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Isolation and Identification of Histamine Producing Bacteria from Indian Mackerel (Rastrelliger Kanagurta)

Gowarthanan Ranganathan*, Ramanathan Thirunganasambantham, Mullaivendhan Ayyanar, Purushothaman Rammoorthy, & Vishnuram Ganapathy

Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University Parangipettai -608502, Tamil Nadu. India

***Corresponding author:** Gowarthanan Ranganathan, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University Parangipettai -608502, Tamil Nadu. India.

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Abstract

Histamine fish poisoning is a major food safety concern caused by fish contaminated with histamine-producing bacteria (HPB). This study isolated and identified HPB from Indian mackerel (Rastrelliger kanagurta) obtained from retail markets in Cuddalore, Tamil Nadu. Given the region's high consumption of histidine-rich fish, this research assessed HPB prevalence, histamine production capacity, and potential transmission by vendors. Bacterial isolation was performed on mackerel muscle samples and vendor hand swabs. Identified isolates underwent biochemical characterization for histamine-producing Enterobacteriaceae, followed by molecular confirmation of the histidine decarboxylase (HDC) gene. Genetic relatedness among isolates was analyzed using partial HDC gene sequencing. Results confirmed HPB presence in fish and vendor hand swabs, indicating contamination during handling and storage. Findings highlight the risk of histamine accumulation due to improper handling and storage. Stringent hygiene practices and temperature control are essential to mitigate contamination and prevent poisoning. This study underscores the need for regulatory interventions to ensure fish product safety.

Keywords: Histamine, Indian Mackerel, Food Safety, Fish Handling, Histidine Decarboxylase, Bacterial Contamination, Tamil Nadu, Public Health

Introduction

Each year, thousands of people experience food poisoning caused by consuming contaminated food or drinks containing bacteria, viruses, or toxins. Symptoms commonly include fever, diarrhea, vomiting, and digestive problems. Foods frequently linked to food poisoning include meat, poultry, unpasteurized dairy products, eggs, raw shellfish, fish, and improperly stored cooked rice. Andrade, S. C. S., et al., (2012) Fish spoils more quickly than red meat due to its high moisture content and abundance of proteins, carbohydrates, minerals, and salts [1]. Its pH level provides a favorable environment for microbial growth, accelerating spoilage and undesirable alterations. Moreover, the delicate texture of fish causes its tissues to break down faster, and its fats oxidize more rapidly than those found in other meats, further contributing to its perishability. Rodrigues, B. L., et al., (2017) Living organisms, including bacteria, exhibit considerable differences in histamine production, primarily influenced by the presence of specific genes responsible for its synthesis. (BIOHAZ) 2011 [2]. Histamine is produced in fish by microorganisms containing the enzyme histamine decarboxylase (HDC). These microorganisms, either Gram-positive or Gram-negative bacteria, convert histidine. Certain fish species contain high concentrations of specific amino acids within their muscle tissues, which can be converted into histamine Bjornsdottir-Butler et al.,

(2010). Biogenic amines, including tricine, cadaverine, and tyramine, are synthesized by both Gram-positive and Gram-negative bacteria, such as Staphylococcus spp. and Photobacterium phosphorus [3]. Various bacterial species have been identified as histamine producers, particularly in fermented seafood products. The ability to generate histamine varies among bacterial species and genera, depending on the specific type of histamine produced Satomi et al., (2011) [4].

Fish, seafood, and their derivatives are frequently linked to elevated levels of histamine-producing bacteria, Poor handling, processing, and storage conditions significantly contribute to histamine formation. This issue can arise during different processing techniques such as salting, smoking, fermentation, and drying, persisting until the final product reaches consumers. Ingesting fish contaminated with histamine can trigger physiological reactions, leading to potential health risks (Fig. 01).

