at 150 rpm for 10 minutes. The organic phase was collected separately and evaporated to dryness [28].

Purification of Antibiotic by Column Chromatography: The column consisted of a 40-cm-long corning glass tube filled with l00–200 µm particle size of silica gel suspended in chloroform as a solid phase. The column was equilibrated with chloroform, and 5ml of the sample passed through the column at a flow rate of 0.2 ml/min with a gradient solvent system consisting of chloroform: methanol in 9:1, 7:3, 1:1 ratios. Finally, the column was washed with methanol [29]. Fractions of 5ml with each solvent system were collected, and TLC analyzed all the individual fractions for homogeneity. Preparative thin-layer chromatography was performed to separate the active compound in each fraction. Sample loaded plate placed in the glass chamber saturated with mobile phase, chloroform, and methanol in a 4:1 ratio.

The chamber was covered with a lid and allowed the solvent to travel along with the sample and left for 60 min. When the mobile phase moved about 90% from the spotted line, the plate was allowed to dry. The dried plate was examined under ultraviolet light. Then the plate was exposed to iodine vapour for 30 min. After 30 minutes, a brown colour spot appeared, and the R_f value was measured using the standard formula. From the R_f values, unstained TLC plates run with the samples were scraped off carefully from the plate, dissolved in methanol, and centrifuged at 10000 rpm for 5 min. The supernatant dried to evaporation and dissolved in water using a further assay. In order to ensure the antibacterial activity of the purified fraction, the TLC scrapping was placed on an MH agar plate swabbed with *P. aeruginosa* (bioautography). The compound at the polar region showing effective antibacterial activity was scraped using a sterile needle under sterile conditions and profiled for TLC to further confirm its purity [30].

Determination of MIC and MBC in Partially Purified Antibiotic: The minimum inhibitory and bactericidal concentrations were determined for partially purified antibiotics from *Streptomyces violascens* vs. A set of eleven sterile test tubes having 2 ml of ISP-2 broth was taken for double dilution of antibiotic, except the first test-tube having 1 ml of broth. 10 mg/ml of purified antibiotic was introduced into the first test tube. After vortexing, 1 ml of diluted suspension was transferred to the next test tube. Similarly, dilutions were carried out until the 11th test tube, where the antibiotic concentration was 1.22 µg/ml. A test tube without an antimicrobial agent and 2ml sterile water was used as the negative control, and a test tube having 2 ml broth with 25 µg of gentamicin was used as the positive control. In each test tube, 10 µl of an overnight broth culture of clinical pathogens, inoculated aseptically and incubated at

37 °C for 24 h [31]. After incubation, turbidity of each tube was determined at 585 nm, and also presence/absence of growth of pathogen was determined by streaking the test suspension on a nutrient agar plate and incubating for (presence or absence) growth for confirmation [32].

RESULTS AND DISCUSSION:

***Streptomyces* sp., from Soil Samples:** Out of five different soil samples collected from unexplored locations, 14 diverse *Streptomyces* sp., were isolated. From the agriculture soil (sample A), three different isolates (named as AC01, 02, and 03), soil samples collected from the bed of a water stream (sample B) six different isolates (AC04, 05, 06, 07, 08, & 09), from the compost one isolate (AC10) and four different isolates (AC11, 12, 13, & 14) from sand collected near a pond. There was no growth of bacteria and actinobacteria recorded from the rock-crushing black sand, as it lacks organic nutrients. These results confirm the role of *Streptomyces* in the recycling of organic nutrients in the soil and are distributed abundantly in high moisture environments [33]. In recent times, undisturbed soil niches have been widely preferred to explore new antibiotic-producing *streptomyces* strains. In this study, colony morphology of the isolates showed that the time is taken for spore formation and the characteristics of spores, including spore colour, shape and number of spores among the isolated *Streptomyces* sp., showed the least variations [34]. As expected in the current study, 6 species out of 14 were isolated from lime soil with a pH of 7.2 **Table 1.**

TABLE 1: LOCATION AND MORPHOLOGICAL FEATURES OF ISOLATED *STREPTOMYCES* SP.

