Free-Living Enterobacterium Pragia fontium 24613: Complete Genome Sequence and Metabolic Profiling

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ABSTRACT: Pragia fontium is one of the few species that belongs to the group of atypical hydrogen sulfide-producing enterobacteria. Unlike other members of this closely related group, P. fontium is not associated with any known host and has been reported as a free-living bacterium. Whole genome sequencing and metabolic fingerprinting confirmed the phylogenetic position of P. fontium inside the group of atypical H2S producers. Genomic data have revealed that P. fontium 24613 has limited pathogenic potential, although there are signs of genome decay. Although the lack of specific virulence factors and no association with a host species suggest a free-living style, the signs of genome decay suggest a process of adaptation to an as-yet-unknown host.

KEYWORDS: Pragia fontium, Enterobacteriaceae, whole genome sequence, phylogeny, free-living bacteria

Introduction

To date, the Enterobacteriaceae family contains 55 genera and 248 species (www.bacterio.net, September 1, 2016). Most of the enterobacteria live in the vertebrate intestine, whereas several other enterobacterial genera/species represent plant pathogens or invertebrate endosymbionts.1 Other enterobacteria are believed to live only in the environment, eg, Pragia, Saccharobacter, Obsumbacterium, Shimwella,2 Mangrovibacter,2 and Biostraticola.3 However, it is possible that their pathogenic/symbiotic potential will be revealed in the future, as it was for Budvicia.4,5

Pragia fontium is a gram-negative, mesophilic, rod-shaped, motile bacterium. The genus Pragia contains only 1 species, P. fontium, which was described in 1988.6 A total of 18 strains were isolated in Czechoslovakia between 1982 and 1986. All strains, except 1, were isolated from water wells and water pipes, whereas 1 strain was obtained from the stool of a healthy woman. Another set of Pragia strains was isolated in Ukraine between 1996 and 1997.7 They were mostly isolated from water (9 strains) and other environmental material (5 strains), although 2 strains came from human clinical material; their relatedness to the Czechoslovakia strains varied from 84% to 95% (based on DNA-DNA hybridization). To date, only strains from these 2 locations have been characterized, and the exact ecological niche and pathogenic potential of Pragia remains unclear.

Pragia fontium, as well as Budvicia spp. and Leminorella spp., is a closely related atypical enterobacterial species. Their common feature is hydrogen sulfide production, with Budvicia diplopodorum being the only known exception.8 These H2S-producing enterobacteria share several metabolic features including reduced metabolic activity that results in utilization of a limited set of substrates. The optimal growth temperature for Pragia and Budvicia is 25°C, whereas Leminorella is capable of growing at temperatures up to 42°C.6 Pragia fontium can be differentiated from Budvicia spp. based on a positive (Simmons) citrate utilization test and from Leminorella spp. by its motility, tartrate utilization, tyrosine clearing, and inability to grow at 42°C.6 In addition, a whole-cell protein pattern analysis of P. fontium, B. aquatica, and Leminorella spp. was determined and the data supported the delineation of these genera.6 On the DNA level, Pragia strains were most closely related to Budvicia (based on DNA-DNA hybridization, relatedness 20%-37%) but barely related to other genera, eg, relatedness to Escherichia coli K12 was about 3%.9

To date, 485 completed enterobacterial genome sequences, covering 21 genera and 47 species, have been deposited in the Genomes OnLine Database (GOLD, https://gold.jgi.doe.gov/). Attention has been focused mainly on clinically and agriculturally important bacteria (eg, Escherichia, Salmonella, Klebsiella, and Yersinia), leaving the remaining genera relatively unexplored.

The whole genome sequence and the pilot assembly of P. fontium 24613 were published in 2015.10 In this study, we characterized P. fontium based on genomic data, including the relationship of Pragia to other genera, and compared metabolic pathways with the results of phenotypic metabolic fingerprinting.
Materials and Methods

Bacterial strains and cultivation conditions

The strains used in this study came from the collection of the Department of Biology, Masaryk University, Brno, Czech Republic (P. fontium 24613, originally stored at the National Institute of Public Health, Prague, Czech Republic); from the Czech National Collection of Type Cultures, Prague, Czech Republic (Budewcia aquatica CNCTC 62857); and from the Czech Collection of Microorganisms, Brno, Czech Republic (Leminorella grimontii CCM 40037). Pragia fontium 24613 came from the same set of strains as P. fontium DSM 556378. Strains were cultivated in TY medium (8 g casein, 5 g yeast extract, 5 g sodium chloride, pH 7.5; HiMedia, Mumbai, India) at 30°C for 24 hours.

