



High Prevalence of *rtxA*, *enhC*, and *mip* Genes in Waterborne *Legionella* Isolates

Su Kaynaklı *Legionella* İzolatlarında *rtxA*, *enhC* ve *mip* Genlerinin Yüksek Prevalansı

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Cite this article as: Bozok T, Kurt CB, Bekçi A, Tezcan Ülger S, Aslan G. High prevalence of *rtxA*, *enhC*, and *mip* genes in waterborne *Legionella* Isolates. FLORA 2025;30(4):466-472.

ABSTRACT

Introduction: *Legionella pneumophila* is an opportunistic, intracellular pathogen found in environmental water sources that can cause Legionnaires' disease-a potentially life-threatening pneumonia if not properly treated, particularly in older adults and individuals with weakened immune systems. Virulence genes such as *rtxA*, *mip* and *enhC* are known to be associated with bacteria's ability to survive under harsh conditions and within a host cell.

Materials and Methods: This study aims to investigate the distribution of virulence genes *rtxA*, *mip* and *enhC* in 61 environmental *Legionella* isolates from environmental water samples. Prevalence and dissemination of these genes among *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2–14, and other *Legionella* species were analyzed via polymerase chain reaction-based molecular detection methods.

Results: In *L. pneumophila* serogroup 1 isolates, *enhC* and *mip* were detected in 100% of samples, while *rtxA* was present in 14.2%. For *L. pneumophila* serogroups 2–14, *mip* was found in 96.4% of isolates, with *rtxA* and *enhC* present in 42.9% and 39.3%, respectively.

Conclusion: The high prevalence of *enhC* and *mip* could suggest their significant roles in the pathogenicity and environmental survival of *L. pneumophila*. The variability in virulence genes among non-*L. pneumophila* species observed in our study suggests that exploring new molecular targets could enhance both detection and differentiation efforts.

Key Words: *Legionella*; *rtxA*; *mip*; *enhC*; Waterborne

Received/Geliş Tarihi: 28/05/2025 - Accepted/Kabul Ediliş Tarihi: 27/08/2025



ÖZ

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Giriş: *Legionella pneumophila* çevresel su kaynaklarında bulunabilen; fırsatçı, hücre içi bir patojendir ve bağışıklık sistemi zayıflamış bireylerde ya da yaşlılarda tedavi edilmediği takdirde hayatı tehdit eden Lejyoner hastalığına sebep olabilir. Virülans ile ilişkili genlerden *rtxA*, *mip* ve *enhC* bakterinin zorlu çevresel koşullarda ve hücre içi ortamlarda hayatta kalma yeteneği ile ilişkilidir.

Materyal ve Metod: Bu çalışmada, çevresel su örneklerinden izole edilmiş 61 *Legionella* izolatında *rtxA*, *mip* ve *enhC* genlerinin dağılımı incelenmiştir. *L. pneumophila* serogrup 1, *L. pneumophila* serogrup 2-14 ve diğer *Legionella* türleri arasında bu genlerin prevalansları ve dağılımları polimeraz zincir reaksiyonu tabanlı moleküler tespit yöntemleri ile yapılmıştır.

Bulgular: *L. pneumophila* serogrup 1 izolatlarında, *enhC* ve *mip* izolatların %100'ünde tespit edilirken *rtxA* %14.2'sinde tespit edildi. *L. pneumophila* serogrup 2-14 izolatlarının %96.4'ünde *mip* pozitifken, *rtxA* ve *enhC* için bu oran sırasıyla %42.9 ve %39.3 olarak tespit edildi.

Sonuç: *enhC* ve *mip* yüksek prevalansı, bu genlerin *L. pneumophila* için çevresel hayatta kalma ve patojenitede oynadıkları önemli rolü ortaya koymaktadır. Virülans genlerinin non-*L. pneumophila* türlerindeki bulunma değişkenliği moleküler tespit için yeni hedeflerin ortaya çıkarılmasının hem tanı hem de ayırmda yararlı olacağını işaret etmektedir.

Anahtar Kelimeler: *Legionella*; *rtxA*; *mip*; *enhC*; Su kaynaklı

INTRODUCTION

The only genus within the family of *Legionellaceae* is *Legionella*^[1]. *Legionella* species are found in environmental freshwater sources and can survive under various chemical and physical conditions^[2]. They are gram-negative, intracellular bacilli responsible for two forms of infections: Legionnaires' disease with a mortality rate of 8-12% and less severe, non-pneumonic and self-limiting Pontiac disease. Moreover, only a small proportion of individuals exposed to *Legionella* spp. develop Legionnaires' disease^[3]. Most of the infections are caused by *Legionella pneumophila* serogroup 1^[4,5].