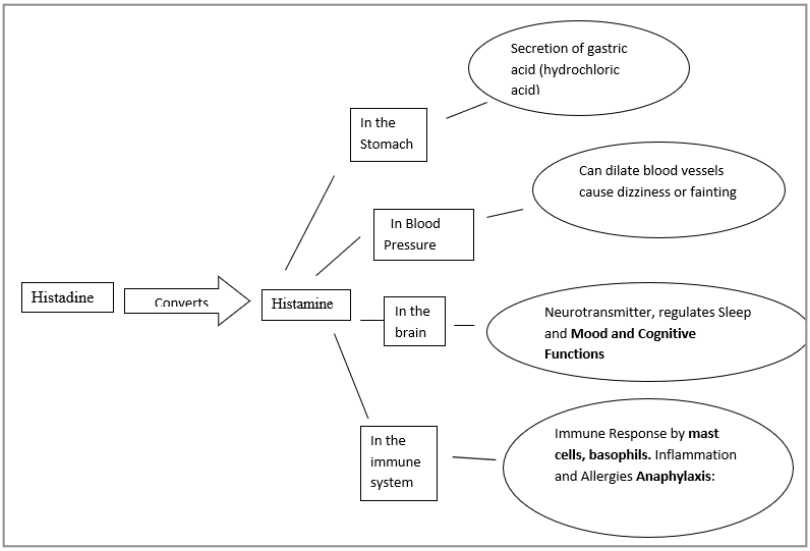

Figure 1: Causing Histamine on Human

Researchers have Categorized Bacteria Isolated from Fish into two Groups:

Those that do not produce histamine and those that do, including histamine-producing strains such as Histidine. These bacteria are moderately thermophilic and cold-resistant species commonly found in marine food products. They contribute to histamine formation in domestically sourced and imported fish Korashy & Farag, (2005) [5]. This study aimed to analyze the bacterial composition of fish and examine its role in histamine production, along with its connection to foodborne illnesses and allergic reactions resulting from fish consumption. The research was undertaken due to the limited number of studies exploring the impact of histamine-producing bacteria isolated from fish.

Materials and Method

Study Area

Mudasalodai, was a coastal village in Cuddalore District, Tamil Nadu, India. A study area is part of the Gulf of Mannar Biosphere Reserve, an ecologically significant area known for its rich biodiversity (Fig.2).

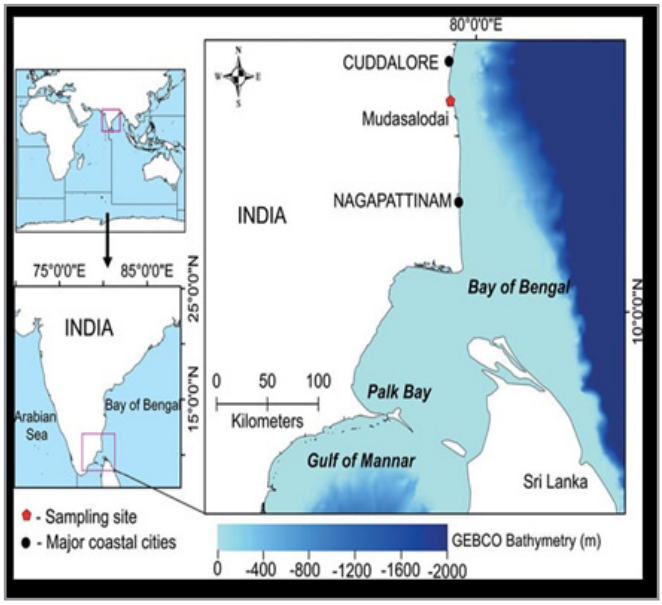

Figure 2: Mudasalodai Landing Area

Study Animal

The Indian mackerel, Rastrelliger kanagurta, is a widely distributed pelagic fish species found in the Indo-Pacific region. Taxonomically, it is classified under the Kingdom Animalia, indicating it is a multicellular organism. It belongs to the Phylum Chordata, which includes animals with a notochord, and is placed in the Class Actinopterygii, the ray-finned fishes. The species is further categorized under the Order Scombriformes, comprising fast-swimming marine fishes. Within this order, it is part of the Family Scombridae, which includes mackerels and tunas. The genus Rastrelliger includes several mackerel species, and the specific species is Rastrelliger kanagurta, known for its ecological and economic importance in coastal fisheries across tropical marine waters [6].

Sample Collection

Fresh Indian Mackerel (Rastrelliger kanagurta Cuvier, 1817) samples were obtained in February 2024 from the Mudasalodai fish landing center. Immediately after collection, the placed samples were in ice and transported to a lab within two hours. For bacteriological analysis, 10 grams of gut tissue from each fish was homogenized with 9 mL of sterile physiological saline for two minutes. These homogenates served as the initial samples for further study.