Soil sample	Location & Geographical distribution	Site of soil sample collection	Soil type & color	pH	Moisture	Isolates with identity
A	Akkarai Kodiveri, Gobichettipalayam 11° 27′ 30″ N 77° 17′ 54″ E	Fertile agricultural land	Peaty &Red	6.9	36	AC01 to AC03
B	Mottanam, Nambiyur 11°21'28.88"N 77°19'14.01"E	Near by a stream	Chalky& White /Cream	6.8	39	AC04 to AC09
C	Punjaipuliampatti, Sathyamangalam 11° 21′ 0″ N 77° 10′ 4.8″ E	Compost	Peaty& Red/Brown	6.5	35	AC10
D	Savakkattupalayam, Avinashi 11°20'24"N 77°18'14"E	Near by a pond	Sandy& Cream	7.2	28	AC11 to AC14
E	Siruvalur, Gobichettipalayam 11° 21′ 58″ N 77° 27′ 38″ E	Construction site	Powdery& Ash/Grey	7.4	22	NIL

These results substantiate previously reported findings that the optimum pH for the growth of *Streptomyces* sp., was neutral to near alkaline pH [35].

Cultural Characteristics: The selected representative colonies were inoculated in ISP-2 medium and variegated colony morphological characteristics such as colour, size, and texture were observed. Due to spore production, the colonies appeared dry to mucoid, ranging from small to large size with different colors (yellow, pink, pale orange, black, grey, chalky white, brown and white). *Streptomyces* sp. is complex bacteria that grow as a branching network of hyphal filaments or like in the form of fungal mycelium. Like fungi, the aerial, branched vegetative hyphae bear a conidia chain containing spores [36, 37]. One or two germ tubes are probably formed after each spore's germination. Later the apical tip extension occurs along with the formation of abundant mycelium. Due to these processes, different kinds of colonies could be observed in the medium [38]. The production of pigments facilitates the spores to adapt and live under unfavourable conditions [39].

Identification of Clinical Pathogen *P. aeruginosa*: The test organism *P. aeruginosa* inoculated on selective media, Pseudomonas isolation agar, and cetrimide agar produced typical colony morphology of *Pseudomonas* sp. Colonies were confluent in growth by the swarm cells in aggregates with green color fluorescent pigmentation and a mawkish odour. Short, motile, Gram-negative, oxidase, and catalase-positive rod-shaped bacteria, utilizing mannitol and glucose non-fermentative organisms were identified. Antibiogram assay confirmed that the isolated *P. aeruginosa* Am^R, Ax^R, Cx^S, Cf^R, Ox^S, Te^R (Ampicillin, Amoxicillin, Cefuroxime, Cefepime, Tetracycline and Ofloxacin) is highly resistant

towards available antibiotics [40]. Still, the pathogen is imperceptible in the development of newer effective drugs and acquires greater adaptability with flexibility by multitudinous virulence elements [41].

Primary Screening: They were intended to examine the efficiency against the test pathogen MDR *Pseudomonas aeruginosa*. One of the classical approaches of the perpendicular streaking method was adopted in our study for primary screening for antibiotic production. The zone of clearance around the perpendicular streak could be considered the efficiency of an antibiotic and produced due to competition [42]. Abundant to normal growth was found near the cross streak, indicating the absence of antibiotic production against MDR *P. aeruginosa*. The isolates AC01, 02, and 04 indicated demarcation in restricting the growth of the pathogen that constitutes around 20% of isolates were potent antibiotic producers distributed in the soil [43] **Fig. 1.**

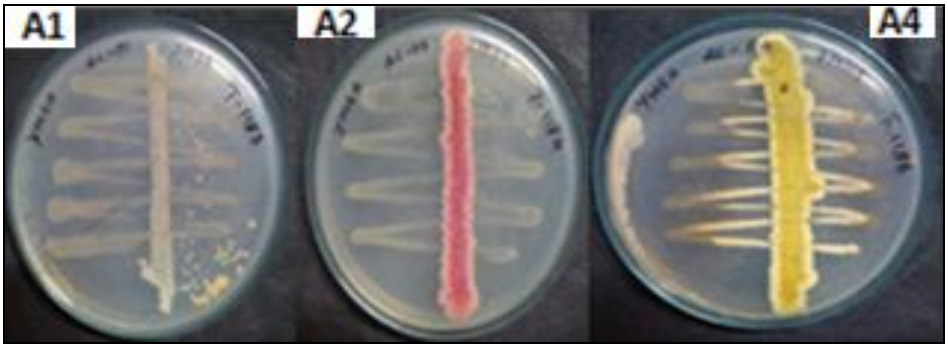

FIG. 1: POSITIVE RESULT IN PRIMARY SCREENING BY *STREPTOMYCES AGAINT* MDR STAIN *P. AERUGINOSA*

In contradiction to the earlier findings, there was no correlation between primary and secondary screening results and also the primary screening technique has not been provided concordant results. Thus, secondary screening was performed with all 14 isolates to confirm antibiotic production.

