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Prevalence and Characterization of *Legionella pneumophila* and Related Species from Water-Based Recreational Sites

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Abstract

Background and Objectives *Legionella* is a genus of gram-negative bacteria common in soil and aquatic systems and is associated with protists. They have emerged as a pathogenic group due to the increased use and poor maintenance of artificial water environments. This study aims at phenotypic and molecular identification of *Legionella* in water and swab samples collected from water-based recreational sites. The biofilm-forming ability of *Legionella* on exposure to various temperatures and iron concentrations was also studied.

Methods A total of 60 samples including 30 swab samples and 30 water samples (decorative fountain ponds, swimming pools, garden sprinklers, drip irrigation system) were collected from in and around Mangalore, Karnataka, India. From each source, swab and water samples were collected as per the Indian standard IS: 1622. The collected samples were processed within 4 hours of collection. The samples were subjected to microbiological and chemical estimation followed by filtration through a 0.2 µm membrane filter. Isolation of *Legionella* from collected samples was performed as per US Centers for Disease Control and Prevention guidelines 2005. The positive isolates were then checked for biofilm-forming ability at various temperatures (25°C, 35°C, and 45°C) and iron concentration (3 mg/L, 30 mg/L, and 300 mg/L) using crystal violet assay.

Results Out of 30, water and swab samples tested, one water sample from a garden sprinkler, swimming pool, and one from both water and swab samples from a decorative fountain showed the presence of *Legionella*. A biofilm study of *Legionella* at various temperatures and iron concentrations categorized the bacteria as a moderate biofilm former.

Conclusion This study revealed that most of the water and swab samples were found to be negative for *Legionella* that is quite encouraging and the contamination of water systems in recreational facilities can be reduced by decontamination techniques and proper hygienic practices.

Keywords
- recreational sites
- biofilm
- *Legionella pneumophila*
- aerosols

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Introduction

Legionellosis is an infection that is transmitted by inhaling aerosolized water particles contaminated with the opportunistic waterborne bacteria *Legionella* spp.[1] *Legionella* was initially discovered in a soldier's blood sample more than 50 years ago, but its importance as a human pathogen was not recognized until there was an unpredicted epidemic of fever including pneumonia among the attendees of an American Legion convention in Philadelphia, in 1976. This resulted in the discovery of a brand new disease known as Legionnaire's disease (LD) and Pontiac fever (PF) being added to the list of bacterial infections.[2] The organism is ubiquitous and widespread in natural aquatic, moist soil and in artificial environments. Since *Legionella* is ubiquitous in aquatic habitats, it is impossible to prevent them from entering man-made water systems completely. Even though the bacteria are largely present in hot water systems, they have also been found in spa pools, decorative fountains, and cooling towers. Marine waters have also been found to contain *Legionella* species. Nutrients required for the survival of bacteria may come from water or dirt entering the system.[3–7] Plumbing fixtures like shower heads and hot water taps serve as a source for the bacteria to colonize and spread.[8] In tertiary hospitals in India, *L. pneumophila* was discovered in 6.66 to 15.2% of the distal outlet waters in patient care areas.[9,10] Any device or system that can store, recirculate, or hold nonsterile water that can be vaporized is a source of legionellosis.[11] Despite these factors, *Legionella* spp. exposure can still occur when people employ recreational water, especially in hot water pools with hydro massage systems. Surveillance of legionellosis is a current public goal. A study conducted at a medical hospital in India from 2015 to 2020 with confirmed pneumonia patients found 14 out of 597 patients tested positive for *Legionella* infection.[12] Infections are caused by inhaling *Legionella*-contaminated aerosols. In the wake of the ongoing crisis due to coronavirus disease 2019, contact with aerosolized water from the reopening of numerous buildings, recreational facilities, and the utilization of previously unused plumbing and cooling systems results in an immersive increase in the cases.[13] The knowledge of the infection status and the worldwide prevalence of *Legionella* is limited. It is rarely reported in many places mainly due to a lack of knowledge, paucity of diagnostics, and unavailability of an active LD surveillance system. The World Health Organization (WHO) claims that because this pathogen is ignored in developing and underdeveloped countries, hence limited reports are available from many countries, and no records from others.[14,15] *Legionella* encounters free-living amoeba underneath water distribution systems and benefits from nutrients, enhanced protection from the climate, antimicrobials, or ultraviolet (UV) radiation.[16] The pathogenicity of *L. pneumophila* is significantly influenced by environmental factors.[17] To regulate the bacteria in artificial water systems and disease control, a thorough understanding of the factors that affect *Legionella* survival and proliferation is essential.[18] The existence of biofilm in recreational facilities pipework and water storage systems is a big problem in inhibiting *Legionella* growth. The biofilm-forming ability of the pathogen could enhance the potential for survival of the bacteria by providing shelter and nutrients and also exhibiting resistance to biocide compounds and chlorination.[19]

