

Heparosan Chain Characterization: Sequential Depolymerization of *E. Coli* K5 Heparosan by a Bacterial Eliminase Heparin Lyase III and a Bacterial Hydrolase Heparanase Bp to Prepare Defined Oligomers

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Heparosan is a non-sulfated polysaccharide and potential applications include, chemoenzymatic synthesis of heparin and heparan sulfates. Heparosan is produced using microbial cells (natural producers or engineered cells). The characterization of heparosan isolated from both natural producers and engineered-cells are critical steps towards the potential applications of heparosan. Heparosan is characterized using 1) analysis of intact chain size and polydispersity, and 2) disaccharide composition. The current paper describes a novel method for heparosan chain characterization, using heparin lyase III (Hep-3, an eliminase from *Flavobacterium heparinum*) and heparanase Bp (Hep-Bp, a hydrolase from *Burkholderia pseudomallei*). The partial digestion of *E. coli* K5 heparosan with purified His-tagged Hep-3 results in oligomers of defined sizes. The oligomers (degree of polymerization from 2 to 8, DP2-DP8) are completely digested with purified GST-tagged Hep-Bp and analyzed using gel permeation chromatography. Hep-Bp specifically cleaves the linkage between D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) but not the linkage between 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid (deltaUA) and GlcNAc, and results in the presence of a minor resistant trisaccharide (GlcNAc-GlcA-GlcNAc). This method successfully demonstrated the substrate selectivity of Hep-BP on heparosan oligomers. This analytical tool could be applied towards heparosan chain mapping and analysis of unnatural sugar moieties in the heparosan chain.

1. Introduction

Heparosan (N-acetyl heparosan) serves as the substrate for the chemoenzymatic synthesis of heparin and heparan sulfate.^[1] Heparosan is a non-sulfated polysaccharide and is composed of $[\rightarrow 4)(\text{GlcA})(1\rightarrow 4)(\text{GlcNAc})(1\rightarrow)_n$ repeating disaccharide units.^[2] Heparosan (from *E. coli* K5) is sequentially modified using a chemoenzymatic process to synthesize biosynthetic heparin.^[1] The critical-to-quality (CTQ) attributes of heparosan include molecular weight (MW) distribution, disaccharide composition, and absence of unnatural sugar moieties in the heparosan chain. The presence of unnatural sugar moieties in the heparosan chain may affect chemoenzymatic processes and subsequently may result in non-anticoagulant heparin or heparin with immunostimulatory potential.

Heparin lyases are enzymes from microorganisms, including, *Flavobacterium* spp.^[3,4] Heparin lyase III (Hep-3, from *Flavobacterium heparinum*) cleaves glycosidic bonds of heparan sulfate through β -elimination of (1 \rightarrow 4) linkage between glucosamine and uronic acid residues.^[5,6] Heparanases are naturally occurring hydrolases.^[7,8] Heparanase Bp (Hep-Bp, from *Burkholderia pseudomallei*) cleaves linkage between D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc)/NS of heparin showing many resistant, highly sulfated domains affording oligosaccharides (4-mer to >28-mer), making it useful for oligosaccharide mapping.^[8]

The current paper describes the production of heparin lyase III (Hep-3) and heparanase Bp (Hep-Bp), and the utilization of these enzymes for heparosan characterization. Recombinant *E. coli* expressing either Hep-3 (from *F. heparinum*) or Hep-Bp (from *B. pseudomallei*) was adapted to a high cell density fermentation and active enzymes were purified. These enzymes were used to develop an analytical method to analyze heparosan chain mapping to characterize heparosan from different sources.