Pragia fontium 24613 genome sequencing and annotation

In our previous study, protocols for DNA extraction, whole genome sequencing, and annotation of P. fontium 24613 were described in detail.10 For additional gene mining and genome comparisons, annotation was manually curated based on results of a RAST (Rapid Annotation using Subsystem Technology) pipeline11 and DOE-JGI (US Department of Energy-Joint Genome Institute) Microbial Genome Annotation Pipeline.12 Detected proteins were assigned to Clusters of Orthologous Group (COG) categories based on DOE-JGI results. Methylocene was characterized using PacBio single-molecule real-time sequencing (1× SMRT cell) of kinetic data collected during the genome sequencing process.13 SMRT analysis version 2.3, using the “RS_Modification_and_Motif_Analysis” protocol, was used for genome-wide base modification and detection of the affected motifs. Regarding sequencing coverage, a default quality score value of 30 (corresponding to a P value of .001) was used for motif determination. The detected motifs were uploaded and further analyzed using the REBASE database.14 The complete genome was also scanned for homologues of restriction-modification system genes (using a Basic Local Alignment Search Tool [BLAST] search, with the BLASTX algorithm) against the REBASE and GenBank databases.

Phylogenetic position of P. fontium

The genome sequence of P. fontium 24613 was compared with other enterobacterial genera on a genome-wide level. Whole genome sequences were downloaded from the GOLD (https://gold.jgi.doe.gov/); their accession numbers are listed in Table S1. Each genus was represented by 1 sequence (except for Pragia where both the type strain DSM 55637 and strain 24613 were used). If available, the sequence of the type strain was used. For genera Biostraticola, Coesenzaea, Gibbsiella, Mangrovebacter, Ochsenumbacterium, Saccharobacter, and Samsonia, no sequences were available. A whole genome phylogenetic analysis was built using PhyloPhAn 0.99,15 which compared more than 400 selected protein sequences conserved across bacterial domains. The genes were identified using an internal PhyloPhAn database by translated mapping with USEARCH 8.1.16 The topology was computed using the neighbor-joining algorithm in conjunction with the Jukes-Cantor evolution model. Moreover, the CAT model, with gamma correction, was used to optimize and rescale the tree. The final tree was reconstructed, using FastTree 2.1,17 from protein subsequences of the genes concatenating their most informative amino acid positions, and each was aligned using MUSCLE 3.8.18 The tree was visualized in MEGA 6.06.19 Dot plot diagrams between genomes were constructed using the Integrated Microbial Genome platform.12 The core genome of P. fontium, B. aquatica, and L. grimontii was determined based on orthologous clusters produced by OrthoVenn20 using a modified OrthoMCL heuristic approach. Default parameters (E-value 1e−5 and inflation value 1.5) were used. Metabolic pathway analysis of P. fontium 24613, Wigglesworthia glossinidias (acc. no. CP003315), and Buchnera aphidicola G002 (acc. no. CP002701) was performed using the KEGG PATHWAY database,21 which is part of KEGG Web services (http://www.genome.jp/kegg/).

Analyses of metagenomics data

Data from the Human Microbiome Project database (http://hmpdacc.org) and EBI Metagenomics database (https://www.ebi.ac.uk/metagenomics/) were searched with BLASTN 2.2.2222 using a consensus sequence of 7 16S ribosomal RNA (rRNA) genes of P. fontium 24613. The first database contained a complete set of human microbiome data (associating data from several human sites), and the latter database covered data from different environmental sources.

Substrate diversity studies

The Biolog GN2 MicroPlate analysis platform (Biolog, Inc., Hayward, CA, USA) was used for determination of the biochemical profiles of P. fontium 24613, B. aquatica CNCTC 62857, and L. grimontii CCM 40037 cultivated on Biolog Universal Growth (BUG) agar at 30°C for 24 hours. Utilization of 95 carbon sources was tested23 (Table S2). Media and all reagents were supplied by Biolog and used according to the manufacturer’s protocol. Plates were incubated in parallel under aerobic and anaerobic conditions and tests were read after 24 hours of incubation.

