Insoluble proteins catch heterologous soluble proteins into inclusion bodies by intermolecular interaction of aggregating peptides.

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Research

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Abstract

Background: Protein aggregation is a biological event observed in expression systems in which the recombinant protein is produced under stressful conditions surpassing the homeostasis of the protein quality control system. In addition, protein aggregation is related to conformational diseases in animals as transmissible prion diseases, and non-transmissible neurodegenerative diseases including Alzheimer, Parkinson's disease, amyloidosis and multiple system atrophy among others. At the molecular level, the presence of aggregating-prone domains in protein molecules act as seeding igniters to induce the accumulation of protein molecules in protease-resistant clusters by intermolecular interactions.

Results: In this work the aggregating-prone performance of a small peptide (L6K2) with additional antimicrobial activity was studied and the relevance of the accompanying scaffold protein to enhance the aggregating profile of the fusion protein has been elucidated. Furthermore, it was demonstrated that the fusion of L6K2 to highly soluble recombinant proteins directs the protein to inclusion bodies (IBs) in *E. coli* through stereospecific interactions in the presence of an insoluble protein displaying the same aggregating-prone peptide (APP).

Conclusions: These data suggest that the molecular bases of protein aggregation are related not only to the presence of aggregation-prone stretches, but to the net balance of protein aggregation potential and not only to the presence of aggregation-prone stretches. This is ultimately presented as a generic platform to generate hybrid protein aggregates in microbial cell factories for biopharmaceutical and biotechnological applications.

Background

Protein aggregation is a phenomenon observed in many living organisms, from bacteria to animals. In bacteria, it has been linked to stress states with the deployment of a complex protein network to compensate for the reduction of the ability of the cells to cope with the conformational stress [1]. In contrast, protein aggregation in yeast is an inheritable adaptive phenomenon [2]. In animals, protein aggregation is observed in pathological states related to conformational diseases [3], as well as associated to the formation of hormone aggregates in secretory granules [4].

As the biopharmaceutical market expands, the need of recombinant proteins increases. However, during recombinant protein production, the detection of protein aggregates is a common outcome and is observed in both eukaryotic and prokaryotic expression systems [5,6]. Therefore, bioinformatic tools are available for the prediction of protein and peptide solubility and the identification of aggregation-prone hot spots in amino acid sequences, which can be modified during the design of recombinant genes to reduce or avoid formation of intermolecular interactions [7–9]. However, changes in the primary structure of the natural proteins may lead to secondary effects as the appearance of immunogenic epitopes [10]. Therefore, the cellular environment and the concentration of the produced protein can be tightly controlled by lowering transcription and translation rates of the gene in order to reduce the frequency and
scope of aggregation as well as maintaining the original amino acid sequence [11]. The main variables to be considered include the media composition, incubation temperature, promoter strength and inductor concentration among others [12].

In prokaryotes, protein solubility is controlled by the protein quality control system, a complex network of protein factors involved in protein folding, unfolding, and degradation [13]. In bacteria, aggregates known as inclusion bodies (IB) are dynamic protein nanoclusters from which solubilized active protein conformers are released under physiological conditions [14–16]. In fact, recent experimental approaches have revealed the ability of active IBs to rescue enzymatic activities in cell cultures and to target cancer stem cells in cancer animal models [17–21]. Therefore, IBs are envisioned as depots of recombinant protein with the capacity to release the protein of interest from a complex and stable scaffold that protects the biological activity of the embed protein over time. Therefore, the enhancement of protein aggregation in this type of nanostructures is gaining interest.

In fact, bioprocess design during recombinant protein production has been shown to impact IB size and physicochemical quality of recombinant protein, achieving constant IB production [22–24]. In addition, aggregation propensity of recombinant proteins in expression systems may be enhanced by the addition of aggregation-prone peptides (APPs) in the design of the coding DNA sequence of the gene [25]. APPs promote the establishment of intermolecular interactions between protein species enhancing the tendency of the resulting complexes to accumulate in the insoluble cell fraction [26]. Aggregation domains have been found in nature as well, in particular, several protein domains have been shown to possess such aggregation capacity, including a variant of the human β-amyloid peptide (Aβ42 (F19D)) [27], a mutant of the maltose binding protein (MalE31) [28], and the cellulose-binding domain of Clostridium cellulovorans (CBDclos) [29], among others. Another domain with high capacity to enhance protein aggregation is VP1, whose amino acid sequence is present in the VP1 structural protein of the Foot-and-mouth disease virus [26]. Due to safety and regulatory purposes, the use of viral protein domains, as VP1, is not suitable for many applications. For that reason, the development of novel APP is of great interest.

