**Paracoccus sordidisoli** sp. nov., isolated from an agricultural field contaminated with hexachlorocyclohexane isomers

Amit Kumar Singh, Puneet Kohli, Nitish Kumar Mahato and Rup Lal*

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**Abstract**

A novel bacterial strain, designated LP91\(^T\), was isolated from an agricultural field contaminated with hexachlorocyclohexane (HCH) isomers at Ummari Village, Lucknow, Uttar Pradesh, India. Cells of the strain were aerobic, short rod or cocoid, Gram-stain-negative and non-motile. Colonies of the strain were initially transparent but with time changed to a creamy white colour. Phylogenetic analysis based on the 16S rRNA marker gene showed that it was closely associated with *Paracoccus aestivaliivorans* GHD-30\(^T\) (99.1\%) and *Paracoccus limosus* NB88\(^T\) (98.0\%), followed by *Paracoccus laevigulosivorans* 43P\(^T\) (97.9\%) and *Paracoccus marinus* KKL-A5\(^T\) (97.0\%). The DNA–DNA hybridization values of strain LP91\(^T\) with the closely related type strains mentioned above were below 51.2±0.64\%\, confirming it as a distinct species from other known species of the genus *Paracoccus*. The major cellular fatty acids of strain LP91\(^T\) were C\(_{18:0}\)ω7c/C\(_{18:0}\)ω6c and C\(_{16:0}\). The major polar lipids were diphasphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and aminophospholipid, along with other lipids including glycolipids, aminolipids and other unknown phosphoglycolipids. Spermine was the major polypeptide, along with putrescine in a minor amount. Ubiquinone (Q-10) was the sole isoprenoid quione. Based on the results of phylogenetic, phenotypic and chemotaxonomic analysis, it is proposed that the isolate represents a new species of the genus *Paracoccus*, for which the name *Paracoccus sordidisoli* sp. nov. is proposed. The type strain is LP91\(^T\) (=KCTC 42938\(^T\)=CCM 8696\(^T\)=MCC 3128\(^T\)).

The genus *Paracoccus* belongs to the alpha subgroup of the proteobacteria, family *Rhodobacteraceae*. This genus was introduced while describing the type species *Paracoccus denitrificans* [1]. The genus *Paracoccus* comprises species that are Gram-stain-negative, short rod or cocoid, non-motile, catalase- and oxidase-positive, having a high G+C DNA content, Q-10 as the major respiratory quinone and summed feature 8 as one of the major fatty acid components. In addition, most members of the genus *Paracoccus* have been reported to accumulate poly-β-hydroxybutyrate granules. At the time of writing, the genus *Paracoccus* consisted of 51 recognized species, including *Paracoccus paracisoli* [2], *Paracoccus sanguinis* [3], *Paracoccus angustae* [4], *Paracoccus laevigulosivorans* [5], *Paracoccus aestivaliivorans* [6], *Paracoccus cavernae* [7] and *Paracoccus contaminans* [8] as the most recently described species. These bacteria have a broad catabolic potential and they can switch between different growth modes according to the environmental conditions, e.g. heterotrophic growth on organic compounds, chemolithoautotrophic growth on reduced sulfur compounds and using hydrogen or ferrous ions as the energy sources [9]. Members of the genus *Paracoccus* have been isolated from a wide range of environmental niches, including soil [4], biofilters [10], water contaminated with dichloromethane [11], a sulphide-oxidizing denitrifying fluidized bed reactor [12] and waste water from a semiconductor manufacturing plant [13]. Most *Paracoccus* strains have been reported to use sulphur compounds as a source of nutrient such as thiosulphate, thiocyanate, carbondisulphide and are also able to degrade these compounds [14]. Thus, strains belonging to genus *Paracoccus* are potential bioremediation candidates because of their ability to metabolize a wide range of organic compounds and use nitrate as an alternative electron acceptor. In the recent past, members of this genus have been used in bioremediation of sites contaminated with polycyclic aromatic hydrocarbons [15].