Although *L. pneumophila* exists freely or within biofilms in aquatic environments, replication occurs only within protozoa or mammalian phagocytic cells. Uptake by host cells occurs via coiling or conventional phagocytosis. Inside the host, *L. pneumophila* forms a unique compartment, the *Legionella*-containing vacuole (LCV), which avoids endosomal maturation. Within the LCV, the bacterium utilizes amino acids for replication. Their depletion triggers 3',5'-bispyrophosphate (ppGpp) accumulation, upregulating RpoS and leading to enhanced motility and inhibition of phagosome-lysosome fusion^[1,6,7].

Several genes contribute to the intracellular biphasic life cycle of *Legionella*, including its ability to inhibit lysosomal degradation, allowing it to remain viable within the *Legionella*-containing vacuole (LCV) and enhancing its overall virulence. Repeats in structural toxin (RTX), encoded by the *rtxA* gene, is involved in β_2 integrin-mediated adherence to the host. Decreased adherence, intracellular replication, cytotoxicity, and pore formation were observed in *L. pneumophila* mutant strains lacking the *rtxA* gene^[8]. The *enhC* gene facilitates cell entry and intracellular growth by reducing Nod1-mediated immune recognition^[9,10]. The macrophage infectivity potentiator (*mip*) gene enhances infection of alveolar macrophages by reducing phagocytosis and promoting chemotaxis of RAW264.7 macrophages^[11,12]. In addition to the symbolic importance of the *mip* gene, which was the first recognized virulence-associated gene of *L. pneumophila*, the *mip* gene is considered to be one of the DNA targets for various polymerase chain reaction (PCR) assays for the detection of *Legionella* nucleic acid^[1,13].

Consequently, detecting the aforementioned virulence-associated genes in *Legionella* strains from environmental water sources could provide critical insights into their pathogenic potential.

Objective

This study aimed to investigate the infective properties of *Legionella* species isolated from environmental water sources by detecting the *mip*, *enhC*, and *rtxA* genes. The second objective was to determine whether the distribution of these three genes differs among *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2–14, and other *Legionella* species.

MATERIALS and METHODS

Isolation, Serogrouping, and Subculturing of Bacterial Strains

In this study, a total of 61 *Legionella* isolates were used, all of which originated from the research team's laboratory collection of environmental water samples obtained in previous years. The sample size was determined based on the number of isolates in the existing collection, and no statistical power analysis was conducted. The isolates were primarily collected from sources such as hospital water systems, cooling towers, and water storage systems of the ships. However, detailed source information was either limited or unavailable in the archived records; therefore, not all source details could be provided in the manuscript. After collection, water samples were concentrated by filtration through a membrane filter with a pore size of 0.2–0.4 µm. The material retained on the filter was then resuspended in sterile water. The suspension was divided into three equal aliquots: the first aliquot was maintained as an untreated control, the second was subjected to thermal treatment, and the third underwent acid treatment. The untreated aliquot served as a reference to assess the efficacy of the thermal and acid treatments in reducing non-target bacterial populations. Following these treatments, each aliquot was inoculated onto supplemented buffered charcoal yeast extract (BCYE) agar (Oxoid, SR0251C). The inoculated Petri dishes were incubated at 36 °C for seven days, and colony formation was monitored daily. Serogroups were determined with the latex agglutination test (Oxoid Legionella Latex Test, Oxoid, United Kingdom), resulting in three groups: *L. pneumophila* serogroup 1, *L. pneumophila* serogroup 2–14, and *Legionella*

spp. Strains were stored at -80 °C in 20% glycerol broth and subcultured from stocks onto BCYE agar, followed by incubation at 37 °C in a humidified environment for 72 hours prior to DNA extraction.