In this study, we have selected a small APP of 8 amino acids in length (L6K2) to study the potential of this type of peptide to enhance the aggregation propensity of soluble proteins [30]. In addition, its aggregating potential was amplified by protein engineering, the anti-microbial activity of this characteristic amphipathic alpha-helix was demonstrated. The role of stereospecific interactions in the aggregation of heterologous recombinant proteins in the presence of L6K2-containing peptides was also analyzed, providing a platform to obtain hybrid IBs inside the recombinant protein-producing cells. These results have relevant implications in the biopharmaceutical and biotechnological applications of IB.

**Results And Discussion**

Modulation of recombinant protein solubility in *ClearColi* cells
In order to study the performance of APP fused to recombinant proteins in the endotoxin free ClearColi™ expression system, two model soluble proteins; iRFP (near-infrared fluorescent protein) and GFP (green fluorescence protein) were selected as scaffolds and fused to the surfactant-like peptide L6K2, previously described as APP (Fig. 1a) [14].

In transformed ClearColi cells, recombinant H6iRFP protein was equally distributed in both soluble and insoluble cell fractions (Fig. 1b and 1c). As expected, upon L6K2 fusion, an altered distribution pattern was observed, with most of the protein located within the insoluble cell fraction, which suggests an increased aggregation tendency for this fusion protein (Fig. 1b and 1c). The change in solubility pattern was observed within 1 hour of induction and was maintained for up to 5 hours (Fig. 1b). In contrast, the recombinant H6GFPL6K2 was mostly detected in the soluble cell fraction (Fig. 1b and 1c). As a model APP, with an appreciable ability to enhance the aggregation tendency of recombinant proteins, VP1 from the capsid protein of the Foot-and-mouth disease virus [26,27] was fused to GFP (Fig. 1a). As expected, most of the protein signal in the sample was detected in the insoluble cell fraction (Fig. 1b and 1c). These results indicate that the aggregation propensity of a recombinant protein may be modulated by an APP, although the solubility tendency of the scaffold protein may play a role in counteracting this effect (compare the solubility of iRFP versus GFP when fused to L6K2 in Fig. 1b and Fig. 1c). In the case of VP1, the strong aggregation tendency overcame the high solubility of the GFP, whereas GFP solubility was not affected by the addition of L6K2 (compare the solubility of GFP when fused to VP1 or L6K2 in Fig. 1b and 1c).

Impact of APP length on protein solubility in ClearColi cells.

As the ability of L6K2 to reduce solubility of GFP was not significant while it was effective in iRFP, the effect of peptide length in the solubility of GFP was subsequently evaluated. To that end, the H6GFPL6K2 recombinant gene was redesigned into several different variations of length and to affix at the C-terminus of the GFP sequence (Fig. 2a). The aggregating potential of L6K2 peptide was amplified by reiteration of leucine and lysine repeats in different positions (see Table 1) and analyzed by AGGRESCAN software [8]. Selected peptides displayed higher hot spot area (HSA) than the original L6K2 peptide. However, only L12K4 and L18K6 showed increased normalized hot spot area (NHSA) and increased average aggregation-propensity hot spot (a4/vAHS).