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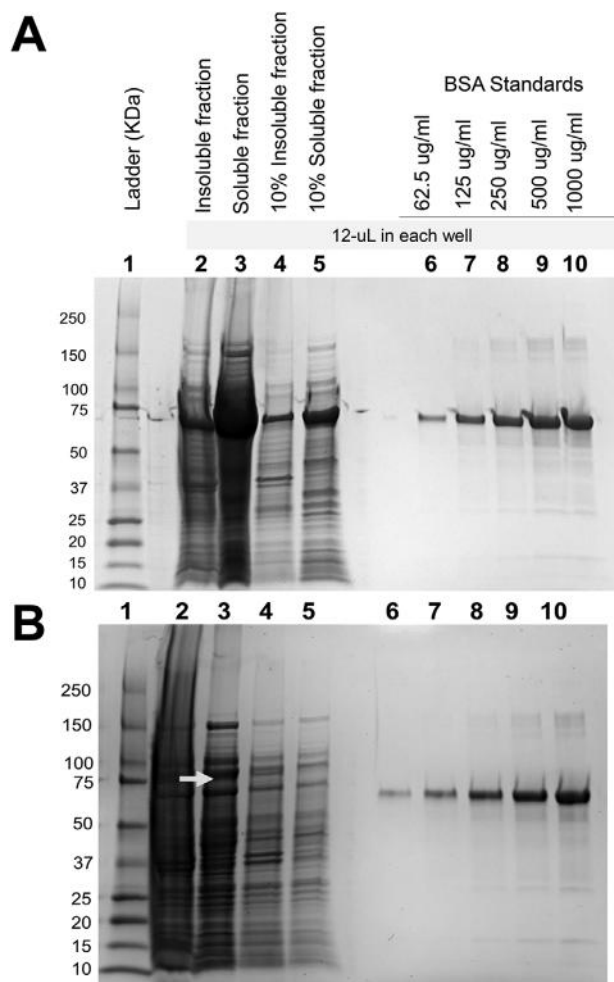


Figure 1. Production of heparin lyase III (Hep-3) and heparanase Bp (Hep-Bp). Fermentation of recombinant *E. coli* expressing Hep-3 and Hep-Bp resulted in the expression of A) His-tagged Hep-3 (75 KDa), and B) GST-tagged Hep-B (72 KDa) in both soluble and insoluble fractions. Of the expressed Hep-3, 72%, and of the expressed Hep-Bp, 34% accumulated in the soluble fraction (Table S2, Supporting Information). Both Hep-3 and Hep-Bp from soluble fractions were active (Figures S3 and S5, Supporting Information).

2. Results and Discussion

2.1. Production of Hep-3 and Hep-Bp

Fed-batch fermentation was used for heparin lyase III (Hep-3) production. The results demonstrate the expression of His-tagged Hep-3 (75 KDa) in soluble and insoluble fractions (Figure 1A). The Hep-3 fermentation resulted in a volumetric yield of 1.2 g enzyme L⁻¹ with the accumulation of 72% of the expressed enzyme in the soluble fraction (Table S2, Supporting Information). Hep-3 was purified from the soluble fraction and a colorimetric assay was used to assess Hep-3 activity.^[9] Results show Hep-3 fermentation gave active enzymes and was comparable to Hep-3 expressed using a shake flask (Figure S3, Supporting Information).

Batch fermentation was used for heparanase Bp (Hep-Bp) production. Results demonstrate the expression of GST-tagged Hep-Bp (72 KDa) in soluble and insoluble fractions (Figure 1B) in a volumetric yield of 213 mg enzyme L⁻¹ with 34% of the expressed enzyme in the soluble fraction (Table S2, Supporting Information). Hep-Bp was purified from the soluble fraction (Figure S4, Supporting Information).^[8] Due to the lack of any colorimetric activity assay for Hep-Bp, a qualitative carbohydrate gel method was used to confirm the activity Hep-Bp produced in the bioreactor. Heparin was digested with Hep-Bp (37 °C, overnight) to form oligomers. Oligomers were resolved using a carbohydrate gel. Intact heparin digested with Hep-Bp (expressed in shake flask) was used as control. Results demonstrate purified Hep-Bp is active (Figure S5, Supporting Information).

We demonstrate active Hep-3 and Hep-Bp were produced in bioreactor fermentations. However, the Hep-Bp fermentation resulted in low soluble enzyme expression, and current efforts focus on improving soluble enzyme expression by altering induction conditions. Plasmid design with other protein fusion partners (e.g., His tag instead of GST tag) will also be evaluated and downstream purification of Hep-Bp, that is, using mild detergents and/or urea refolding will be evaluated.