Though *Legionella* is reflected as a problem in developed countries, the WHO considers it as underestimated in developing countries. Due to the paucity of information in this geographical location, the goal of the study was to determine the prevalence of *Legionella pneumophila* and related species from recreational locations in this area.

Methodology

Study Population

The study population includes water-based recreational sites, such as decorative fountain ponds, swimming pools, garden sprinklers, and drip irrigation systems. The sites were randomly selected based on the important recreational spots where there is more human intervention to ensure a representative sample.

Sample Collection

Water samples will be collected from the selected recreational sites in 1-L volume sterile wide-mouth bottles in accordance with Indian Standard IS 1622: method of sampling and testing for water and wastewater guidelines.[20] Polyester swabs were used to collect the swab samples from the same sources and each swab was individually placed in 1 mL of sterile water and labelled on the tube and transported to the lab. A total of 60 samples were collected, which included 30 swab samples and 30 water samples. The sample size was determined based on the incidence of *Legionella* in water distribution systems that varied between 15 and 27%. Thus, to isolate at least one *Legionella* strain at a 90% confidence interval, the sample size is 30. Hence, 30 water samples and 30 corresponding swab samples were taken.

Sample Processing

The samples were processed within 4 hours of collection using standard microbiological methods. Both water and swab samples were collected from in and around Mangalore city, Karnataka.

Isolation of *Legionella pneumophila*

Isolation of *L. pneumophila* and related species from the samples was performed as per US Centers for Disease Control and Prevention guidelines (2005).[21] The water samples were filter concentrated under the biological safety cabinet using a sterile membrane filtration funnel assembly containing a 0.2 µm, 45 mm diameter polycarbonate membrane filter (Millex, Merck, India). After filtration, the filter was placed into sterile 50 mL screw-capped tubes (Tarsons, India) having 5 mL sterile water. Further, the tubes were vortexed for a minute to dislodge microorganisms and organic compounds from the filter. Before using the swabs, the suspension was vortexed for 5 minutes. *Legionella* was isolated

using Buffered Charcoal Yeast Extract (BCYE, HiMedia FD142) agar supplemented with 0.1% alpha-ketoglutarate, glycine, vancomycin, cycloheximide, and polymyxin B (GVCP, HiMedia FD143). BCYE and GVCP agar were spread with 100 µL suspension and incubated in a candle jar at 35 °C for 72 hours.

Confirmation of *Legionella* Using BCYE Agar Base Medium with and without Cysteine

The isolated bacteria were further confirmed as *Legionella* by testing survival in absence of amino acid cysteine. Cultures that are cysteine dependent were subjected to gram staining followed by a catalase test. Once the isolates were phenotypically confirmed were then subjected to confirm as *Legionella* by polymerase chain reaction (PCR).

Genotypic Identification Using PCR Assay

The isolates that thrived on GVPC were further enriched in BCYE broth before being subjected to the cetyl trimethylammonium bromide method of DNA extraction.[22] The quality and quantity of the extracted DNA were quantified using a UV nanodrop spectrophotometer (IMPLEN) and used as a template for molecular detection.

The obtained isolates were confirmed as *Legionella* by PCR using genus (16S rDNA) and species-specific (dot gene) primer. As an alternative, the swab suspension was directly used as a template for PCR-based culture-independent detection. Details of the primers are given in ►**Table 1**.

Estimation of Total Chlorine Content

Iodometric titration method was used to estimate total chlorine in water samples.[23] Briefly, 10 mL of glacial acetic acid, 1 gram of potassium iodide crystals, and 8 drops of ammonium molybdate solution were added to 200 mL of the sample. This mixture was then titrated against 0.1N sodium thiosulfate to faint yellow. Starch indicator was added and titrated again until the blue color disappears.