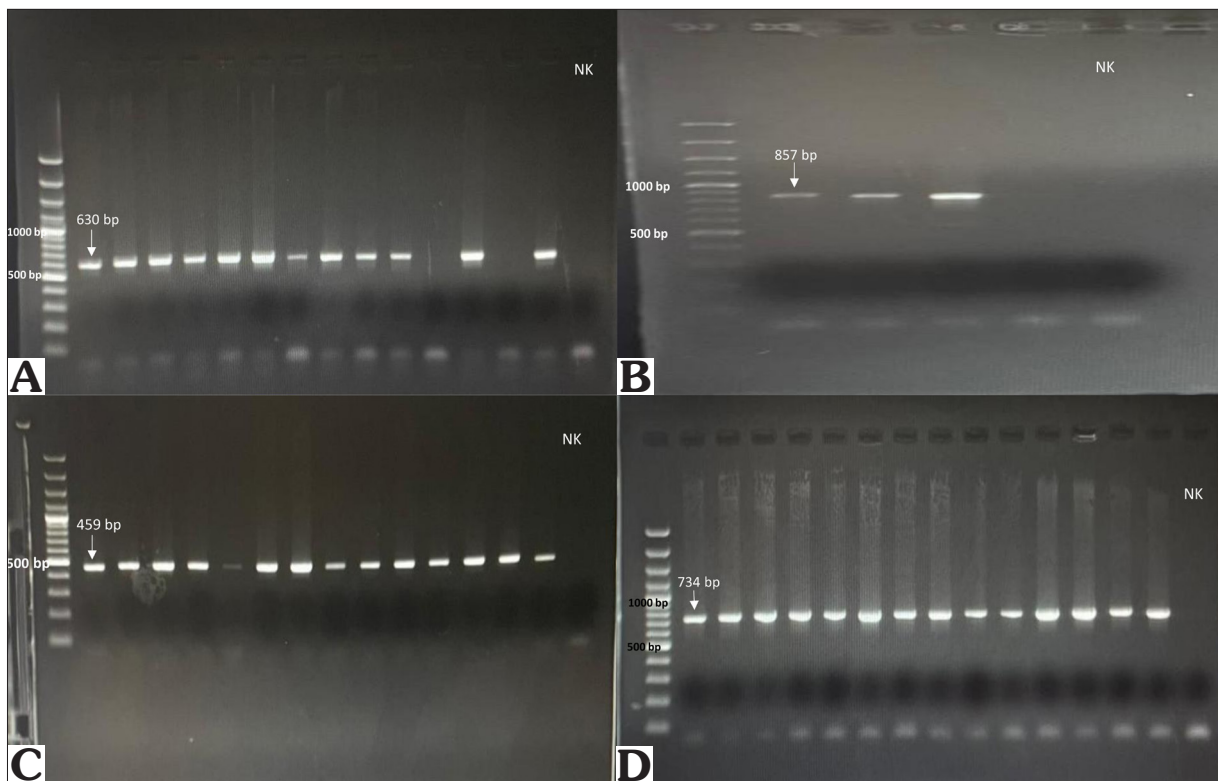
DNA Isolation, Primer Design, and PCR Conditions

DNA from *Legionella* isolates was extracted using a chloroform-based method, which was adapted from Green and Sambrook^[14]. Pure colonies from BCYE agar were collected and suspended in 1 ml of sterile water. Bacterial pellets were suspended in lysis buffer containing SDS and proteinase K at 55 °C for 30 minutes. Equal volumes of phenol/chloroform were added and centrifuged at 13,000 rpm for 10 minutes. DNA was precipitated with cold ethanol, centrifuged again at 13,000 rpm for 10 minutes. The resulting DNA pellet was washed with 70% ethanol. After being left to air-dry, it was resuspended in nuclease-free water. Primers used in this study were designed based on the sequence in the GenBank with accession number NZ_CP015941. Primers were designed with an online primer design program^[15]. Primer pairs in this study are summarized in Table 1. Denaturation of the DNA template was performed at 95 °C for five minutes, followed by 30 cycles of PCR reaction. Each reaction contained 0.5 µM primer, 1.5 mM MgCl₂, 200 µM deoxynucleotide triphosphates, one unit of Taq DNA polymerase, 5 µL PCR buffer, 37.25 µL distilled water, and 3 µL of extracted bacterial DNA. To analyze DNA fragments, 1% agarose gel was prepared using agarose powder and Tris-acetate-ethylenediaminetetraacetic acid buffer. Electrophoresis was conducted for 45 minutes at a constant electric potential (90 milliamperes and 100 volts). Ethidium bromide was used as the DNA dye. After electrophoresis, gel images were captured using a ultraviolet transilluminator. The size of DNA fragments was determined by comparing their migration distance to the DNA marker. Fluorescence intensity was accepted as an indicator of the presence of the fragment. Expected base pair outcomes aligned with our designed primers; therefore, further sequencing was not deemed necessary (Figure 1).