Results

Genome analyses of P. fontium 24613

Complete genome sequence of P. fontium 24613. A complete genome sequence for P. fontium 24613 represents a single circular chromosome with a length of 4 094 629 bp.10 The P. fontium 24613 genome was compared with 3 draft genomes
of related bacteria, including the draft genome of *P. fontium* DSM 5563T (Table 1). Both the *B. aquatica* DSM 5075T genome and the *L. grimontii* DSM 5078T genome were larger in size and gene count compared with the complete genome sequence of *P. fontium* 24613. Moreover, the proportion of pseudogenes was larger in *P. fontium* (4.1%) than in the draft genomes of other *H*$_2$S-producing enterobacteria (ie, 2.8% and 1.6% for *B. aquatica* and *L. grimontii*, respectively), suggesting genome decay in *P. fontium*. In addition, a clearly higher GC content was found in the *L. grimontii* DSM 5078T genome. The draft status was likely responsible for the lower number of predicted rRNA and transfer RNA genes in the *P. fontium* DSM 5563 T, *B. aquatica*, and *L. grimontii* genomes.

**Phylogenetic position of *P. fontium***. A whole genome phylogenetic approach was used to compare the genome sequence of *P. fontium* 24613 with genome sequences of other enterobacterial genera. The relevant part of the *Enterobacteriaceae* tree is shown in Figure 1. Strong support was found for a close relationship among *Pragia* and other atypical *H*$_2$S producers, including *Budvicia* and *Leminorella*. The high similarity among genomes was also supported by a dot plot analysis of *H*$_2$S producer genomes (Figure S1). Another related genus was *Plesiomonas*, an oxidase-positive genus recently reclassified into the *Enterobacteriaceae* family. A sister clade contains a cluster of genera occurring frequently in the environment (*Providencia*, *Moellerella*, *Proteus*, and *Morganella*), (2) genera associated with nematodes (*Xenorhabdus*, *Photorhabdus*), and (3) endosymbionts (*Arsenophonus*, *Buchnera*, and *Wigglesworthia*). Except for the delineation of *Proteus* vs *Morganella* and endosymbionts *Buchnera* vs *Wigglesworthia*, all other branches were supported by bootstrap values higher than 99%.

### Table 1. Genome features of *Pragia fontium* 24613 in comparison with the draft genome of the type strain and the draft genomes of closely related hydrogen sulfide producers.

| FEATURE                      | *P. FONTIUM 24613* | *P. FONTIUM DSM 5563T* | *B. AQUATICA DSM 5075T* | *L. GRIMONTII DSM 5078T* |
|------------------------------|--------------------|-------------------------|--------------------------|---------------------------|
| Genome status                | Complete           | Draft                   | Draft                    | Draft                     |
| Genome size                  | 4 094 629 bp       | 3 950 845 bp            | 5 670 930 bp             | 4 222 128 bp              |
| GC content                   | 45.38%             | 45.23%                  | 45.68%                   | 53.86%                    |
| No. of CDS                   | 3579               | 3464                    | 5130                     | 3878                      |
| No. of rRNA genes            | 22 (8–7–7)         | 10 (2–5–3)              | 7 (5–2–0)                | 16 (8–6–2)                |
| No. of tRNA genes            | 72                 | 58                      | 57                       | 57                        |
| No. of pseudogenes           | 146 (4.1%)         | NA                      | 144 (2.8%)               | 62 (1.6%)                 |
| No. of genes with predicted function | 2809 (78.49%) | 2862 (82.62%) | 3896 (75.95%) | 3083 (79.50%) |
| No. of genes assigned to COG | 2601 (72.67%)      | 2613 (75.43%)           | 2601 (72.67%)            | 2804 (72.31%)             |
| No. of genes assigned to KEGG pathways | 1160 (32.41%) | 1172 (33.83%) | 1419 (27.66%) | 1217 (31.38%) |

Abbreviations: CDS, coding sequences; COG, Clusters of Orthologous Group; KEGG, Kyoto Encyclopedia of Genes and Genomes; rRNA, ribosomal RNA; tRNA, transfer RNA.

