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PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Detection of *Legionella pneumophila* by PCR-ELISA Method in Industrial Cooling Tower Water

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Abstract: Water supply and Cooling Tower Water (CTW) are among the most common sources of *Legionella pneumophila* (LP) contamination. A nonradio active method is described to detect LP in industrial CTW samples. DNA was purified and amplified by nested-PCR with amplimers specific for the 16S rRNA gene of LP. The 5' end biotinylated oligomer probe was immobilized on streptavidin B coated microtiter plates. The nested-PCR product was labeled with digoxigenin and then hybridized with 5'-biotinylated probes. The amplification products were detected by using peroxidase-labeled anti digoxigenin antibody in a colorimetric reaction. The assay detected LP present in 1 L of 5 CTW samples examined. All of the samples were *Legionella* positive in both culture and PCR-ELISA methods. The PCR-ELISA assay appears to exhibit high specificity and is a more rapid technique in comparison with bacterial culture method. Thus could prove suitable for use in the routine examination of industrial CTW contamination.

Key words: *Legionella pneumophila*, nested PCR, industrial cooling tower, PCR-ELISA, 16S rRNA

INTRODUCTION

Legionella pneumophila (LP), the causative agent of Legionnaires' disease, was first recognized in 1976 following an epidemic of acute pneumonia in Philadelphia Lisby and Dessau (1994) and Fraser *et al.* (1977). Since then, a total of 41 *Legionella* species containing 62 serogroups have been characterized (Stranbach *et al.*, 1989; Atlas, 1999). Twenty-one of *Legionella* species have been reported as pathogens in humans (Stranbach *et al.*, 1989; Wilkinson *et al.*, 1986; Fang *et al.*, 1989). Legionnaires' disease is normally acquired by inhalation or aspiration of *Legionella* from a contaminated environmental source. Several reports have shown a clear association between the presence of LP in hot water systems and the occurrence of legionellosis (Fiume *et al.*, 2005; Hiroshi and Hiroyuki, 1997; Fields and Benson,

2002; Vincent-Houdek *et al.*, 1993). Pneumonia caused by *Legionella* has a poor prognosis unless it is diagnosed early and treated with specific antibiotics. Eighty-five percent of these infections are caused by LP (Jonas *et al.*, 1995). Cultivation of *Legionella* organism from appropriate samples represents the definitive method for diagnosis and has a sensitivity of 50 to 90.5% (Lisby and Dessau, 1994; Fraser *et al.*, 1977; Thacker and Robert, 1991). However, colonies become macroscopically visible after 3 to 4 days of culture (Hiroshi and Hiroyuki, 1997; Bej and Mahbubani, 1991; Aslani *et al.*, 1997). More recently, PCR has been used to amplify LP DNA. Amplification products are detected in stained agarose gels or by hybridization with specific oligonucleotide probes. Different DNA sequences have been selected for amplification (Declerck *et al.*, 2006; Hiroshi and Hiroyuki, 1997; Jonas *et al.*, 1995; Reggam and Leitner, 2002;

Edelstein *et al.*, 1987). The DNA sequences of the macrophage infectivity potentiator (*mip*) gene as a determinant of pathogenicity have been used for detection of LP and different *Legionella* species (Fiume *et al.*, 2005; Reggam and Leitner, 2002; Michio and Astushi, 1993; Bej *et al.*, 1990). Amplification of genes coding for r-RNA is also feasible and published protocols employ the 5S rRNA gene alone or combined with *mip* gene (Hiroshi and Hiroyuki, 1997; Jonas *et al.*, 1995; Michio and Astushi, 1993). Recently, amplification of the 16S rRNA gene has enabled detection of 1 cfu mL⁻¹ in water sample or 10 cfu mL⁻¹ in stimulated bronchial fluid (Joly *et al.*, 2006; Jonas *et al.*, 1995; Wellinhouse and Cathrin, 2001; Michio and Astushi, 1993). Amplification of the 16S rRNA gene has well defined advantages. In particular, a large number of 16S rRNA sequences are now available and suitable primers can thus be selected for gene amplification (Horng *et al.*, 2006; Lisby and Dessau, 1994; Jonas *et al.*, 1995). Here we describe a simple and generally applicable method for molecular diagnosis of LP using specific 16S rRNA PCR amplification in combination with Enzyme Linked Immunosorbent Assay (ELISA). DNA was purified from CTW samples. A 301 bp sequence of the 16S rRNA gene was amplified using Digoxigenin (Dig) labeled nucleotides. The PCR products were hybridized to a biotinylated specific probe immobilized onto streptavidin coated ELISA plates. The reaction was developed using Peroxidase Labeled anti-Dig antibodies.