During a survey to explore the microbial diversity of an area contaminated with hexachlorocyclohexane (HCH) [16–21], one novel bacterial strain, LP91\(^T\), was isolated and selected for detailed polyphasic taxonomic study. Strain LP91\(^T\) was isolated from an agricultural field contaminated with HCH isomers near a HCH dumpsite at Ummari Village, Lucknow, Uttar Pradesh, India (27° 2’ 5.673” N and 81° 8’ 18.253” E). The soil samples were collected from this site on (14 May 2014) and transported to the laboratory at 4°C.
Strain LP91\textsuperscript{T} was isolated and cultured using a mineral salt medium, with the following constituents: (NH\textsubscript{4})\textsubscript{2}HPO\textsubscript{4} 0.5 g l\textsuperscript{−1}, MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O 0.2 g l\textsuperscript{−1}, FeSO\textsubscript{4} \cdot 7H\textsubscript{2}O 0.01 g l\textsuperscript{−1}, K\textsubscript{2}HPO\textsubscript{4} 0.1 g l\textsuperscript{−1}, Ca(NO\textsubscript{3})\textsubscript{2} 0.01 g l\textsuperscript{−1} and supplemented with 0.3 % glucose (pH 7.5) [22]. Serial dilutions were spread on Luria–Bertani (LB) agar plates containing streptomycin (10 µg ml\textsuperscript{−1}) and nystatin (20 µg ml\textsuperscript{−1}). The single bacterial colonies that appeared were streaked several times onto the growth media at 28 °C in an attempt to obtain a pure culture. The isolated strains were routinely cultured on LB agar plates, and also maintained as glycerol stock (20 %, v/v) at −80 °C for long-term storage. A cream-coloured colony that appeared after 3–4 days was designated strain LP91\textsuperscript{T}. The characterization of this bacterial strain was done by using a polyphasic approach [23, 24].

Cell morphology was analysed using a light microscope (Olympus) and a transmission electron microscope (TEM 269D; Morgangni, Fei) after growing the strain on LB agar at 28 °C for 2 days (Fig. S1, available with the online Supplementary Material). Cells of the strain were Gram-stain-negative, aerobic, rod-shaped (0.4–0.7 µm in diameter and 0.8–1.3 µm long) and non-motile.