Accession numbers of the whole genome sequences of the type strains are listed in Table S1. Order of the rRNA genes in parentheses: 5S-16S-23S. NA—data not available in the GenBank and Genomes OnLine databases.
metabolism compared with the \textit{L. grimontii} and \textit{B. aquatica} genomes (Table S3).

\section*{Genome methylation pattern}

Analysis of PacBio sequencing data revealed 24,814 methylated positions of the \textit{m6A} type, but only a single sequence motif (GATC) was found in all these modifications. More than 80\% (21,735 of 26,606) of the GATC positions in the genome were methylated. Methylation type \textit{m4C} was not found. Kinetic signatures of \textit{m5C} were subtler than signatures of \textit{m6A} and \textit{m4C} and harder to detect using PacBio SMRT sequencing\textsuperscript{25}; therefore, they were not assessed. The results of \textit{P. fontium} genome methylation were deposited in the REBASE PacBio database (http://rebase.neb.com/cgi-bin/pachliost).\textsuperscript{14} In total, 8 different putative restriction-modification systems, all of them type II, were predicted in the genome (Table S4). Seven of them consisted of only methyltransferases, whereas the last one modifying \textit{m5C} consisted of methyltransferase, mismatch repair endonuclease, and restriction endonuclease.

\section*{Virulence and antimicrobial genes in the \textit{P. fontium} genome}

In silico analysis of virulence determinants of the \textit{P. fontium} genome revealed genes involved in iron acquisition (encoding Fe\textit{2+} and Fe\textit{3+} transport systems), adhesion (encoding P pilii and type I pilii), secretion systems (TISS and T6SS), and antibiotic resistance (encoding AmpC \beta-lactamase and several efflux pump) (see Table S5). Production of tailocins, ie, R-type and F-type bacteriocins resembling phage tails, was previously detected in several \textit{Pragia} strains.\textsuperscript{26} Gene clusters similar to the phage genes were described as being responsible for production of these antimicrobial compounds.\textsuperscript{27} A total of 6 clusters homologous to phage genes were predicted in the \textit{P. fontium} genome, and one of them was likely responsible for tailocin production (see Table S5). The genome search also detected a gene encoding a colicin-like bacteriocin, a homologue of pyocon S3.

\section*{Metabolic profiling of \textit{P. fontium} 24613}

The carbohydrate utilization pattern resulting from the testing of various saccharides, carboxylic acids, alcohols, amino acids, aromatic compounds, and their derivatives was determined for \textit{P. fontium} 24613, \textit{B. aquatica} CNCTC 6285\textsuperscript{T}, and \textit{L. grimontii} CCM 4003\textsuperscript{7}. In general, the data obtained from the Biolog assay revealed low levels of metabolic activity in all tested strains. Substrate utilization profiles differed for the 3 tested H\textsubscript{2}S producers in 17 substrates (Table S2). \textit{Pragia fontium} 24613 was able to utilize 15 substrates (out of 95; 16\%) under aerobic conditions and 22 (out of 95; 23\%) under anaerobic conditions. \textit{Pragia} utilized monosaccharides and their derivatives (\textalpha-d-glucose, \textalpha-d-glucose-1-phosphate, d-glucose-6-phosphate, N-acetyl-d-glucosamine, and \textbeta-methyl-d-glucoside), mono-carboxylic acids (d,l-lactic acid, and d-gluconic acid), dicarboxylic acids (\textalpha-keto-glutaric acid, and l-glutamic acid), alcohols and their derivatives (glycerol, d,l-\alpha-glycerol phosphate, myo-inositol, and xylitol), amino acids (d-serine), and aromatic compounds (uridine and thymidine). In addition to substrates

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{The Venn diagram represents the core genome and pangenome of \textit{Pragia} and the closely related atypical H\textsubscript{2}S producers. The numbers represent the gene clusters shared by corresponding group of genera. The diagram shows the close relationships among those inside the group of atypical H\textsubscript{2}S producers.}
\end{figure}

\section*{The core genome of enterobacterial hydrogen sulfide producers}

The core genomes of \textit{P. fontium}, \textit{B. aquatica}, and \textit{L. grimontii} contain 2327 gene clusters (ie, at least 1 gene from each cluster was found in each genome; Figure 2). The number of gene clusters exclusively shared by 2 genomes was higher for the \textit{P. fontium} and \textit{B. aquatica} genomes (325) compared with the \textit{P. fontium} and \textit{L. grimontii} genomes (109), whereas there were 494 clusters shared by the \textit{L. grimontii} and \textit{B. aquatica} genomes. These data indicate a higher degree of relatedness between \textit{P. fontium} and \textit{B. aquatica} compared with \textit{P. fontium} and \textit{L. grimontii}. A set of 30 gene clusters was unique for the \textit{P. fontium} genome; these clusters encoded homologues to fimbrial genes found in \textit{Serratia} spp. and \textit{Pragia} spp. and also homologues to pyocin S3 and its immunity protein–encoding genes. In total, 10 clusters encoded genes for hypothetical proteins.