Table 1. Primers used in the study

Gene	Primer Name	Primer Sequence (5'-3')	Product Size (bp)	Source
<i>rtxA_1</i>	Forward	GAT CCG CAA GTA GCG CTC AC	630	[20]
	Reverse	TGT AAT GCT GGC ATT AGG CG		
<i>rtxA_2</i>	Forward	GCA GGA CAA GCA AGA ACA GC	857	This study
	Reverse	TCA ACC GCT TCA TCA TGG CT		
<i>enhC</i>	Forward	TGG ACA GGA GAT TGG AAG CG	734	This study
	Reverse	TAC ATG GCC TTG GCT GGA TC		
<i>mip</i>	Forward	ACC GAT GCC ACA TCA TTA GC	459	This study
	Reverse	CTG GAA CGT TGC TGG CTT AC		

bp: Base pair.

**Figure 1. A. *rtxA_1* B. *rtxA_2* C. *mip* D. *enhC*.**

RESULTS

PCR analysis revealed the presence of three key virulence genes, *rtxA*, *enhC*, and *mip*, in isolates of *Legionella* species (Table 2). Among *L. pneumophila* serogroup 1 isolates, the *enhC* and *mip* genes were detected in 100% of the samples, while *rtxA* was present in only 14.3% (4/28) of the samples. A combination of *rtxA*, *enhC* and *mip* was observed in 14.3% of the *L. pneumophila* serogroup 1 samples. In *L.*

pneumophila serogroups 2-14, the presence of the *mip* gene was 96.4% (27/28), with the *rtxA* and *enhC* genes being found in 42.9% (12/28) and 39.3% (11/28), respectively. Coexistence of *rtxA* with either *enhC* or *mip* was found in 32.1% and all three aforementioned genes were present in 14.3% (4/28) among the samples. For *Legionella* spp., the distribution of genes was more variable. Among the five non-*L. pneumophila* isolates, *rtxA* was detected in 40% (2/5) of

Table 2. Dissemination of virulence genes among *Legionella* species

Group	Total number of bacteria isolates	<i>rtxA</i> % (n)	<i>enhC</i> % (n)	<i>mip</i> % (n)	<i>rtxA</i> + <i>enhC</i> % (n)	<i>rtxA</i> + <i>mip</i> % (n)	<i>enhC</i> + <i>mip</i> % (n)	<i>rtxA</i> + <i>enhC</i> + <i>mip</i> % (n)
<i>L. pneumophila</i> serogroup 1	28	14.3 (4)*	100 (28)	100 (28)	14.3 (4)	14.3 (4)	100 (28)	14.3 (4)
<i>L. pneumophila</i> serogroup 2-14	28	42.9 (12)*	39.3 (11)	96.4 (27)	14.3 (4)	32.1 (9)	39.3 (11)	14.3 (4)
<i>Legionella</i> spp.	5	40 (2)	60 (3)	40 (2)	40 (2)	20 (1)	40 (2)	20 (1)

*Two primer sets were used and total sum was written.

the cases, while *enhC* was present in 60% (3/5) of the samples. The *mip* gene was also detected in two of the five isolates (40%). Interestingly, the co-occurrence of all three genes was relatively rare, observed in only 20% (1/5) of cases. These results demonstrate that *L. pneumophila* serogroup 1 and serogroups 2–14 exhibit a high prevalence of virulence gene combinations, suggesting greater pathogenic potential compared to other *Legionella* species.

DISCUSSION

The study demonstrates significant variability in the distribution of the *rtxA*, *enhC*, and *mip* virulence genes among environmental *Legionella* species. The majority of the isolates in this study were identified as *L. pneumophila*, including both serogroup 1 and serogroups 2–14, while only a small number of non-*L. pneumophila* *Legionella* species were included. This limited number of non-*L. pneumophila* strains restricts the generalizability of the findings related to this group.

Notably, the *mip* and *enhC* genes were highly prevalent among *L. pneumophila* serogroup 1 and serogroups 2–14 (Table 2), highlighting their potential roles in the pathogenicity and environmental persistence under stress. Consistent with this, the *enhC* gene may contribute to survival under environmental stress by maintaining cell envelope integrity, as shown by Aurass et al.^[16]. In our experiments, the high prevalence of the *enhC* gene among environmental *L. pneumophila* serogroup 1 and serogroups 2–14 isolates suggests that its presence is not only common but also crucial for maintaining cell envelope integrity under harsh conditions. Our findings raise the possibility that *enhC* may play a role in clinical