Bacterial Isolation (Falkow's Method)

To isolate bacteria, homogenized muscle samples—including skin, gills, and intestines—were subjected to serial dilution and spread onto Modified Niven's Medium (MNM) plates using the spread plate technique. The plates were incubated at 30°C for 18–24 hours. Colonies displaying a pink halo on MNM were identified as potential histamine-producing bacteria. These colonies were then streaked onto trypticase soy agar (TSA) supplemented with 0.1% L-histidine for further purification. The bacterial isolates were preserved in glycerol broth at −80°C for long-term storage.

To Assess Histidine Decarboxylase Activity, the Purified Isolates were Tested Using four Different Media:

1. Histidine Decarboxylase Broth (Medium I)

This medium was prepared following Falkow's method, with a control tube containing only the basal medium (without histidine) to prevent false positives. The tubes were sealed with sterile liquid paraffin and incubated at 30°C for 48 hours. A color change from purple to yellow, followed by a return to purple, was considered a positive result, indicating pH fluctuations.

2. Histidine Decarboxylase Broth with Durham's Tube (Medium II)

Similar to Medium I, this broth contained an inverted Durham's tube to detect gas production. A control tube without histidine was included for reference. After incubation at 30°C for 24–48 hours, the presence of gas bubbles in the Durham's tube signified a positive reaction.

3. Histidine Decarboxylase Agar Medium (Medium III)

This medium was prepared based on the composition of MNM. After boiling and distributing into 5 mL test tubes, it was sterilized. Purified bacterial cultures were stab-inoculated and incubated at 30°C for 18–24 hours. A control tube lacking histidine was used for comparison. A distinct red color change indicated a positive reaction.

4. Modified Niven's Medium (MNM) (Medium IV)

Bacterial isolates were streaked onto MNM plates and incubated at 30°C for 24 hours. A pronounced red color shift in the medium confirmed a positive reaction Torido et al., 2012; Falkow, (2004) [7, 8].

Identification of Bacteria

(a) Morphological Characteristics

The newly isolated bacterial strains exhibited Gram-positive staining, were rod-shaped, spore-forming, and aerobic. They demonstrated motility and tested positive for both oxidase and catalase enzymes. These characteristics are consistent with the morphological and physiological traits typical of the Bacillus genus Claus & Berkeley, (1986) [9].

(b) Gram Staining

Gram staining is an essential microbiological technique used to classify bacteria based on differences in their cell wall composition. Gram-positive bacteria retain the crystal violet stain, appearing purple or blue under a microscope, whereas Gram-negative bacteria lose the stain and take up the counterstain, appearing red or pink. These staining differences result from variations in cell wall structure and membrane composition.

(c) Biochemical Analysis

1. Indole Test

Peptone broth was prepared, sterilized, and dispensed into five test tubes, with duplicates assigned for each bacterial strain. A set of uninoculated tubes served as controls. Each tube was inoculated with 0.1 mL of bacterial culture and incubated at 37°C for 24 hours. After incubation, two drops of Kovac's reagent were added. The appearance of a cherry-red ring at the surface indicated a positive result, confirming that tryptophan had been hydrolyzed to indole. A yellow coloration indicated a negative result, signifying the absence of indole production Kirthi et al., (2021) [10].

2. Methyl Red (MR) Test

Methyl Red-Voges Proskauer (MR-VP) broth was prepared and autoclaved at 121°C for 10 minutes to ensure sterility. After sterilization, 5 mL of the broth was aliquoted into test tubes, with duplicates assigned for each bacterial strain. A control tube without bacterial inoculation was included for reference. Each test tube was inoculated with 0.1 mL of bacterial culture and incubated at 37°C for 24 hours. Following incubation, five to six drops of methyl red indicator were added. A red color change signified a positive result, indicating the presence of stable acidic byproducts, whereas a yellow coloration indicated a negative reaction Kirthi et al., (2021).