\section*{Genome-based metabolic and virulence analyses}

\textit{Analysis of metabolic pathways in the \textit{P. fontium} genome.} Based on the genomic data analysis from KEGG PATHWAY and DOE-JGI, aerobic and facultative anaerobic metabolic of \textit{P. fontium} 24613 was predicted. Oxidized nitrogen and sulfur compounds were capable of serving as alternative terminal electron acceptors under anaerobic conditions. Identification of thiosulfate reductase, responsible for H\textsubscript{2}S production, corresponded to previously detected enzyme activity.\textsuperscript{6} The genes involved in glycolysis, citrate cycle, and pentose phosphate pathway could also be found in the \textit{P. fontium} genome in addition to genes responsible for amino acid, fatty acid synthesis, lipid, and nucleotide metabolism. \textit{Pragia} was found to be auxotrophic for l-tryptophan, l-histidine, and l-leucine and deficient in biotin synthesis. Compared with \textit{Budvicia} and \textit{Leminorella}, \textit{Pragia} was able to synthesize l-arginine but lacked the genes for fatty acid degradation. In addition, the \textit{P. fontium} genome contained fewer genes involved in carbohydrate
utilized under aerobic conditions, anaerobically cultivated "Pragia" utilized l-arabinose, pyruvic acid methyl ester, β-glucuronic acid, bromosuccinic acid, l-aspartic acid, glycyl-l-aspartic acid, and l-serine. Although Budvicia utilized 16 substrates (17%) aerobically and 24 (25%) anaerobically, Leminorella utilized only 13 substrates (14%) aerobically and 18 (19%) anaerobically. Budvicia and Leminorella were able to metabolize several amino acids and their derivatives (l-asparagine, l-aspartic acid, and glycyl-l-aspartic acid) as well as derivatives of organic acids from Krebs cycle (pyruvic acid methyl ester, bromosuccinic acid), which were not utilized by Pragia. The complete results of this assay are shown in Table S2. In most of the substrate tests, which differed among H2S producers, the genes encoding corresponding catabolic enzymes or enzymes possibly involved in metabolism of these compounds were found (Table S6). The only exception was the B. aquatica genome, where some of the genes responsible for catabolism of uridine were not found.

**Discussion**

*Pragia* belongs to a relatively small group of H2S-producing enterobacteria containing *P. fontium*, *Budvicia* spp., and *Leminorella* spp. Although all members of this small group are closely related and have a relatively similar biochemical profile, they occupy quite different ecological niches. Although *Budvicia* was originally isolated from freshwater,28 several other isolates have been described from the intestinal microflora of insects,26,29 *Diplopoda*,5 and salmonids.30 A possible clinical relevance for *B. aquatica* was reported by Corbin et al4 when this bacterium was isolated from a human clinical sample. *Leminorella* spp. have been exclusively isolated from human clinical specimens and no environmental sources have been reported. Although its clinical significance is unclear,1 although the presence of *Pragia* has been detected in the intestines of freshwater salmon,35 the much more frequent isolation from deepwater wells6 tends to support a free-living lifestyle of *Pragia*. Both detected secretion systems, T1SS and T6SS, are widely distributed in gram-negative bacteria,36,37 and could mediate interaction with a host or with another bacterium.38 Although the contribution of T6SS to pathogenesis has been described for several bacteria, eg, *Pseudomonas*39 and *E. coli*,40 T6SS has also been found in saprophytic bacteria, where it was involved in interactions across the microbial community.38 Several bacteriocin types have been suggested as putative virulence factors, whereas the importance of others was demonstrated in interactions across microbial community.41 Although the function of *P. fontium* bacteriocins remains unknown, both tailocins and colicin-like homologues were found in the *Pragia* genome. The GATC methylation motif was found in the *P. fontium* genome, and because the corresponding gene for the restriction enzyme recognizing this motif was not found, methylation appears to be more connected to gene expression regulation43 and not to degradation of foreign nucleic acid molecules.