infections, given its consistent detection in *L. pneumophila* serogroup 1 isolates, which are frequently implicated in clinical cases. Moreover, the high prevalence of the *mip* gene observed in our study among *L. pneumophila* serogroup 1 and serogroups 2–14 aligns with the findings of Aurass et al., suggesting that this gene enables *L. pneumophila* to persist in aquatic habitats while retaining its pathogenic potential. In our experiments, the prevalence of *mip* among *L. pneumophila* isolates aligns with the findings of Carvalho et al., who examined the presence of *L. pneumophila* in man-made water systems using *mip*-based PCR and sequencing. Their study found that *L. pneumophila* serogroup 1 was predominant, suggesting that artificial environments create favorable conditions for the persistence of *L. pneumophila*. Our data further support the stability and reliability of *mip* as a diagnostic target. The consistently high prevalence of the *mip* gene observed among *L. pneumophila* serogroup 1 and *L. pneumophila* serogroups 2 to 14 isolates in this study underscores its critical role in the virulence of these serogroups. Compared to other virulence genes like *rtxA*, the higher detection rate of *mip* further indicates its fundamental importance for diagnostic value. While Katsiaflaka et al. reported a 95% detection rate of *rtxA* in *L. pneumophila* serogroups 2–14 isolated from water distribution systems in Greece, our study demonstrated a detection rate of 14.3% in *L. pneumophila* serogroup 1 and 42.9% in serogroups 2–14^[17]. This discrepancy may be attributed to strain-specific differences, as well as environmental factors such as selective pressure, which may promote a higher prevalence of *rtxA* in water systems. Zhan

et al. emphasized the role of *rtxA* in mediating cell attachment and invasion, indicating that environmental conditions favorable for these functions may also lead to gene retention^[18]. Together, these findings suggest that the variability in *rtxA* detection across studies may reflect differences in local ecological conditions, genetic diversity, or strain-specific characteristics among isolates. Among non-*L. pneumophila* strains, the distribution of virulence genes was more variable, with detection rates of 40% for *rtxA*, 60% for *enhC*, and 40% for *mip*. Although the *mip* gene and 16S rRNA sequencing-based studies have been conducted for the identification of *Legionella*, new molecular targets should be considered, especially for non-*L. pneumophila* strains. New molecular targets, including genes involved in LPS biosynthesis and specific virulence factors, offer certain advantages over *mip* gene-based detection. These targets have the potential to differentiate species and serogroups without the need for post-PCR sequencing^[19]. This would enable a faster diagnostic process and more precise detection of non-*L. pneumophila* strains.

In conclusion, our study highlights the importance of *mip* and *enhC* as key markers for both virulence and environmental adaptability in *L. pneumophila*. The variability in *rtxA* detection across different environments points to the influence of ecological conditions and strain diversity. From a public health perspective, identifying stable and highly conserved targets such as *mip* and *enhC* is essential for early detection of potentially virulent strains in artificial water systems, ultimately reducing the burden of Legionnaires' disease in both community and healthcare settings, along with the development of more accurate surveillance strategies to prevent Legionnaires' disease outbreaks. Future research should focus on additional genetic markers and the development of refined diagnostic methods to better capture the genetic diversity and ecological adaptations of *Legionella* spp. eventually improving surveillance and control efforts in both natural and artificial environments.

Limitations

The sample size, particularly for non-*L. pneumophila* species was relatively small, which may

restrict the ability to generalize the findings to broader environmental populations. Additionally, the sample size was not determined based on a formal statistical power analysis but was instead limited by the number of isolates available in the existing collection. Future studies employing gene expression analysis or functional assays could help clarify the roles of *rtxA*, *enhC*, and *mip* in environmental contexts. Another limitation is the lack of next-generation sequencing and RNA-seq which would allow for a more comprehensive analysis of the genomic landscape surrounding these virulence genes and their expression at a certain time. Such methods could reveal additional regulatory elements, including insertion sequences, which were not captured in this study.

Since virulence is influenced by multiple factors, including other genes and various host-pathogen interactions, it would be worthwhile to conduct further animal model studies to clarify the specific roles of *enhC*, *rtxA*, and *mip* genes in the pathogenesis of Legionnaires' disease.

Publication and Presentation Status

Preliminary results of this study were partially presented at a national scientific congress; however, the full dataset and comprehensive analysis presented in this article have not been published previously.

ETHICS COMMITTEE APPROVAL

This study does not require ethics committee approval.

CONFLICT of INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHORSHIP CONTRIBUTIONS

Concept and Design: All of authors

Analysis/Interpretation: All of authors

Data Collection or Processing: All of authors

Writing: All of authors

Review and Correction: TB, CBK, GA, AB

Final Approval: GA, TB

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