MATERIALS AND METHODS

This study was conducted and supported by grant from avesina research institute (Thran, Iran) from July 2003 to May 2004.

Bacterial strains and culture condition: Serogroup 1 strain of *L. pneumophila* (ATCC 33152) was used as a standard LP strain. To achieve pure colonies of LP, bacteria were grown on enrichment broth and were spread on to α BCYE agar and MWY α BCYE agar (Hiroshi and Hiroyuki, 1997; Jonas *et al.*, 1995; Aslani *et al.*, 1997). Oligonucleotide probe was synthesized and 5' biotinylated by Cybergene, AB (Stockholm, Sweden). Oligonucleotide primers were synthesized by Cinagene (Tehran, Iran). A PCR core kit including PCR buffer, MgCl₂, dNTPs and Taq DNA polymerase were purchased from Roche (Roche Diagnostics, Mannheim, Germany). Culture media were purchased from Himedia (Tehran, Iran); different chemicals were from Sigma, BBL, Merck and Roche.

Handling of CTW samples for culture and identification

of LP: CTW samples used in this study were taken mainly from the industrial units around Tehran, south and central Parts of Iran. After treatment with acid reagents (0.2 M KCl/HCl pH 2.2) by mixing equal volume of samples and acid treatment reagent and centrifugation (6000 rpm 30 min), samples were plated on specific (α BCYE agar) and selective [α BCYE agar+Legianella 040 (selective agar containing Glycine, polymixin B, Vancomycin and Amisomycin)] media. Nine different biochemical tests (mobility, Hippurate hydrolysis, urease, carbohydrate fermentation, gram staining, Sudan Black B staining, gelatin liquefaction, nitrate reduction and catalase) were used to identify colonies primarily (Jonas *et al.*, 1995; Michio and Astushi, 1993).

DNA preparation from CTW samples: The chromosomal DNA extraction was performed according to Nejadmoghaddam *et al.* (2007) procedure with slight alteration: in that 10 mL on samples were centrifuged for 10 min at 5000 rpm. The pellets were washed twice with PBS and finally resuspended in 120 μ L ice-cold SNE lysis buffer (10% Sucrose, 0.1 M NaCl, 0.1 M EDTA, pH = 8, containing 4 mg mL⁻¹ Lysozyme) and incubated for 30 min at 0°C and then 30 μ L TESS lysis buffer (10 mM Tris/HCl, pH = 7.4, 1 mM EDTA, 100 mM Sodium acetate, 50 μ L of 10% SDS) was added. It was incubated for 15 min at 70°C. After addition of 20 μ L distilled water, the lysate was incubated for 30 min at 37°C with 10 μ L RnaseA (1 μ g μ L⁻¹-Roche). Three microliter of proteinase K (20 mg mL⁻¹-Roche) was added to the solution and incubated at 37°C for 30 min. Phenol/chloroform extraction was carried out and upper aqueous phase transferred into a fresh microtube. Then sodium acetate (pH 5.2) equal to 0.1 volume and cooled Isopropanol equal to 2 volume of the transferred fraction were added and mixed gently. Chromosomal DNA was precipitated by centrifugation for 10 min at 14000 rpm (Eppendorf). DNA pellet washed with 70% ethanol and dried for 30 min and resuspended in 50 μ L distilled water.

DNA electrophoresis: One microliter of extracted DNA was loaded onto ethidium bromide stained 1% agarose gel in 1 \times TBE buffer pH 8.0 and the electrophoresis was performed at 100 volts for 1 h. The size of extracted DNA was estimated by comparison with size marker (marker III from Roche) Fig. 1.