Estimation of Iron Content

The iron content of water samples was estimated by the 1,10-phenanthroline method as outlined by IS 3025(Part 53).[23]

Biofilm Formation at Various Temperatures

The biofilm-forming ability of the isolated *Legionella* was tested in 96 well microtiter plates inoculated with *Legionella* grown in BCYE using crystal violet assay.[24] One-hundred ninety microliter of BCYE broth and 10 µL of bacterial culture were added to 96 well microtiter plates. BCYE broth without bacterial culture was used as a control. Plates were incubated for 7 days at temperatures of 25°C, 35°C, and 45°C. After incubation, wells with culture, as well as control, were decanted and washed with 0.85% saline three times. Plates were left to dry at room temperature. Once dried, 200 µL of 1% crystal violet solution was added followed by 10 to 15 minutes of incubation. After incubation, wells were washed and allowed to dry at room temperature. Then 200 µL of 33% glacial acetic acid was added and then transferred to a fresh plate. Absorbance was recorded at 630 nm using a spectrophotometer. True O.D. values were obtained by subtracting the control value. Classification of isolates was done as follows: D≤O.D.c = non-biofilm producer, O.D.c< O.D= strong biofilm Producer.

Biofilm Formation at Various Iron Concentrations

The biofilm-forming ability of the isolated *Legionella* was tested in 96 well microtiter plates. Plates were incubated for 7 days at a varied iron concentration (3mg/L, 30mg/L, and 300 mg/L). After incubation, plates were subjected to staining and washing before taking the O.D. value.

Results

Phenotypic Characterization

The presence of *Legionella* in water and swab samples was carried out by detecting the presence of characteristic round, shiny and white-colored colonies on BCYE agar supplemented with GVPC after 72 hours incubation at 35°C (►**Fig. 1**). In this culture medium, we observed a higher percentage of recovery than in the nonselective BCYE agar. Cysteine-deficient GVPC plates were used as a negative control since cysteine supplement is essential for *Legionella* growth. Obtained isolates were confirmed by PCR using genus and species-specific primers. Results were represented in ►**Table 2**.

Table 1 Primers used in this study

Primer	Sequence(5′-3′)	Amplicon size (bp)	Reference
JFP	AGGGTTGATAGGTTAAGAGC	386	34
JRP	CCAACAGCTAGTTGACATCG		
dotF	ATTGTCTCGCGCGATTGC	440	35
dotRM	CTTCCATTGAGTTTCACCAAATCA		
dotfk	GGTGATGGTTAATAATGATCCGGC	387	35
dotrm	CTTCCATTGAGTTTCACCAAATCA		
LPmipF	GCAATGTCAACAGCAA	153	36
LPmipR	CATAGCGTCTTGCATG		

Among the 60 samples analyzed, three samples (5%) provided the desired amplification product for the conserved region 16SrDNA and *Jfp* gene thereby confirming the presence of *Legionella* in recreational water.

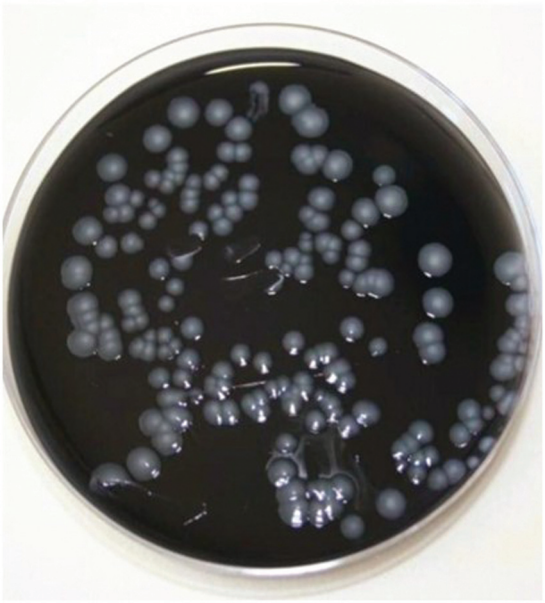

Fig. 1 Representative image of *Legionella* isolates showing growth on GYPC agar.