2.2. Applications of Hep-3 and Hep-Bp as an Analytical Tool for Characterizing Heparosan

Purified heparosan (from *E. coli* K5) was partially digested with heparin lyase III (Hep-3) to form different sized heparosan oligomers. The oligomers of defined sizes were completely digested with heparanase Bp (Hep-Bp). Heparosan dp2-dp8 oligosaccharides were separated by GPC. The GPC analysis demonstrated 2–3 peaks, and the ratio of the peak area correlated to the size of the initial heparosan oligomer substrate. Furthermore, these peaks were resolved using MS. Evaluation of peak 1 revealed three signals for Δ UA-GlcNAc-GlcA; GlcNAc-GlcA-GlcNAc; and a process impurity peak of glutathione (Figure 2). The presence or absence of [Δ UA-GlcNAc-GlcA] and [GlcNAc-GlcA-GlcNAc] correlated with the size and composition of starting heparosan substrate. Hep-Bp treatment of the heparosan 4-mer, Δ UA-GlcNAc-GlcA-GlcNAc, resulted in the formation of [Δ UA-GlcNAc-GlcA] (Figure 2A). Hep-Bp treatment of heparosan 6-mer, Δ UA-GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc, resulted in Δ UA-GlcNAc-GlcA and GlcNAc-GlcA-GlcNAc (Figure 2B). Hep-Bp treatment of heparosan 5-mer GlcNAc-GlcA-GlcNAc-GlcA-GlcNAc resulted in GlcNAc-GlcA-GlcNAc (Figure 2C). Evaluation of peak 2 revealed one signal, GlcNAc-GlcA (Figure 2B-C). The presence of GlcNAc-GlcA also correlated with the size and composition of starting heparosan oligomer substrate (Figures 2, 3). Heparosan dp2 (Δ UA-GlcNAc) could not be digested by Hep-BP but heparosan dp4 (Δ UA-GlcNAc-GlcA-GlcNAc) could be digested to (Δ UA-GlcNAc-GlcA) and (GlcNAc). This indicates that the Hep-BP can cut the GlcA-GlcNAc linkage but not the Δ UA-GlcNAc linkage. Heparosan dp6 and dp8 gave trisaccharide (Δ UA-GlcNAc-GlcA), disaccharide (GlcNAc-GlcA), and monosaccharides (GlcNAc) after the digestion with a minor amount of GlcNAc-GlcA-GlcNAc remaining. Heparosan dp3, dp5, and dp7 showed similar cleavage after treatment with Hep-BP. The cleavage of these heparosan oligosaccharides by