3. Voges-Proskauer (VP) Test

MR-VP broth was prepared, sterilized at 121°C for 10 minutes, and distributed into test tubes, with duplicates for each isolate. An uninoculated test tube served as a control. After inoculation with 0.5 mL of bacterial culture, the tubes were incubated at 37°C for 24 hours. Following incubation, 0.1 mL of 40% (w/v) potassium hydroxide and 3 mL of 5% (w/v) α-naphthol were added. A deep rose color development 15 minutes indicated a positive result, signifying acetoin production. The absence of pink or rose coloration was considered negative and adverse reaction Kirthi et al., (2021).

4. Citrate Utilization Test

Simmon's citrate agar slants were prepared and inoculated with bacterial cultures using stab and streak methods. The slants were then incubated at 37°C for 24–48 hours. A color change from green to Prussian blue indicated a positive result, confirming the organism's ability to utilize citrate as its sole carbon source. The result was considered negative if no color change occurred and the medium remained green Kirthi et al., (2021).

(d) Molecular Identification

Polymerase Chain Reaction (PCR) was carried out using universal 16S rRNA forward and reverse primers. The amplified products were then sequenced using the ABI 3500 Genetic Analyzer. The obtained sequences were processed, aligned, and edited using MEGA software version 11. Using the NCBI-BLAST tool, a similarity search was performed by comparing the aligned sequences against available sequences in the NCBI database (Table1).

Table 1: NCBI

PRIMERS USED	SEQUENCE (5'- 3')
>533 F	GTGCCAGCAGCCGCGGTAA
>1100 R	AGGGTTGCGCTCGTTG

Sequence Analysis

The user analyzed sequence quality using Sequence Scanner Software v1 from Applied Biosystems. For sequence alignment and editing, they employed Geneious Pro v5.6 Drummond et al., (2012) [11]. Additionally, a Basic Local Alignment Search Tool (BLAST) analysis was performed to compare the obtained sequences with those available in the National Center for Biotechnology Information (NCBI) database Zhang et al., (2000) [12].

Construction of Phylogenetic Tree Montieri et al., (2010)

The user constructed a phylogenetic tree using partial 16S rRNA gene sequences from the isolates and closely related sequences obtained from the NCBI database, aligned using Clustal Omega for multiple sequence alignment [13]. The tree was created using the neighbor-joining method with bootstrapping at 10,000 trials, applying the Kimura-2 parameter, in MEGA 11 software.

Results

Collection and Isolation of Bacteria

The fish sample, specifically Indian mackerel, was collected from the Mudasalodai landing center in Cuddalore District, Tamil Nadu, India. (Figure 3) The collected Indian mackerel was in frozen condition but decomposed within three hours. The fish muscle was dissected, homogenized using a mortar and pestle, and centrifuged at 4000 rpm to prepare serial dilutions. The 10^-2 dilution factor was then spread-plated on TSA agar plates (Figure 4).

Figure 3: Indian Mackrel

The results showed the total bacterial count (TBC) and histamine-forming bacterial counts (HFC) in the fish gut, as depicted in Figure 3. Notably, the fish gut exhibited the highest total bacterial load, ranging from 10^4 to 10^6 cfu/g. (Figure .4)

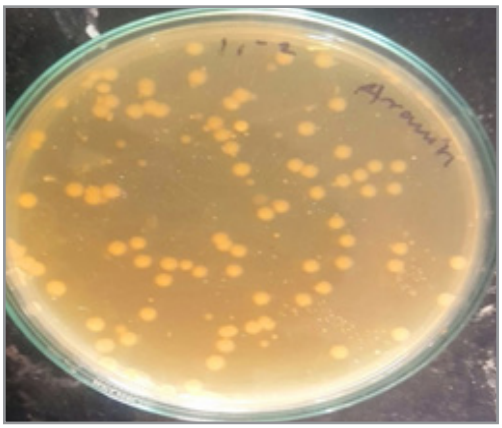

Figure 4: TSA Agar plate

The loop of the colony was streaked in a TSA agar plate for pure culture to incubate at 24 hrs at 37°C to isolate the pure colony, as shown in (Figure 5).