For phylogenetic analysis of strain LP91\textsuperscript{T}, genomic DNA was isolated by following the standard protocol described by Sambrook et al. [25]. 16S rRNA gene amplification and sequencing was carried out using universal primers UNI16S-L (ATTCTAGAGTTTGATCATGGCTCA) and UNI16S-R (ATGGTACCGTGATGACGGCGGTGTGTA) [26, 27] at the University of Delhi, South Campus, Delhi. PCR was carried out in a thermocycler (ABI 2700; Applied Biosystems) under the following conditions: denaturation at 95 °C for 5 min, denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min 30 s, final extension at 72 °C for 7 min and hold at 4 °C. The 16S rRNA gene sequence was assembled using Sequencing Analysis, version 5.1.1 (Applied Biosystems), and Clone Manager software version 5 (Sci-Ed Software). A nucleotide sequence of length 1400 bp of the 16S rRNA gene of strain LP91\textsuperscript{T} was obtained. The closest phylogenetic neighbours were identified by using the BLAST program of the NCBI (www.ncbi.nlm.nih.gov), and confirmed by using the seq match tool of the RDP (https://rdp.cme.msu.edu/) and EzTaxon-e-Server (http://www.ezbiocloud.net/) [28]. Strain LP91\textsuperscript{T} showed highest 16S rRNA gene sequence similarity to P. aesteruarivivens GHD-30\textsuperscript{T} (99.1 %), followed by P. limosus NB88\textsuperscript{T} (98.0 %), P. laeviglucosivorans 43P\textsuperscript{T} (97.9 %) and P. marinus KKL-A5\textsuperscript{T} (97.0 %). The degree of similarity with other members of Paracoccus was in the range of 93.4–96.9 %. Nearly complete 16S rRNA gene sequences that were closely related to strain LP91\textsuperscript{T} were obtained from GenBank for the reconstruction of a phylogenetic tree. The 16S rRNA gene sequences of Rhodobacter capsulatus ATCC 11166\textsuperscript{T} and Rhodobacter sulphidophilum DSM 1374\textsuperscript{T} were used as out-groups. The CLUSTAL\_X program was used for alignment of 16S rRNA gene sequence of strain LP91\textsuperscript{T}, along with corresponding sequences belonging to genus Paracoccus. Reconstruction of phylogenetic trees was carried out by using the neighbour-joining (NJ) (Fig. 1) [29], maximum-likelihood (ML) (Fig. S2) and maximum-parsimony (MP) (Fig. S3) [30] algorithms using MEGA 7.0 software [31]. The resultant tree topologies were figured out by bootstrap strap analysis [32] based on 1000 replications. Strain LP91\textsuperscript{T} clustered with GHD-30\textsuperscript{T} in the NJ and ML phylogenetic trees, but in the MP tree the clustering was somewhat different. It is possible that MP method is not statistically consistent when a large number of taxa is considered due to its dependency on character-based phylogeny. Strain LP91\textsuperscript{T}, P. aesteruarivivens GHD-30\textsuperscript{T}, P. laeviglucosivorans 43P\textsuperscript{T} and P. limosus NB88\textsuperscript{T} were very closely related to each other as they formed a very tight cluster in the NJ and ML phylogenetic trees. The phylogenetic analysis further indicated that strain LP91\textsuperscript{T} belongs to the genus Paracoccus.

Gliding motility of the organism was examined using fresh LB broth culture following the hanging-drop method [33] along with motility agar. The growth of strain LP91\textsuperscript{T} was checked on different media, including marine agar (HiMedia), LB agar (HiMedia), brain heart infusion agar (HiMedia), R2A agar (HiMedia), nutrient agar (HiMedia), tryptone soya yeast extract agar (HiMedia) and brilliant green agar (HiMedia). Gram-staining was performed using Gram staining kit (bioMérieux). Oxidase activity was measured using 1 % (w/v) N,N,N,N-tetramethyl-1,4-phenylenediamine reagent (bioMérieux) according to the manual provided with the kit. Catalase activity was performed by analysing bubble production after the application of drops of 3 % (v/v) hydrogen peroxide solution on freshly grown colonies on LB agar medium. Hydrolysis of DNA was tested using DNase test agar (SRL). Citrate utilization was tested using Simmons’ citrate agar (HiMedia). Hydrolysis of casein and starch was determined as described by Cowans and Steel [34]. Hydrolysis of Tween 20 and 80 was determined according to Arden-Jones et al. [35]. The presence of flexi-ubin-type pigments was examined using a 20 % (w/v) KOH solution [36, 37]. Degradation of HCH isomers by strain LP91\textsuperscript{T} was examined as this strain was isolated from an area containing high levels of HCH (0.7 mg g\textsuperscript{−1} HCH) [38, 39] following the protocol described by Kumari et al. [40]. For other biochemical tests and carbon source utilization, API 20NE and API 50CH kits were used according to the manufacturer’s instructions (bioMérieux). Antibiotic susceptibility was checked according to Kirby–Bauer’s method [41] by using Mueller–Hinton II agar with readymade antibiotic discs (HiMedia) containing varying amounts of antibiotics (µg per disc): streptomycin (10), ampicillin (10), chloramphenicol (30), nalidixic acid (30), vancomycin (30), penicillin (10), polymyxin B (300), kanamycin (30), oxytetracyclin (30), amikacin (30), tetracycline (30), gentamycin (10), rifampicin (5) and ciprofloxacin (5). Growth of the strain at different temperatures (4, 8, 15, 24, 30, 37, 45 and 55 °C) was measured by incubating the strain LP91\textsuperscript{T} in LB broth followed by measuring the OD\textsubscript{600} at respective temperatures. The pH range (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0) for growth was examined as described by Ardens-
Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequence data showing the evolutionary relationship of strain LP91T and other representative members of genus Paracoccus. The tree was reconstructed by using neighbour-joining method in MEGA 7.0 software and the rooting was done by using Rhodobacter capsulatus ATCC 11166T and Rhodobacter sulfidophilum DSM 1374T as outgroups. Bar, 0.01 nucleotide substitutions per 1000 nucleotide positions. Bootstrap values > 70% based on 1000 replications are shown at branch points. The Genbank accession numbers for the 16S rRNA gene sequences of each strain are shown in parentheses.
Jones et al. [35]. The following compositions of buffered media were used to restrict the change in pH after autoclaving the media: sodium acetate buffer for pH 3–4; K$_2$HPO$_4$, KH$_2$PO$_4$ buffer for pH 5–8; NaHCO$_3$, NaOH buffer for pH 9–11; and Na$_2$CO$_3$-NaOH buffer for pH 12. Growth at different salt concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 %, w/v, NaCl) was measured by using manually prepared LB broth, with varying amounts NaCl.