Nested PCR amplification of DNA: Oligomers were selected from the published full-length sequence of the LP 16S rRNA gene (NCBI) nucleotide sequence database: (Accession No. M59157). For the first step

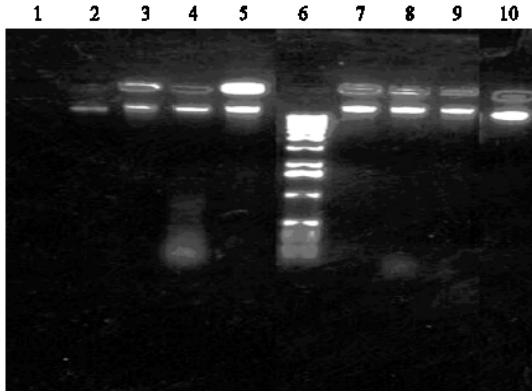


Fig. 1: Agarose gel electrophoresis of extracted DNA. Extracted DNA were electrophoretically separated on 1% agarose gel and visualized by ethidium bromide staining. Lane 1, Negative control was from D.D water, Lane 2, positive control (ATCC 33152), Lanes 3,4,5, three CTW samples (Parsoil refinery, Tehran oil refinery respectively), Lane 6 size marker III from Roche, Lane 7, 8, two CTW samples (Arak petrochemical company, Behran oil refinery respectively) and Lanes 9, 10, Non Legionella bacteria (*Streptococcus* H46A and *Chlamydia pneumoniae*, respectively)

PCR, the sense oligonucleotide primer p1.2 (5'-AGGGTTGATAGGTTAAGAGC-3') was located at position 451 to 470 and the antisense primer cp3.2 (5'- CCAACAGCTAGTTGACATCG-3') was complementary to position 836 to 817. For the second step nested PCR, Lpnest F (5'-GCTGATTAAGTGGACGTTACCC-3') sense primer was located at position 469 to 490 and LpnestR (5'-CTTTCGTGCCCTCAGTGTCAG-3') antisense primer was complementary to position 769 to 749. The nested PCR amplified a 301 bp fragment of the 16S-rRNA gene. The 5'-biotinylated 20-mer cp₂ (5'-CAACCAGTATTATCTGACCG-3'), complementary to positions 630 to 649 was used as the probing oligomer. In the first step PCR; DNA samples (1 µL each) were added to microtubes containing 24 µL of PCR mixture. The PCR mixture contained 1U of taq DNA polymerase (Roche), 20 pmol of each primer (p1.2, cp3), 2.5 µL 10X PCR buffer, 1.5 mM MgCl₂, 0.4 mM dNTPs (each). The PCR profile included an initial denaturation at 95°C for 5 min followed by 40 cycle of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 90 sec. The last extension step was extended for another 10 min at 72°C. In the second-step PCR samples were diluted 1:2000 and were added (1 µL each) to microtubes containing 24 µL of PCR mixture as in the first PCR but containing 20 pmol of

nested PCR primers (LpnestF, LpnestR). The nested PCR was run with the same profile as the first PCR for 40 cycles except that the dNTP mixture contained 0.04 mM Dig-dUTP as well as the same amount of the other dNTPS as above.

Gel electrophoresis: Ten microliter of the PCR product was loaded onto ethidium bromide stained 2% agarose gel in 1×TBE buffer pH 8.0 and the electrophoresis was performed at 100 volts for 45 min. The sizes of DNA fragments were estimated by comparison with size markers (100 bp, Fermentas and marker VIII from Roche).

Enzyme-Linked Immunosorbent Assay (ELISA) for detection of PCR products: Streptavidin-coated microplates were washed with 100 µL PBS-TWEEN 20. Hundred µL of 20 nmol L⁻¹ of biotinylated probe was pipetted into each well. After 1 h incubation at 37°C, the wells were washed three times with PBS-Tween. Next 100 µL hybridization solution (33 µL 10× SSC, 0.5 µL 10% SDS, 10 µL 20% PEG 1500, 56.5 µL D water) was added to each well. The double stranded DNA products were denatured at 95°C for 5 min and were added to each well as quickly as possible in 2 µL volumes and incubated at 50°C for 3 h. The wells were then washed by 3×washing with PBS-Tween. Two microlitres Horseradish peroxidase conjugated sheep anti Digoxigenin F(ab')₂ fragments (150 µmol µL⁻¹) were then added to the wells and incubated at 37°C for 1 h in 100 µL conjugation solution (100 µL Tris/HCl 1M pH 8.3, 100 µL NaCl 1M). The unbound conjugate was removed by three washes with 100 µL of PBS-Tween. Then 100 µL of TMB solution were added and the OD was measured in an ELISA reader at 440 nm.

RESULTS

Isolation of LP colonies from CTW samples by bacterial culture: To clarify the morphology of LP colonies, standard LP (ATCC33152) was cultured on Legionella selective supplement agar (Fig. 2A). CTW samples were then cultured on Legionella specific agar plates. Among the different colonies that grew (Fig. 2B), those that morphologically resembled the LP standard colonies on Legionella selective supplement agar were selected and recultured on Legionella selective supplement agar (Fig. 2C). The resulting colonies were biochemically characterized using different tests (Table 1) and proved that all the CTW samples were contaminated with LP.