Table 2 Detection by molecular methods

Sl. no	Source	Total number of samples	Presence of virulence gene		
			16S rDNA	mip	dot A
1	Garden sprinkler	36	1	1	–
2	Decorative fountain	20	1	1	–
3	Swimming pool	4	1	1	–
Total		60	3	3	–

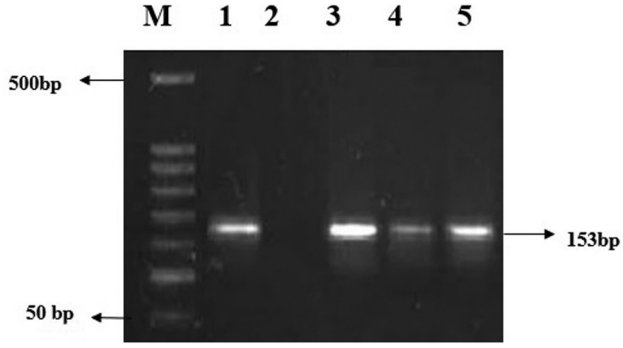

Fig. 2 : Representative agarose gel electrophoresis for *mip* showing amplicon of 153 bp. Lane M: DNA molecular weight marker (50 bp) Lane 1: Positive control (ATCC 33152). Lane 2: Negative control; Lane3-5: Representative positive isolates.

Detection of Virulence Genes

The isolates obtained in this study were used for screening of the virulence genes (*dot* and *mip*). To enhance the sensitivity of detection, nested PCR was performed. All the samples were found negative for virulence gene dot. However, two water and one swab sample showed the presence of the macrophage infectivity potentiator (*mip*) gene (►**Fig. 2**).

Estimation of Total Chlorine

The total chlorine concentration of the water samples was measured using iodometric titration. The total chlorine content of water samples was estimated to be below 0.2 mg/L.

Iron Content

Iron content in samples was estimated by the absorbance obtained at 510 nm by the 1,10-phenanthroline method. Out of 30 water samples analyzed, 11 samples showed iron content below 75 µg/L, 15 samples showed iron content between 75 and 300 µg/L, and four water samples showed iron content above 300 µg/L. Water with iron content above 300 µg/L is usually considered objectionable. In this study, three water samples from the garden sprinkler and one water sample from the decorative fountain showed iron content above 300 µg/L.

Biofilm Formation at Various Temperatures

The *Legionella* American type culture collection (ATCC) culture was compared with obtained *Legionella* isolates for the biofilm formation potential of the bacteria by crystal violet staining method. The ATCC culture showed optical density between 2 and 4 at 630 nm indicating the bacteria as a moderate biofilm former. Similarly, all isolates showed an O.D. between 2 and 4 on exposure to the different temperatures of 25°C, 35°C, and 45°C infers that all four isolates are moderate biofilm formers. All four isolates showed a gradual increase in the biofilm-forming ability with respect to an increase in temperature with maximum biofilm formation at 45°C and least at 25°C (►**Fig 3**).

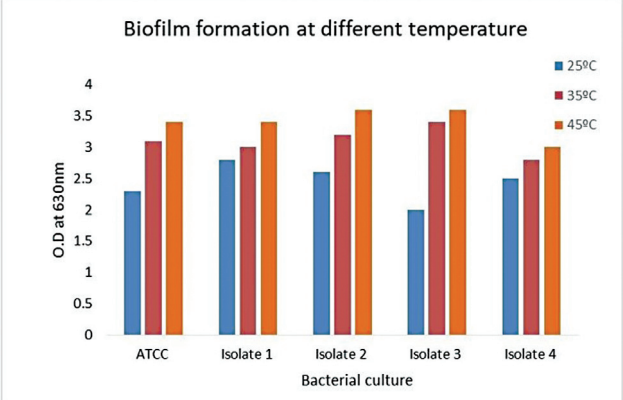

Fig. 3 Bar diagram showing comparative biofilm formation by standard ATCC and isolates of *Legionella* at various temperatures (25°C, 35°C, and 45°C).