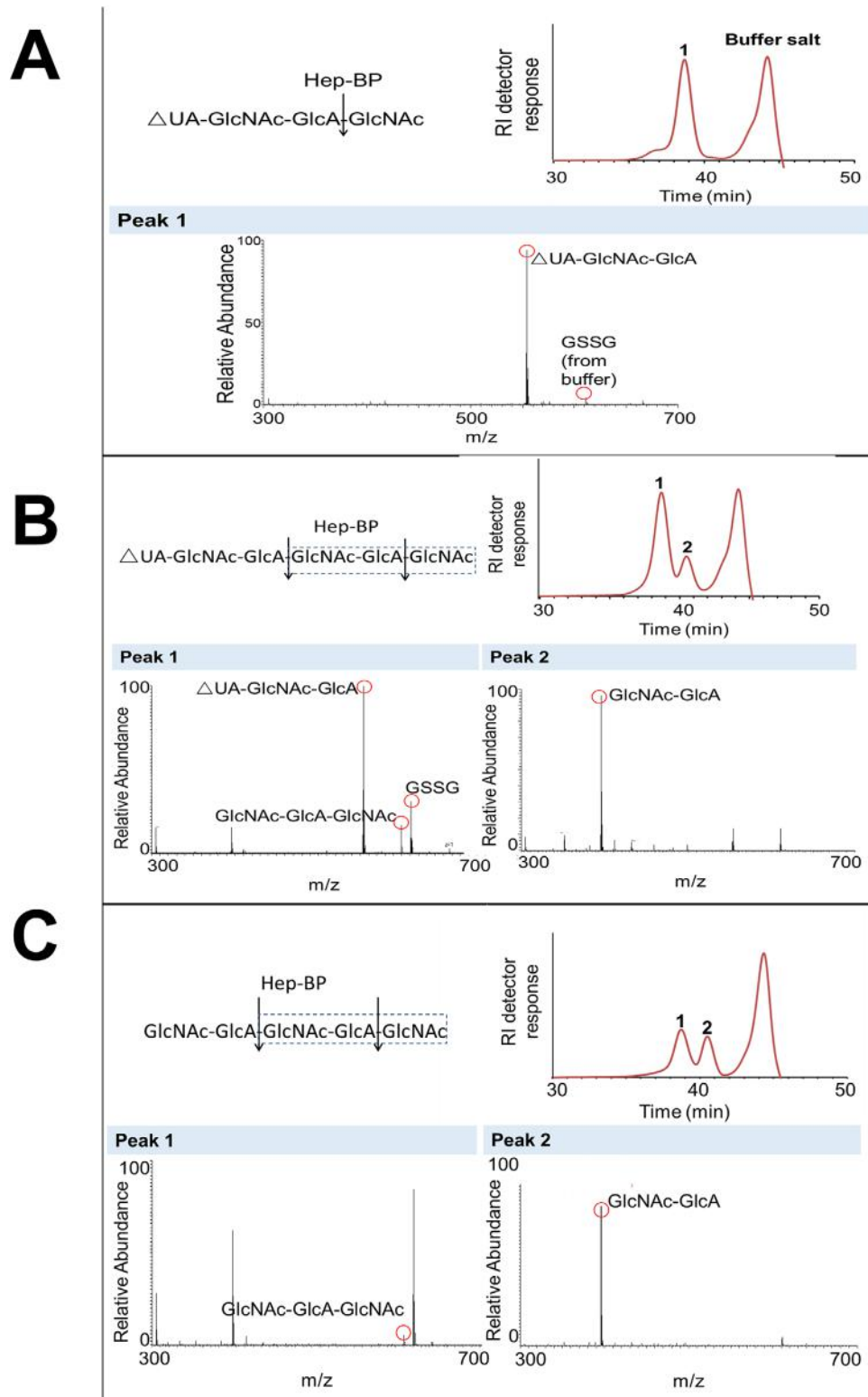
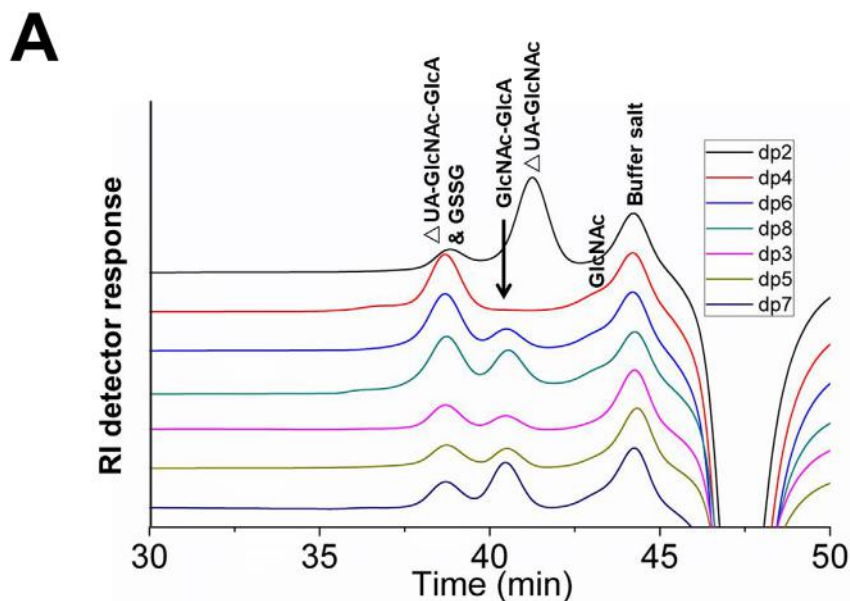