As previously observed, H6GFPL6K2 was detected in the soluble cell fraction of transformed E. coli cells (Fig. 2b and 2c) and consequently, emitted fluorescence that was homogenously distributed throughout the cytosol (Fig. 2d). The addition of the L6K2 derived peptides had a positive impact in protein aggregation. As expected, the distribution of fluorescence in the transformed cells was detected in protein clusters (IB; Fig. 2d and Additional file 1: Fig S1a). In fact, two different aggregation patterns in the L6K2 derived constructs were detected. The proteins containing serial L6K2 repeats ((L6K2)x2 and (L6K2)x3) were preferentially detected in periplasmic areas around the cells, while the constructs containing longer non-repetitive Leucine/Lysine tracks (L12K4 and L18K6) were detected as fluorescent cellular pole aggregates. Therefore, the serial L6K2 repeats acted both as APPs and periplasm localization signals,
deduced from the fluorescence pattern which revealed signal clustering in discrete aggregates on the periphery of the cell cytoplasm. In addition, the aggregation tendency of L6K2 repeats increased with the number of repeats, while L12K4 presented an aggregation pattern similar to the one observed in cells expressing the positive control, VP1GFP. This aggregation tendency was not recorded in Western Blot analysis of the soluble and insoluble cell fractions (Fig. 2b and 2c), indicating that the aggregation modulatory effects of the L6K2-derived peptides may be sensitive to the tested experimental conditions of protein extraction. This was not the case of the aggregation pattern of VP1GFP construct that was perfectly replicated under confocal laser scanning microscopy and Western Blot analysis (Compare VP1GFP data in Fig. 2b, 2c and 2d).

Antimicrobial activity of L6K2-containing recombinant proteins

During recombinant gene expression experiments of L6K2-containing constructs, the growth of transformed *E. coli* cells was compromised, especially in the case of L18K6 (data not shown). At that point, it was questioned whether or not the peptides were toxic to the cell because of the previously discussed antimicrobial activity. The modeling of the L6K2 derived peptides with PEP-FOLD 3 [31–33] displayed amphipathic alpha helices in all cases (Additional file 1: Fig. S2a). The preferred conformation of the L6K2-containing peptides was maintained in the presence of the PT linker, which has been described as a flexible peptide for separating protein domains (Fig. S2b) [34]. This configuration has been described in naturally produced or synthetic cationic antimicrobial peptides (AMPs) which have been proposed as a potential new class of antimicrobial drugs [35].

However, the production of small peptides is difficult to achieve through recombinant technologies due to reduced stability, and alternative strategies have been utilized to overcome such a hindrance [36]. One possibility to obtain recombinant peptides is through fusion to partner proteins for a potential dual effect on the final product. On the one hand, the reduction of the toxicity of the AMP over the expressing host, and on the other, the improvement in the stability of the peptide in expression systems [37]. However, the study of the resulting biological activity when fused to reporter proteins by genetic engineering has not been explored in depth. Examples of this strategy include the fusion between GWH1 (an AMP with amphipathic alpha helical structure) [38] and GFPH6 (as partner protein) [14,39] and the secretory production of AMP-containing fusion partners [40]. In these studies, the fusion of the AMP to the N-terminus of recombinant proteins preserved the AMP bactericidal activity despite the C-terminus being anchored by the fusion protein. Therefore, the putative antimicrobial activity of the purified soluble versions of H6GFPL6K2 and H6GFP(L6K2)x2 proteins was analyzed and subsequently compared to the data that was obtained from the purified GWH1-GFPH6 in this work. The results indicated that the antimicrobial activity of L6K2-containing recombinant proteins is strain specific (Fig. 3), being comparable to the antimicrobial activity of GWH1 peptide fused to GFP in *E. coli* cultures (Fig. 3b). In addition, the position of the peptide at each end of the scaffold protein did not appear to be relevant to the antimicrobial activity. The incubation of *S. aureus* with the proteins containing amphipathic alpha-helices had only a slight effect on cell viability under the tested conditions (Fig. 3a). Interestingly, the antimicrobial activity of the recombinant proteins was completely different when *Micrococcus luteus*
cells were challenged. The addition of the purified proteins had a positive effect on cell viability at lower concentrations while at the highest protein concentration (8 mmol/L) the cell viability dropped drastically (Fig. 3c).

As observed in Figure 3b, the antimicrobial activity of the L6K2-containing constructs was detected in E. coli cultures at low protein concentrations. This mechanism may explain the cell growth inhibition observed in ClearColi cultures transformed with expression vectors with cloned L6K2-derived genes. Small cationic or amphipathic molecules, similar to the ones described in this work, have been described as produced by prokaryotes and eukaryotic organisms as defense against infectious agents. These molecules belong to a non-specific ancient system of innate immunity and they perform their activity through direct interaction with membranes, nucleic acids, proteins or even activate autolysins [41–44]. In the case of membrane interaction, they cause the destabilization of the cytoplasmic membrane by forming pores or by their arrangement parallel to the membrane surface, disrupting the proton motive force and provoking the leakage of vital molecules which leads to cell death. However, even though their mechanism of action is nonspecific, a differential efficacy of the same antimicrobial peptide between Gram-negative and Gram-positive bacteria has been described [45,46]. In the case of Gram-positive bacteria, apart from membrane disruption, the reaction requires further interactions with the cell wall [45].