The bacterial strain LP91$^T$ was able to grow at the following range of temperature, pH and % NaCl: 8–40 °C (optimum 28 °C), pH 5–11 (6–9) and with 0–6 % NaCl (0–4 %). It was able to grow on marine agar, brain heart infusion agar, LB agar, R2A agar, nutrient agar and tryptone soya yeast extract agar. It was able to metabolize/hydrolyse Tweens 20 and 80, DNA, urea, aesculin, ferric citrate, D-glucose, L-arabinose, D-mannose, N-acetyl-glucosamine, adpic acid, malic acid and trisodium citrate. Although this strain was isolated from a highly contaminated HCH dumpsite (0.7 mg g$^{-1}$) [38], it was not able to degrade any of the HCH isomers as confirmed by degradation assay on a gas chromatograph.

For analysis of fatty acids, cell biomasses of strain LP91$^T$ and the reference type strains were harvested from an LB agar plate after incubation at 28 °C for 2 days. The physiological age of strain LP91$^T$ and the reference strains were standardized using the standard protocol as described in the MIDI manual (www.microbialid.com/PDF/Technote_101.pdf). The fatty acids were analysed using the standard method of RTSBA6 by using the Sherlock Microbial Identification System, version 6.2, at Royal Life Science, Secunderabad, India (Table S1).

The bacterial cell mass for study of polar lipids, quinones and polyamines was obtained by growing cultures in LB broth at 28 °C followed by lyophilization and storing at −20 °C for further analysis. Analysis of polar lipids of strain LP91$^T$ was performed using the protocol described by Bligh and Dyer [42]. The total lipids were identified using molybdatephosphoric acid (Fig. S4a), while characteristic lipids were identified using specific reagents, which included molybdenum blue (Fig. S4b) and ninhydrin (Fig. S4c). The major polar lipids were diphasphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and aminophospholipid, along with other lipids including two glycolipids, three aminolipids, two unknown lipids and another unknown phosphoglycolipid.

Quinones were extracted by using the standard method [43]. The extracted quinones were further analysed by a reverse phase thin-layer chromatography [44]. For analysis of quinones, strain P. limosus NB88$^T$ was used as a reference strain. Ubiquinone Q-10 was the sole isoprenoid quinone of strain LP91$^T$, which was in congruence with the description of the genus.