Figure 2. A) Complete digestion of heparosan dp4, B) heparosan dp6, and C) heparosan dp5. Purified heparosan was digested with heparin lyase III (Hep-3) to form different sized oligomers. Oligomers of defined sizes (dp4, dp6, and dp5) were completely digested with heparanase Bp.



Substrate molar ratio, dp2: dp4: dp6: dp8: dp3: dp5: dp7= 10: 1: 1: 1: 1: 1: 1

B Molar ratio of products from *E. coli* K5 heparosan oligosaccharides digested by heparanase Bp (Hep-Bp, a bacterial hydrolase)

	Δ UA-GlcNAc	Δ UA-GlcNAc-GlcA	GlcNAc-GlcA	GlcNAc	GlcNAc-GlcA-GlcNAc
dp2	1	/	/	/	/
dp4	/	1	/	1	/
dp6	/	1	1	1	minor
dp8	/	1	2	1	minor
dp3	/	/	1	1	minor
dp5	/	/	2	1	minor
dp7	/	/	3	1	minor

Figure 3. GPC analysis of heparosan dp2-dp8 digested with heparanase Bp (Hep-Bp). The GPC analysis of dp2-dp8 (degree of polymerization from 2 to 8) confirms Hep-Bp cleaves the linkage between GlcA and GlcNAc but not between Δ UA and GlcNAc. Data also demonstrate a resistant trisaccharide GlcNAc-GlcA-GlcNAc. (A) GPC analysis (B) Molar ratio of products.

Hep-BP and the molar ratio of the resulting oligosaccharides are summarized in **Figure 3B**. These data suggest that Hep-Bp specifically cleaves the linkage between GlcA and GlcNAc but not the linkage between Δ UA and GlcNAc, with the presence of a resistant trisaccharide (GlcNAc-GlcA-GlcNAc).

3. Conclusion

Heparin (average MW \approx 20 kDa) and low-molecular-weight heparins (average MW \approx 4–6 kDa), produced from porcine intestinal mucosa,^[12–14] are clinical anticoagulants.^[12] Safety concerns for animal-derived heparin include, 1) prion contamination, 2) process impurities, and 3) adulteration with over-sulfated

chondroitin sulfate.^[12] The availability of heparins is impacted by an unstable supply chain, leading to the need for biosynthetic heparins from non-animal sources.^[1,9,13–16]

Heparosan is the substrate for chemoenzymatic synthesis of heparin.^[1] Heparosan has potential applications as an inert drug delivery vehicle.^[17,18] Heparosan is produced microbially from *E. coli* K5, *E. coli* Nissle, *Pasteurella multocoda*,^[2,19,20] and from engineered *E. coli* and *Bacillus* sps.^[21–23] Characterization of heparosan isolated from natural producers and engineered-cells is important for its potential applications. The presence of Un-natural sugar moieties present in heparosan may result in adverse effects. Heparosan is characterized by an analysis of intact heparosan MW distribution or heparinase digestion and

disaccharide analysis.^[2,24,25] Here we describe a novel method where heparin lyase (Hep-3) and heparanase (Hep-Bp) are used to depolymerize and characterize heparosan. This analytical tool can be used to map and analyze unnatural sugar moieties in heparosan.