Pull-down effect on aggregation tendency of H6GFPL6K2

Aggregation of different proteins may be enhanced by the stereospecific interaction of APP in bacteria [26]. Therefore, the aggregation ability of a recombinant protein fused to L6K2 may enhance the aggregation tendency of H6GFPL6K2 when produced simultaneously in cells. For that purpose, a dual expression vector including the gene encoding for H6iRFPL6K2 was generated, which displayed a high tendency to aggregate beside the gene coding for H6GFPL6K2 to be simultaneously expressed. In cells expressing the aggregation prone H6iRFPL6K2 construct and the soluble H6GFPL6K2 construct at the same time, the fluorescence of the GFP shifted from the cytoplasm to polar protein aggregates (IBs) (Fig. 4a). The green fluorescence distribution in expressing cells was similar to the pattern observed when co-expressing VP1GFP and H6iRFPL6K2 (Fig. 4b).

The change in the aggregation propensity of the H6GFPL6K2 seemed to be directed by the pull-down ability of the L6K2 peptide present in the H6iRFPL6K2 construct. The intermolecular interactions between L6K2 present in the two proteins enhances the aggregation tendency of GFP. In the expressing cells, the newly formed H6GFPL6K2, when interacting with H6iRFPL6K2 with a high tendency to aggregate was dragged to the insoluble cell fraction. Therefore, it may be hypothesized that when two different proteins share aggregation prone domains, even if one of the proteins is still soluble, the protein with the highly aggregation propensity may lead the accompanying soluble protein to the insoluble cell fraction through co-expression. However, although the secondary structures of the iRFP and GFP proteins are not similar (Additional file 2: Fig. S3), the effect of the iRFP scaffold protein in the aggregation enhancement of H6GFPL6K2 may not be ruled out. For that reason, a spectral variant of GFP (EBFP2; highly similar in amino acid sequence and secondary structure) was fused to VP1 domain generating VP1EBFP2H6
construct (Additional file 3: Fig. S4). Predictably, when produced recombinantly, this protein was mainly accumulated in the insoluble cell fraction (Additional file 5: Fig. S5).

The distribution of the GFP fluorescence in cells simultaneously transformed with plasmids coding H6GFPL6K2 and VP1EBFP2H6 was homogeneously distributed in the cytoplasm of the cells, in agreement with the data obtained in the expression experiment of H6GFPL6K2 alone (compare the distribution of GFP fluorescence in Fig. 2c, Fig. 5a and Additional file: Fig. S1b). On the other hand, the fluorescence emitted by EBFP2 fused to VP1 in those cells was mainly detected in polar IBs as expected. When VP1GFPH6 was expressed along with VP1EBFP2H6, the GFP fluorescence was located exclusively at the poles of the cells, as IBs (Fig. 5b and Additional file: Fig. S1b). The colocalization analysis of the fluorescence emission from both proteins indicated the preference of H6GFPL6K2 to aggregate in the presence of the same APP (Fig. 5c) ruling out an aggregating role of the scaffold protein in this process. Therefore, this result has a direct application for biopharmaceutical and biotechnological applications through protein engineering. In fact, these protein nanoclusters have been described as a source of soluble active protein obtained upon incubation in non-denaturing conditions [14–16] and have also been administered as biocompatible depots for tumor targeting of therapeutic proteins [17–21]. Furthermore, protein aggregation seems to be a common mechanism described in most of the expression systems [47–49] that opens up the possibility of expanding this type of strategy to proteins that are difficult to produce in prokaryotes. Therefore, the fusion of common APP to different therapeutic recombinant proteins can induce the colocalization of two recombinant proteins in IBs, obtaining protein formulations with potential synergic activities.