Secondary Screening: In secondary screening for fourteen different *Streptomyces sp.*, gentamicin (HLG120) as the positive control, and distilled water was considered the negative control used for the analysis. The isolated AC01 and the positive control HLG120 restricted the growth of the pathogen and produced 14 mm and 10 mm of the zone of clearance, respectively **Fig. 2.**

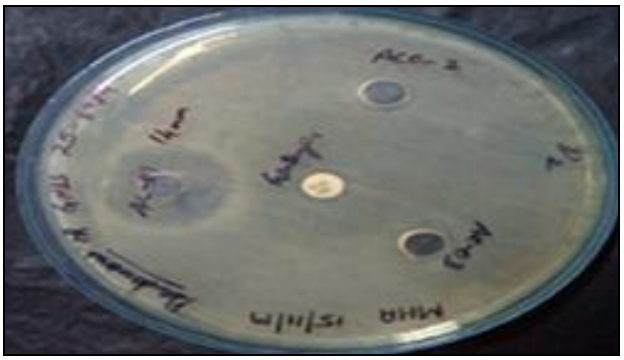

FIG. 2: ZONE OF INHIBITION BY AC01 BY AGAR WELL DIFFUSION ASSAY

The inhibition zone exhibited by the AC01 was much higher in secondary screening than the primary screening. Zone of inhibition was completely absent in AC02 and AC04, which were recorded as positive in primary screening. Our results bear a partial resemblance to recent research [44], where the zone of inhibition of some isolates in primary and secondary screening varied significantly. This rather contradictory result was still not clear, but there are several possible explanations to justify it. Compared to YEMA used in primary screening, MHA facilitates the better diffusion of secondary metabolites [45]. The zone of inhibition or restricting the growth of the pathogen depends on the rate of diffusion or penetration of antibiotics in the media. Moreover, antibiotic production may be induced only in the presence of the pathogen, which is impossible to commercialize the strain for antibiotic mass production [46].

Identification of the Isolated *Streptomyces* sp.: The selected *Streptomyces* sp., AC01 was subjected to microscopic observation revealed the presence of aseptate vegetative mycelial growth of the isolate with small, spherical flexuous sporophores arranged in chains.

Soluble melanin pigment production was absent on the ISP-2 medium instead of producing light yellow color spores [47]. This utilized sugars like glucose, fructose, maltose, and lactose. Molecular identification using genus-specific probe confirmed the isolate belonging to *Streptomyces* sp., and from the multiple sequence analysis and by the construction of NJ-plot revealed that the isolate belonging to the new strain showed characteristics very close (99.9%) to *Streptomyces violascens* hence the isolated strain named accordingly followed by authors initials and submitted in the NCBI repository as *Streptomyces violascens* strain VS (MW365353.1) **Fig. 3** under the family Streptomycetaceae and class Actinobacteria.