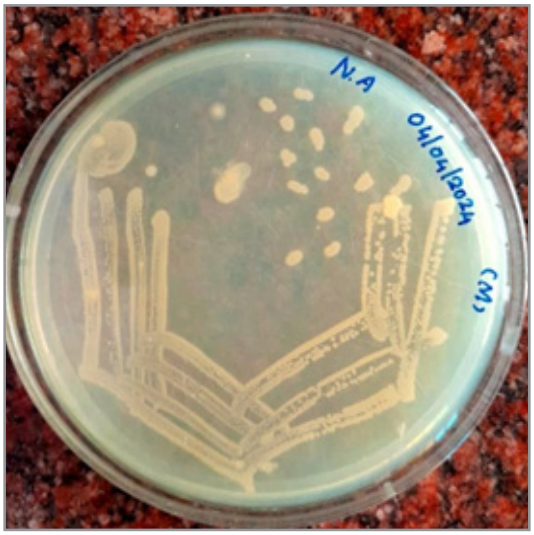

Figure 5: TSA Streaking

Gram Staining

The isolated bacteria were morphologically identified using Gram staining and observed under a photographic microscope at 40X magnification, as depicted in (Figure 6). The study revealed that the isolated bacteria were Gram-positive (+) and had short, curved, rod-shaped morphology.

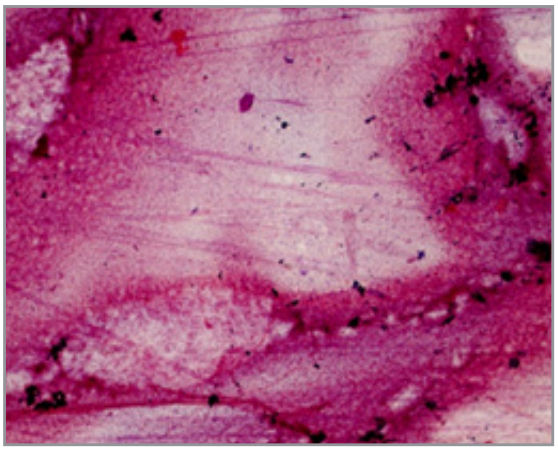

Figure 6: Gram Staining Result

Modiefied Nivens Medium

A loop of a pure colony was taken on a pure plate of TSA and also streaked into MNM agar to identify purple colonies, indicating the potential histamine-forming ability of our bacteria (Figure 7&8).

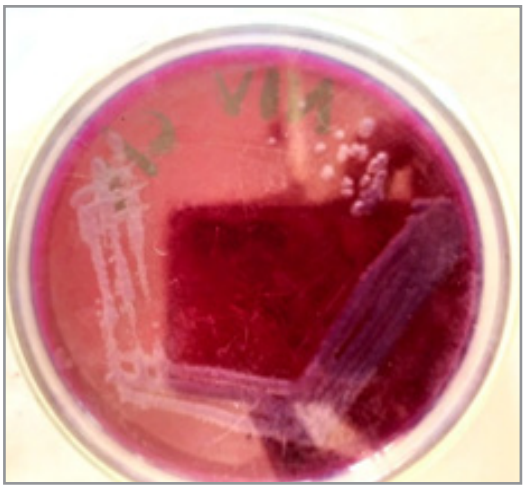

Figure 7&8: MNM Pure Colony

Biochemical Test

The biochemical test results provide valuable insights into the organism's metabolic and physiological characteristics. A negative indole test indicates the absence of the enzyme tryptophanase, meaning the organism cannot break down tryptophan to produce indole. Similarly, the negative Voges-Proskauer and citrate utilization tests suggest that the organism was not glucose fermented via the pathway of butylene glycol to produce acetoin and cannot utilize citrate as its sole source of carbon, respectively. On the other hand, the positive motility test confirms the organism's ability to move, likely through the presence of flagella, aiding in environmental adaptation. A positive oxidase test indicates the presence of cytochrome c oxidase, an enzyme that plays a crucial role in the electron transport chain and cellular respiration. Oxygen-dependent energy production indicating aerobic respiration, the positive catalase test also demonstrates the organism's ability to break down hydrogen peroxide into water and oxygen, allowing it to survive in oxygen-rich conditions by neutralizing harmful reactive oxygen species. These results collectively contribute to identifying and differentiating the organism, and the results are recorded in (Table 2).