**Conclusions**

Protein aggregation is a universal event which is associated to conformational diseases in eukaryotes. In bacteria, although it has been described as a symptom of metabolic stress resistance, some studies suggest the relevance of protein aggregation in physiological adaptation to stress [1]. In most of the recombinant protein production experiments described so far, a variable portion of the protein accumulated in bacterial inclusion bodies (IBs). In recent years, the use of IBs as active protein deposits has begun to be explored for biopharmaceutical and biotechnological applications [22,50]. The current study highlighted the ability to enhance protein aggregation by the fusion of aggregation prone peptides (APP) to recombinant proteins used as baits for the capture of soluble proteins. This effect was even observed for highly soluble proteins as GFP. In addition, hybrid IBs, enriched in two different recombinant proteins, were formed through stereospecific interactions between common APP. Therefore, the presented data described the potential of APP in the control of the aggregation propensity of recombinant proteins in biological formulations based on IBs and open up the possible exploration of synergic activities of hybrid protein aggregates, produced in bacterial cell factories, for biomedical and nanobiotechnological purposes.

**Methods**
Molecular cloning

All protein designs were cloned in pETDuet™-1 plasmid (Novagen), except for H6iRFP, GFPH6 and GWH1GFPH6, which were cloned into Ndel and HindIII sites of plasmid pET22b (Novagen). For all pETDuet™-1 derived expression vectors, protein-coding DNA fragments were inserted in either, MCS1 or MCS2 of pETDuet™-1 plasmid. In the case of H6GFPL6K2, H6GFP(L6K2)x2, H6GFP(L6K2)x3, H6GFPL12K4, H6GFPL18K6 and VP1GFPH6, digestion was performed with Ndel and XhoI and insertion into the MCS2. On the other hand, H6iRFPL6K2 and VP1EBFP2H6 were digested with Ncol and HindIII and inserted into the MCS1. For dual expression plasmids, H6iRFPL6K2 + H6GFPL6K2, H6iRFPL6K2 + VP1GFPH6, VP1EBFP2H6 + VP1GFPH6 and VP1EBFP2H6 + H6GFPL6K2 a two-step cloning strategy was followed. After the generation of the MCS2 cloning plasmids (pETDuet-H6GFPL6K2 and pETDuet-VP1GFPH6), H6iRFPL6K2 and VP1EBFP2H6 fragments were inserted into the MCS1 after digestion with Ncol and HindIII. All L6K2-containing protein versions included a linker (PT) between GFP and the L6K2 peptide or derivatives as previously described [30].

Expression of recombinant proteins in *ClearColi* cells

*ClearColi* BL21 (DE3) was selected as expression host for the different versions of the fluorescent proteins. The same conditions were applied in all cases. Briefly, after transformation with the corresponding expression vector, bacterial cells were allowed to grow in lysogenic broth (LB) medium supplemented with 100 µg/ml ampicillin in a shake flask (250 rpm) at 37 °C. When cultures reached an optical density of approximately 0.5-0.6, protein expression was induced by adding 1mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). Protein samples were taken at the indicated times (h) postinduction. In all cases, bacterial OD was measured and adjusted to 1, subsequently cells were collected by centrifugation (5 minutes, 1,200 g). Resuspended cells were processed for confocal microscopy visualization or to evaluate the relative protein distribution between the insoluble and soluble cell fractions, in those cases, the expression time was set at 3 h.

Evaluation of protein aggregation propensity

Bacterial pellets harboring the expressed proteins were resuspended in 1 mL of PBS until a homogeneous suspension was achieved. Bacterial cell disruption was carried out by sonication (1 round of 1 min at 10 % amplitude and 1 round of 1 min at 15 % amplitude). Then, the soluble and insoluble cell fractions were separated by centrifugation (15 min, 15,000 g at 4 °C). The insoluble cell fraction, containing the cell debris, was resuspended in 1 mL of PBS, after that, a small aliquot of both fractions, soluble and insoluble, was mixed (1:1) with Laemmli buffer. Soluble samples were boiled at 90 °C for 10 min, while the insoluble samples were boiled for 40 min. The processed samples were charged on SDS-PAGE gels and analyzed by Western Blotting with an anti-His monoclonal antibody (His Tag Antibody, mAb, Mouse, Genscript). Images were acquired with the ChemiDoc™ Touch Imaging System (Bio-Rad) and further processing was performed with Image Lab Software. Percentage of aggregation was calculated based on the numerical band intensity value obtained from blotting membrane images. For each expression time,
the total amount of protein (100 %) was considered as the sum of the band intensities in both, soluble and insoluble cell fractions. Therefore, percentage of aggregation can be estimated from the band intensity value in the insoluble cell fraction.