Metabolic profiling revealed a metabolic pattern for *Pragia*, *Budvicia*, and *Leminorella*, which was quite distinct from other enterobacteria,44 supporting the distinctness of enterobacterial H2S producers and also the close relationship of these bacteria within this group. Despite their overall similarity, H2S-producing enterobacteria revealed several differences in their ability to utilize substrates. Analyses of genomic data supported the metabolic findings, with only 1 case in which some of the genes encoding expected enzymatic activity were not found. This is likely a result of an incomplete genomic sequence in *Budvicia*. Surprisingly, all species were able to degrade multiple substrates under anaerobic conditions suggesting that alternative electron acceptors (nitrate, reduced sulfur compounds) could be used under anaerobic conditions. Nitrogen oxidation could be carried out using the “nitrite reduction to ammonium pathway” for which the corresponding genes were found in the *P. fontium* genome. This pathway is preferred for respiration under anaerobic conditions, and it is common across *Enterobacteriaceae* and in other facultatively anaerobic bacteria.45

A comparative genomics approach revealed that almost 80% of the gene clusters were shared by H2S-producing enterobacteria, whereas only 49% were shared when *E. coli* K12 was added to the analysis. Analysis of the complete genome sequence of *Pragia* revealed that the genome contains genes involved in essential metabolic pathways, in nutrient metabolism, and also in the synthesis of most of the amino acids.
However, the “fatty acid degradation pathway” is missing from the *P. fontium* 24613 genome. This pathway is present in most enterobacterial genomes but not in invertebrate endosymbionts with a reduced genome, such as *Wigglesworthia* and *Buchnera*. Nevertheless, when compared with these endosymbionts, the *P. fontium* genome is relatively large and also contains an additional set of genes, eg, those responsible for degradation of more complex polysaccharides. However, *P. fontium* 24613 has a relatively small genome in comparison with other enterobacteria, even in comparison with the genus *Budvicia*. In addition, the proportion of pseudogenes was larger in *Pragia* compared with other closely related bacteria (despite their draft status, which is prone to assembly errors).

Larger proportions of pseudogenes have also been observed in bacteria that were associated with or dependent on eukaryotic hosts. Nevertheless, this analysis comes from a limited number of genome sequences per species and it is known that the prevalence of pseudogenes is quite variable even among closely related strains. A reduction in genome size and an increased number of pseudogenes are common signs of bacterial adaptation to a eukaryotic host. In addition, the *P. fontium* genome contains fewer genes involved in carbohydrate utilization compared with other *H. S.* producers; a large battery of degradation enzymes is important mainly for free-living bacteria. The traces of genome decay (ie, small genome, absence of fatty acid degradation pathways, the small number of genes associated with carbohydrate utilization, and a larger proportion of pseudogenes) suggest an ongoing process of adaptation to a particular host organism. Although no such host has been identified for *P. fontium*, the recent progress in metagenome studies could help to answer this question in the near future.

**Conclusions**

Analysis of the complete genome sequence of *P. fontium* 24613 and metabolic profiling confirmed the close relatedness of this bacterium to other *H. S.*-producing enterobacteria, *Budvicia* spp. and *Leminorella* spp., although for each genus a different environmental niche has been described. Virulence gene mining and the absence of *Pragia* 16S rDNA sequences in the human metagenomics data suggest limited pathogenic potential for *Pragia*, consistent with the previously described free-living lifestyle of this bacterium. On the contrary, reduced genome size, limited number of encoded enzymes for carbohydrate and fatty acid degradation, and frequent presence of pseudogenes suggest a process of adaptation to an as-yet-unknown host.

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**Author Contributions**

KSn and DS conceived, designed, and performed the experiments (genome sequencing). IS performed phenotypical characterization. KSn, KSe, and IP analyzed the data. KSn wrote the first draft of the manuscript. DS and JB contributed to the writing of the manuscript. All authors reviewed and approved the final manuscript.

**Disclosures and Ethics**

As a requirement of publication, author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including, but not limited to, the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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