Table 2: Biochemical test results of Bacteria

S.No.	Tests	Result
I.	Indole	-
II.	VogesProskauer	-
III.	Citrate utilization	-
IV.	Motality	Motile
V.	Oxidase test	+
VI.	Catalase test	+

Molecular Identification, Phylogenetic Tree

Based on rRNA 16S gene sequencing, this study's findings confirmed the identity of the isolated bacteria as Bacillus safensis (Gene Bank accession No; PP754995). NCBI-BLAST analysis revealed 100% similarity with other bacterial strains. Subsequently, a phylogenetic tree was constructed, as shown in (Figure 9).

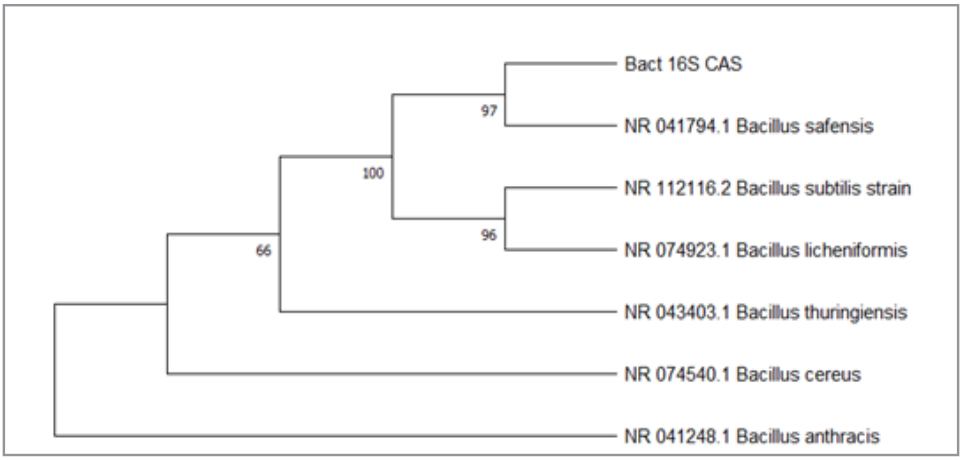

Figure 9: Phylogenetic Tree

Discussion

Rastrelliger kanagurta (Indian mackerel) has not previously been linked to scombroid poisoning, despite its high histamine content. Studies have shown that histamine-producing bacteria (HPB) are present in a significant proportion of mackerel (53%), with a large majority (85%) belonging to the Enterobacteriaceae family. These findings highlight the considerable presence of HPB in mackerel, with a notably higher incidence than other fish species. (Sabry, M.A., et al., 2019) [14]. Similar results have been reported in previous studies, indicating that 53% of mackerel samples contained HPB, and 85% of these bacteria were from the Enterobacteriaceae family. This consistency suggests that Enterobacteriaceae play a crucial role in fish histamine production. In particular, sardines have shown an even higher prevalence of HPB than mackerel. [15, 16]. These findings emphasize the significance of Enterobacteriaceae in histamine formation, which can contribute to scombroid poisoning across various fish species. [17, 18]. In this study, histamine-producing bacterial species were isolated and analyzed for their ability to produce histamine. Gram-negative bacteria outside the Enterobacteriaceae family were also observed on the VRBG medium. According to Eden and Arbon (2014), VRBG medium is more effective in recovering Enterobacteriaceae than other media like MacConkey, though it can also support the growth on non-Enterobacteriaceae. The most commonly identified Enterobacteriaceae species included Citrobacter freundii, Enterobacter cloacae, Enterobacter aerogenes and Raoultella planticola, among the mackerel and salmon isolates examined. In previous studies, these species have been associated with human histamine fish poisoning (HFP). [19, 20]

Conclusion

In conclusion, this study successfully isolated and identified histamine-producing bacteria from Indian mackerel collected at the Mudasalodai landing center on the southeast coast. The diverse fish caught from this center reflect the rich marine biodiversity

of the Bay of Bengal. Through 16S rRNA sequence analysis, the histamine-producing bacterial strain was identified, providing valuable insights into the microbial composition and potential health risks linked to fish consumption from this region.

Ethics Approval: Not Applicable
Constant to Participate: Not applicable
Constant to Publish: Not applicable
Availability of Data and Materials: Not applicable

Competing Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Author Contributions

The first draft of the manuscript was written by Gowarthanan Ranganathan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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