Polyamines were extracted and analysed using the protocol described by Busse and Auling [45]. Standards for polyamines (spermine, spermidine, and putrescine) were obtained from Sigma-Aldrich and activated as described earlier [26]. The internal standard was 1,8-diamino-octane (Himedia). The extracted polyamines were analysed by high-performance liquid chromatography (Agilent Technologies 1220 infinity LC) using an Agilent Zorbax SB18-C18 column (250×0.5 mm, 5 µm) with 60 % methanol as the mobile phase at a flow rate of 0.3 ml min$^{-1}$. The analysis was carried out by using a UV/Vis detector set at 234 nm. Spermidine was the major polyamine detected, along with a minor amount of putrescine in strain LP91$^T$, while strain P. limosus NB88$^T$ contained a major amount of spermidine.

The DNA–DNA hybridization was performed by using a fluorimetric method [46] to determine the level of genomic relatedness among the bacterial type strains whose 16S rRNA gene sequence was greater than 97.0 %. This procedure utilizes spectrophotometric DNA reassociation kinetics as described by De Ley et al. [47] The DNA–DNA hybridization values were calculated using the De Ley equation [47]. The hybridization was performed in five replicates, but the actual result reported was the mean of three intermediate values excluding the extreme values. The results obtained for DNA–DNA hybridization were well below the 70 % recommended for delineation of new species [48] (Table S2). The DNA G+C content was determined according to the procedure described by Gonzalez and Saiz-Jimenez [49] by using an Applied Biosystems 7500 Real-Time PCR system along with a control to determine the authenticity of results. The G+C content of strain LP91$^T$ was found to be 67.2 mol%.

The phenotypic differences between strain LP91$^T$ and P. aestuariiivivens GHD-30$^T$ included their different results for growth temperature, pH and salinity (Table 1). Growth of strain LP91$^T$ occurred at 8–40 °C, 0–6 % NaCl (w/v) and pH 5–11, while GHD-30$^T$ grew at 15–45 °C, 0–8 % NaCl and pH 5–7.5. Apart from these results, their differential ability to utilize various carbon sources are as follows: strain LP91$^T$ was able to metabolize starch, Tween 80, N-acetyl-glucosamine and adpic acid, whereas it was unable to metabolize casein, cellobiose, trehalose and reduce nitrate to nitrite. On the other hand strain, GHD-30$^T$ was able to metabolize casein, cellobiose, trehalose and reduce nitrate to nitrite, but unable to metabolize starch, Tween 80, N-acetyl-glucosamine and adpic acid. However, strain LP91$^T$ and GHD-30$^T$ produced similar results for the following tests (+, positive; −, negative): oxidase (+), indole production (−), urease (+), aesculin (+), gelatin (−), β-galactosidase (−), potassium gluconate (−), trisodium citrate (+), L-arabinose (+), D-galactose (+), D-glucose (+), D-fructose (+), D-mannose (+), esculin ferric citrate (+). Moreover, the strains shared several chemotaxonomic characteristics, i.e. the major fatty acids, polar lipids and quinones, which corresponded to the description of the genus. Strain LP91$^T$ showed significant differences when compared to its closest four phylogenetic relatives, i.e. P. aestuariiivivens GHD-30$^T$, P. laevigilcosivorans 43B$^T$, P. limosus NB88$^T$ and P. marinus KKL A5$^T$, as it was able to grow at a broad range of temperature (8–40 °C) and pH (5–11). The phenotypic differences
mentioned above, along with phylogenetic and genetic differences of strain LP91\(^T\), suggest that the novel strain is separated from other species of the genus *Paracoccus*. On the basis of phylogenetic, genotypic and phenotypic data, strain LP91\(^T\) should be assigned to genus *Paracoccus*, for which the name *Paracoccus sordidisoli* sp. nov is proposed.

**DESCRIPTION OF PARACOCCUS SORDIDISOLI SP. NOV.**

*Paracoccus sordidisoli* (sor.di.di.so’li. L. adj. sordidus, dirty; L. n. solum, soil; N.L. gen. n. sordidisoli, from dirty soil, referring to the source of isolation).