4. Experimental Section

Production of Hep-3 and Hep-Bp: Recombinant *E. coli* BL21 Star (DE3) strain (Novagen, Cambridge, MA) expressing heparin lyase III (Hep-3, from *F. heparinum*) was used for the production of His-tagged Hep-3 (gift from Prof. Jian Liu at UNC, USA). Recombinant *E. coli* BL21 Star (DE3) strain (Novagen, Cambridge, MA) expressing heparanase Bp (Hep-Bp, from *B. pseudomallei* K96243) was used for the production of GST-tagged Hep-Bp.^[8] Shake flask fermentations were performed as previously described.^[8] Bioreactor fermentation conditions are described in Table S1, Supporting Information. Enzyme purification and analyses are shown in Figure S1, Supporting Information. The His-tagged Hep-3 was purified and analyzed as described previously.^[9] The GST-tagged active Hep-Bp was purified as described previously.^[8] Enzyme concentrations were evaluated as previously described.^[10] Hep-Bp activity measurement used a carbohydrate gel^[2] (Figure S2, Supporting Information).

Preparation of Heparosan Oligomers Using Hep-3: *E. coli* K5 heparosan was prepared as described previously.^[2] Heparosan (MW \approx 41.0 kDa, 100 mg) was dissolved in 5 mL digestion buffer (50 mM $\text{NH}_4\text{OCOCH}_3$, pH 7.2), and partially digested with heparin lyase III (Hep-3, 2–5 U). The reaction was incubated for 0.5–2 h at 37 °C, resulting in unsaturated oligomers with an even-number saccharide-residues. Post-digestion the solution was heated to deactivate and precipitate the enzyme. Mixed oligomers were separated by a gel filtration chromatography (GPC) on a 1.0 \times 120 cm column (BIO-RAD, Moscow, Russian Federation) packed with Superdex 30 Prep Grade resin (GE Healthcare, MA, USA). The Superdex 30 eluent was 0.2 M NH_4HCO_3 . Heparosan oligomers with odd numbers of saccharide residues were prepared by removing the non-reducing terminal Δ UA residue from the oligomers using $\text{Hg}(\text{OCOCH}_3)_2$.^[11]

Substrate Selectivity of Hep-BP on Heparosan Oligomers: Heparosan oligomers (100 μL of 1 μM , dp3-dp8, and 10 μM dp2) were dissolved in 50 mM NH_4COCH_3 (pH 4.5) and digested overnight at 37 °C with 15–25 μg of heparanase Bp (Hep-Bp). The supernatant was profiled by gel filtration chromatography (GPC), and using a column (10 \times 300 mm) packed with Superdex peptide 10/300 GL (GE Healthcare, Chicago, IL). The Superdex 30 column was eluted with 0.2 M NH_4HCO_3 (flow rate of 0.4 mL min^{-1}), monitoring with a refractive index detector. Peaks were collected and analyzed by mass spectroscopy.

Mass Spectroscopy: Samples were dissolved in water (0.2–0.5 μg μL^{-1}), and 5 μL was directly injected to standard electrospray ionization (ESI) source of LTQ-Orbitrap XL Fourier transform mass spectrometer (FTMS) (Thermo Fisher Scientific, San-Jose, CA). LC parameters include mobile phase A, 5 mM aqueous $\text{NH}_4\text{OCOCH}_3$, and mobile phase B, 5 mM $\text{NH}_4\text{OCOCH}_3$ in 98% acetonitrile with 2% water. The flow used was 50% A and 50% B (250 μL min^{-1}). The source parameters for FTMS were in negative-ion mode, a spray voltage of 4.2 kV, a capillary voltage of –40 V, a tube lens voltage of –50 V, a capillary temperature of 275 °C, a sheath flow rate of 30 L min^{-1} , and an auxiliary gas flow rate of 6 L min^{-1} . All FT mass spectra were acquired at a resolution 60 000 with a 200–2000 Da mass range.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

Payel Datta: conceptualization-Equal, investigation-Equal; Lufeng Yan: investigation-Equal; Adeola Awofranye: investigation-Equal; Jonathan Dordick: conceptualization-Equal, funding acquisition-Equal, project administration-Equal; Robert Linhardt: conceptualization-Equal, funding acquisition-Equal, project administration-Equal. P.D. equally contributed to conceptualization and investigation; L.Y. and A.A. equally contributed to investigation; J.D. and R.L. equally contributed to conceptualization, funding acquisition, and project administration.

Keywords

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