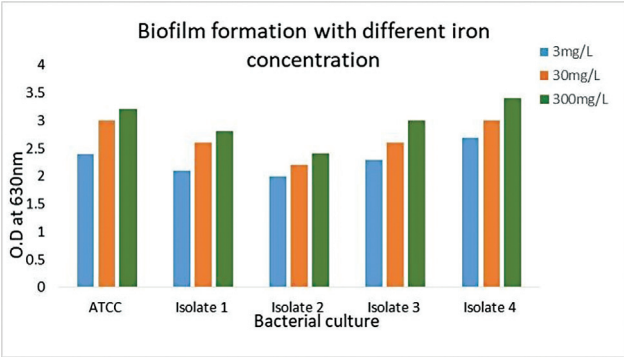

Fig. 4 Bar diagram showing comparative biofilm formation by standard ATCC and isolates of *Legionella* at various iron concentrations of 3 mg/L, 30 mg/L, and 300 mg/L.

Biofilm Formation at Various Iron Concentrations

All isolates showed an O.D between 2 and 4 on exposure to various iron concentrations (3mg/L, 30mg/L, and 300mg/L) inferring that all four isolates are moderate biofilm formers. All four isolates showed a gradual increase in the biofilm-forming ability with respect to increasing iron concentrations with maximum biofilm formation at the iron concentration of 300 mg/L and least at the iron concentration of 3 mg/L (►**Fig. 4**).

Discussion

Legionella pneumophila and related species have been considered causal agents of a new world disease. Showers, whirlpool spas, cooling towers, and other artificial water systems and appliances that emit polluted aerosols are the main sources of Legionella infections. Inhalation of these aerosols, especially by those with compromised immune systems, leads to infection.[2] Since the prevalence of infections caused by the genus *Legionella* is high in developed countries, strict surveillance systems are in place to monitor and control any outbreaks.[25,26] The exact incidence of legionellosis worldwide is unknown because of differences in national policies in reporting such cases and due to the inadequacy of knowledge pertaining to isolation and identification of the organism.

A study was conducted to find *L. pneumophila* and identify Lp1 in the water systems of a tertiary healthcare facility in northern India that offers cancer treatment and organ transplantation services. Out of 79 water samples (10 potable and 11 nonpotable), 21 (27%) showed the presence of Legionella spp by conventional method and 28 isolates from the 79 samples were confirmed as *L. pneumophila* by molecular technique. Among them, four water samples taken from patient locations also showed the presence of L. *pneumophila* leads to the raising possibility of nosocomial infection.[9] A similar study was conducted to investigate Legionella spp from a water system of tertiary care hospital, India. Among the 201 water samples collected, 38 (19—potable and 19—nonpotable) samples were positive for the Legionella by traditional method. The samples were collected from patient areas, residential areas, and general hospital areas, and from AC cooling towers and these results concur with a previous study from India that reported a positivity of 15%.[27]

Most of the few reports on *Legionella pneumophila* incidence from India are from clinical samples. Although preemptive environmental surveillance of Legionella and routine cooling tower installations treatment are advised in many countries, neither of these practices is common in India, and there have been studies conducted in this country for monitoring Legionella contamination from hospital waters.[18]

Environmental monitoring for the same is not routine. Many studies have looked for *Legionella* in recreational centers and hospitals. In a study conducted in Chicago, United States, 41 out of 160 samples were positive for *Legionella* from 12 of 36 recreational centers.[25] North Carolina recently faced an outbreak of LD among the people who attended the NC Mountain State Fair held in 2019. The investigation found that the outbreak of LD was caused by exposure to *Legionella* in aerosolized water from a hot tub that was kept for display during the fair.[28]