Cells are Gram-stain-negative. 0.4–0.7 μm in diameter and 0.8–1.3 μm long. Catalase- and oxidase-positive. Colonies are circular, smooth, mucoid and creamy white in colour. Poly-β-hydroxy-butyrate granules are present in the cells. Growth occurs at 8–40 °C (optimum 28 °C), pH 5–11 (6–9) and with 0–6 % NaCl (0–4 %). Strain LP91\(^T\) is able to grow on marine agar, brain heart infusion agar, LB agar, R2A agar, nutrient agar and tryptone soya yeast extract agar. Cells can hydrolyse Tween 20 and 80, and DNA, but not casein. Sensitive to streptomycin, chloramphenicol, vancomycin, penicillin-G, polymyxin B, kanamycin, oxytetracycline, amikacin, tetracycline, gentamycin, rifampicin and ciprofloxacin, but resistant to ampicillin and nalidixic acid. In the API 20 NE test, negative for reduction of nitrates to nitrites, arginine dihydrolase, gelatinase, β-galactosidase, D-mannitol, maltose, potassium gluconate, capric acid and phenylacetic acid. Positive results are obtained for utilization of D-glucose, urea, aesculin ferric citrate, fermentation of D-glucose, L-arabinose, D-mannose, α-acetamido-3-glucosaminic acid, succinic acid and sodium acetate. According to the API 50CH test, positive results are obtained for the following sugars, D-arabinose, starch, L-arabinose, D-ribose, D-xylene, L-xylene, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, isonitrose, aesculin ferric citrate, D-lyxose, D-fucose and D-and fucose. Negative results are obtained for glycerol, erythritol, D-adonitol, methyl-β-D-xlyopyranoside, L-sorbose, dulcitol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellubiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melizitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-tagatose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and

**Table 1. Differential morphological and physiological characteristics of strain *P. sordidisoli* LP91\(^T\), *P. aestuariivivens* GHD-30\(^T\), *P. laevigatosivorans* 43P\(^T\), *P. limosus* NB88\(^T\), *P. marinus* KKL-A5\(^T\), *P. contaminans* WPAno2\(^T\), *P. pacificus* F14\(^T\) and *P. denitrificans* KCTC 2530\(^T\).**

<table>
<thead>
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<th>Characteristics</th>
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<tr>
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<td>Short rods/Coccoid</td>
<td>Coccoid</td>
<td>Short rods</td>
<td>Short rods</td>
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<tr>
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<td>Yellowish white</td>
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<td>Dull orange</td>
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<td>1–4</td>
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<td><strong>Reduction of nitrates</strong></td>
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<td><strong>Hydrolysis of</strong></td>
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<tr>
<td><strong>DNA G+C content (mol%)</strong></td>
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<td>62.0</td>
<td>64.1</td>
<td>66.4</td>
<td>69</td>
<td>66.1</td>
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</table>

*All data from this study.
potassium 5-ketogluconate. Strain LP91T produces acid with the following sugars: L-arabinose, D-xylene, D-galactose, D-glucose, D-mannose and D-fucose. Flexirubin-type pigments are absent. Growth takes place on marine agar, brain heart infusion agar, nutrient agar and brilliant green agar, and limited growth occurs on R2A agar. The major isoprenoid quinone is Ubiquinone Q-10. The major fatty acids are summed feature 8, which includes C18:0 3ω7c/ω6c and C16:0. The major polar lipids are diphasophatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and aminophospholipid, along with other lipids including glycolipids, aminolipids and other unknown phosphoglycolipids commonly reported for the genus Paracoccus. Spermidine is the major polyamine along with a minor amount of putrescine. The DNA G+C content of the strain is 67.2 mol%. The type strain, LP91T (CCTC 42938T =CCM 8696 =MCC 3128T), was isolated from an agricultural field contaminated with HCH, at Ummari village, Lucknow, Uttar Pradesh, India.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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