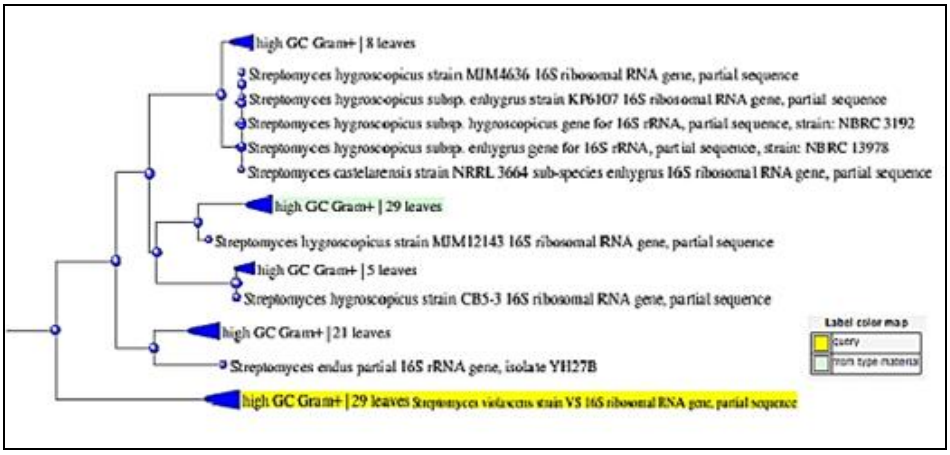

FIG. 3: PHYLOGENETIC TREE REPRESENTING S. *VIOLASCENS VS* HIGH SIMILARITY (97%) WITH *S. HYGROSCOPICUS*

Phylogenetic analysis showed the isolate has around 97% relatedness to the *S. hygroscopicus* and *S. endus.*

Purification of Antibiotics: Isolated *S. violascens* strain VS was mass cultured and partially extracted with ethyl acetate. 1 liter of mass cultured media resulted in 2.3 g of partially purified antibiotic. Complete antibiotic purification resulted in 1.25 g/l of the mass production media. Column chromatography and preparative thin-layer chromatographic techniques resulted in the homogeneous compound [48]. A white color, crystalline, highly hygroscopic biomass with high solubility in water and ethanol was obtained. The high degree of solubility of the antibiotic in water indicated the similarity with strongly polar polycationic aminoglycoside (primary cationic amines) antibiotics, which can effectively kill the highly charged Gram-negative bacteria such as *P. aeruginosa* by internalization of antibiotics and interfering with cell wall integrity [49, 50].

Determination of MIC and MBC: The lowest concentration of antibiotics that inhibits the growth is known as minimum inhibitory (MIC). The lowest concentration where there is no growth by metabolic inactivation of the bacteria is called minimum bactericidal concentration (MBC) of the drug. To determine the MIC and MBC of purified drug from *Streptomyces violascens* strain VS evaluated at concentrations of 1024 µg/ml to 1 µg/ml *in-vitro* condition. In contrast, measuring the turbidity of each tube at 585 nm, at the concentrations of no difference in turbidity from the initial inoculation to after incubation were recorded at 2500 µg/ml, and no turbidity or growth of MDR *P. aeruginosa* were observed from 5000 µg/ml. No turbidity was observed in the tube containing positive control of gentamicin at 25 µg/ml. The test pathogen MDR *P. aeruginosa* is considered as the negative control, showing very good turbidity. These tubes were further sub-cultured in nutrient agar plates to confirm the results. The MIC and MBC values are reconsidered as the tool to determine the resistance of the pathogen and assess the activity of new antimicrobial drugs [51].

The MIC scores of an antibiotic streamline the prescription of rational antibiotics against specific infections and partake in the deterrence of emerging drug-resistant strains. *Pseudomonas aeruginosa* acquires both greater adaptability and flexibility by multitudinous virulence elements.

Still, it is imperceptible in developing newer effective drugs against MDR *Pseudomonas aeruginosa* [52]. Over three decades, the resistance to most classes of antibiotics has been developing at a faster rate by the clinical pathogens. This might be due to the currently available drugs being the modified formulations of the existing class of antibiotics. Therefore, the identification of promising novel drug compounds may successfully help in combating such emerging pathogens.

CONCLUSION: *Streptomyces* species are well-known for their property in the synthesis of various antibiotics, and many more such drug candidates are commercialized. Still, the evolution of multidrug-resistant P. aeruginosa like pathogens is paramount in the clinical domain, which burdens ascertain radical drugs. So, from the weird sites, diverse *Streptomyces* sp., isolated and assessed for their ability to produce unusual and prospective antibiotics. The current study produced an effective antibiotic by *S. violascens* strain VS which effectively controlled the pathogen at minimum concentration. Further progressive studies on chemical characterization and toxicity assay of the antibiotic may result in a suitable drug contender to treat Gram-negative MDR strains such as *P. aeruginosa.*

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Authors' Contribution: V. Viswapriya: Performed the experiments on antimicrobial screening of an antibiotic from the genus *streptomyces*. P. Saravana Kumari: Formulated the project, provided overall guidance, and prepared the manuscript.

CONFLICTS OF INTEREST: The author declares no conflicts of interest.

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