A study conducted in southern Italy during the period of 2001 to 2017 regarding the LD outbreaks in recreational facilities revealed that areas with high temperatures and a high prevalence of *Legionella* increase the risk of LD.[29] Among 30 water and swab samples analyzed, one water sample from a swimming pool, garden sprinkler, and one swab sample from a decorative fountain was found positive for virulence gene *mip* of *Legionella* during PCR amplification. The *mip* gene has been proven to be a virulence factor in previous investigations, which could give genetic evidence for the high incidence of *L. pneumophila* strains in man-made systems. This factor protects mammalian and protozoan phagocytic cells against intracellular apoptosis.[30] One water sample from a decorative fountain showed prominent amplification for the conserved region of 16S rRNA (*jfp* gene). This study showed a higher incidence of *Legionella* in water than in swab samples. In Europe, out of 518 water samples collected from swimming pools, decorative fountains, hot tubs, drip irrigation, and garden sprinklers, 67 tested positive for *L. pneumophila* and nonpneumophila species.[31] This study observed that obtained isolates were moderate biofilm former. This could pose a major health risk in hospitals and healthcare facilities as biofilm renders antibiotic resistance to bacteria. Once the *Legionella* is protected within the biofilm, it becomes difficult for disinfection procedures to destroy them as biofilms in the pipework and water storage systems. It further increases the potential for the survival of the bacteria by resisting biocidal compounds. A study published in the journal of microbiological research regarding the replication of *Legionella* in biofilms of water distribution pipes revealed that protozoa like amoebae increase the risk of spread of biofilm-associated *Legionell*a in aquatic systems and thus in the occurrence of LD.[32] Hence, preventive measures need to focus more on the biofilm-forming ability of the bacteria and not just on eliminating their growth. Biofilm forming ability of the bacteria gradually increased with increasing temperature and iron concentration which indicates that maintaining water systems at low temperature

and regularly checking the iron concentration of water may help to overcome the resistance offered by biofilm. Inadequate water management programs, dense biofilm formations, and high ambient temperatures create an ideal environment for *Legionella* spp, proliferation.[26]

A study revealed the rate of biofilm development and growth of *Legionella* varies according to the material used to make the plumbing system.[28] There have been recent reports that cast iron rust is a significant contributor to the growth of *Legionella* in water distribution systems. Thus, it was postulated that iron may be used in routine water testing as an indicator for *Legionella*. *Legionella* is reported to be associated with at least 0.095 mg Fe/L.[33] Out of 30 water samples analyzed, 11 water samples had iron concentrations below 75 µg/L, 15 water samples had iron concentrations between 75 and 300 µg/L, and four water samples had an iron concentration above 300 µg/L. The highest iron concentration of more than or equal to 300 µg/L was found in three water samples from the garden sprinkler and one water sample from the decorative fountain that would enhance the biofilm-forming ability of *Legionella*. The total chlorine content of all water samples was estimated to be below 0.2mg/L indicating a low intensity of chlorine. Even though the prevalence of *Legionella* was found to be low in recreational sites of Mangalore, the detection of *Legionella* in three water samples and one swab sample indicates poor monitoring and irregular cleansing practices being followed. The purpose of environmental intervention measures is to lessen the amount of *Legionella* present in water systems or to make sure that the bacteria cannot be released into the air where people will breathe it, hence lowering the health risk brought on by *Legionella*. Thus, spreading awareness and educating people about this pathogen are important in a region where the use of artificial water systems is on the increase.

In conclusion, though *Legionella* is often designated as a problem in developed countries, the WHO considers it to be underestimated in developing countries. This study was taken up to determine the prevalence of *Legionella pneumophila* and related species in water-based recreational sites in Mangalore, Karnataka. The prevalence of Legionella was found to be low in recreational sites of Mangalore included in this study. The main objective of this study was to determine the prevalence of Legionella pneumophila and related species in this geographical location since such information were not available. However, the presence of Legionella was detected in 6% of the total 60 samples collected from swimming pools, decorative fountains, and garden sprinklers. Out of 30 water samples tested, 10% of them showed the presence of Legionella. Out of 30 swab samples tested, 3% of them showed the presence of Legionella. This points out the need for improving microbiological surveillance and risk analysis in water-based recreational sites of Mangalore. Even though environmental surveillance and regular treatment of water distribution systems are recommended in many countries, these rules are not followed routinely in India, and limited studies have been conducted in India for monitoring Legionella contamination in recreational water systems. To the best of our knowledge, this is the first work on reactional facilities from this part of India. Since Legionella is found to be a moderate biofilm former, preventive measures need to focus more on their biofilm forming ability along with inhibiting the multiplication of bacteria. Maintaining good hygiene, regular microbiological surveillance, and disinfection procedures like hyper chlorination and UV disinfection can help in eliminating the bacteria from water systems of recreational sites. A general water safety plan as a part of infection control addressing microbial growth in addition to control of external contamination by Legionella should be a part of recreational facilities even in countries that do not fall in the category of the developed nation.

Conflict of Interest
None declared.

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