Visualization of recombinant proteins in ClearColi cells

Bacterial pellets harboring the expressed proteins were resuspended in 500 µL of PBS containing 4 % formaldehyde. Then, resuspended samples were incubated 10 minutes at RT and washed twice with PBS. In a glass slide, a small drop of ProLong™ Gold Antifade Mountant (Thermo) was mixed with 5 µL of the bacterial suspension. The resultant solution was covered with a coverslip and fixed to avoid dehydration. The observation of the fluorescent proteins inside bacteria was recorded by TCS-SP5 confocal laser scanning microscopy (Leica Microsystems). Images were processed using the ImageJ software. Colocalization analysis of fluorescent proteins in ClearColi cells were performed by measuring the overlap coefficients of 10 regions of interest (ROIs) which were compared by one-way analysis of variance (ANOVA).

Purification of soluble recombinant proteins fused to APP

For purification of H6GFPL6K2, H6GFP(L6K2)x2 and GWH1GFPH6, protein expression was induced with 0.1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) at 20 ºC, overnight. The cell pellet was collected (6,000 g, 4 ºC, 15 minutes) and resuspended in wash buffer (20 mmol/L Tris-HCl, pH 8.0, 500 mmol/L NaCl, 10 mmol/L imidazole) with ethylenediamine tetra-acetic acid-free protease-inhibitor (complete EDTA-Free, Roche). Cells were then disrupted by sonication (1 round of 2 min at 10 % amplitude and 10 rounds of 2 min at 15 % of amplitude) and cell debris was separated from soluble fraction by centrifugation (15,000 g at 4 ºC, 45 minutes). After filtration (0.22 µm), the His-tagged proteins were purified from the soluble fraction by His tag affinity chromatography using HiTrap Chelatin HP 1 ml column (GE Healthcare) in an ÄKTA purifier FPLC (GE Healthcare). The purified fraction was obtained after elution with a linear gradient of 20 mmol/L Tris-HCl pH 8.0, 500 mmol/L NaCl, 500 mmol/L imidazole. The purity of the different samples was analyzed by TGX gel chemistry and Western Blotting. The selected fractions were mixed and dialyzed against sodium bicarbonate buffer with salt (166 mmol/L NaHCO3, pH 8.0, 333 mmol/L NaCl) and protein amounts were quantified by Bradford assay.

Antimicrobial activity of APP-containing recombinant proteins

The antimicrobial activity of H6GFPL6K2, H6GFP(L6K2)x2 and GWH1GFPH6 was evaluated against three bacterial species, E. coli, S. aureus and M. luteus, using the broth micro-dilution method. Different two-fold dilutions of the proteins, ranging from 0.06 to 8 µmol/L, were seeded in 96-well plates for each bacterial species. After that, 10⁶ CFU/mL of the corresponding bacteria were inoculated in each well. Maximal growth was achieved in control wells with no protein. Each concentration was evaluated in technical duplicates. Wells with 100 µL of Mueller Hinton Broth Cation-adjusted medium (MHB-2, Sigma-Aldrich) were considered as blank solution. Growth conditions were established in 18 hours at 37 ºC. The bacterial viability was evaluated using the commercially available BacTiter-Glo™ Microbial Cell Viability
Assay (Promega) following the manufacturer's instructions. Luminescence was measured using the Multilabel Plater Reader VICTOR3 (PerkinElmer).

Abbreviations

IB, Inclusion body; APP, aggregation-prone peptide; iRFP, near-infrared fluorescent protein; GFP, green fluorescence protein; HS, hot spot; HAS, hot spot area; NHSA, normalized HAS; a^4vAHS, average aggregation-propensity in each HS; AMP, antimicrobial peptide

Declarations

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Authors’ contributions

JVC and NFM designed the experiments. JVC, AC and EH performed the experiments. JVC and NFM analyzed and interpreted the data. NFM wrote the manuscript. AVC and JVC revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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