Specificity of the nested PCR: Specificity of the LP nested PCR was investigated for standard LP (ATCC33152) and isolated colonies from 5 industrial

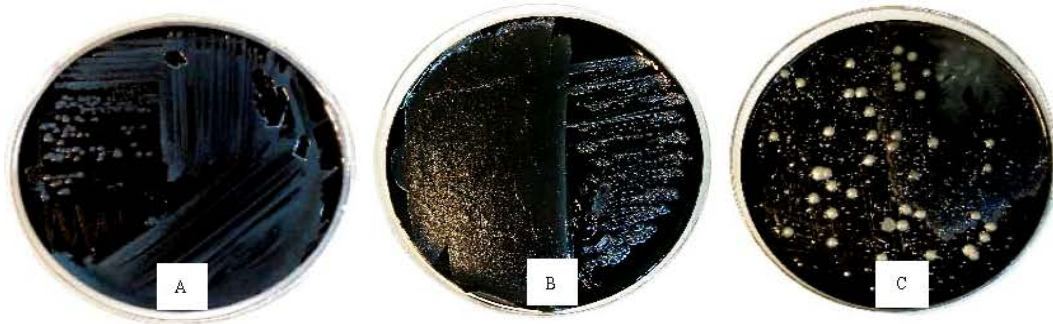


Fig. 2: (A) Pure colonies of standard LP (ATCC33152) on Legionella selective supplement agar, (B) Different colonies of Arak petrochemical company grown on Legionella specific agar plate and (C) Pure colonies of Arak petrochemical company on Legionella selective supplement agar

Table 1: Biochemical characterization of colonies selected on Legionella selective supplement agar

Samples	Gram staining	Sudan black B	Hippurate hydrolysis	Gelatin liquefaction	Urease test	Carbohydrate fermentation	Motility	Nitrate reduction	Catalase
Arak petrochemical	G ⁻	+	+	+	-	-	+	-	+
Pars oil company	G ⁻	+	+	-	-	-	+	-	+
Behran oil company	G ⁻	+	+	+	-	-	+	-	+
LP standard	G ⁻	+	+	+	-	-	+	-	+

G⁻: Gram Negative, +: Test is Positive, -: Test is Negative

Table 2: PCR amplification of DNA from standard LP, LP isolated from CTW and non-Lp bacteria

Bacteria	Amplicon size	
	386 bp ^a	301 bp ^b
Standard LP(ATCC 33152)	+	+
Isolated colony from Arak petrochemical company	-	+
Isolated colony from Bandar Imam petrochemical company	-	+
Isolated colony from Behran oil refinery	+	+
Isolated colony from Pars oil refinery	+	+
Isolated colony from Tehran oil refinery	+	+
Streptococcus H46A	-	-
Chlamydia pneumonia	-	-
SRB bacteria	+	-
Enterobacteriaceae culture	-	-

+: PCR is Positive, -: PCR is Negative, ^a: PCR was performed using primers p1.2 and cp3.2 followed by detection of the amplified product by electrophoresis on an ethidium bromide-stained agarose gel, ^b: PCR was first performed using primers p1.2 and cp3.2 followed by nested PCR primers LpnestF and LpnestR. The detection of the final amplified product was performed by electrophoresis on an ethidium bromide stained agarose gel

cooling towers as well as 4 other non-LP bacterial strains by running the primary and the nested PCRs. Table 2 shows, results of these PCRs on isolated colonies of LP and some non-LP strains. While some of the LP that was isolated from CTW did not show any amplification in the primary PCR, they were all proved to be positive in the nested PCR. None of the non-LP bacteria showed amplification by the nested PCR. However, the SRB bacteria was the only non-LP that showed amplification in the primary PCR which was not confirmed by the nested PCR (Table 2).

Several PCR product sizes resulted from the first-step PCR in most of the samples (Fig. 3). However, in nested

PCR the specific band (301 bp) was detected only in standard LP as well as in our isolated LP colonies and none of the non-LP bacteria produced such products (Fig. 4).

Specific LP detection in CTW by PCR-ELISA: The 16Sr-RNA based PCR amplification products were detected after hybridization with a 5'-end-biotinylated oligomer probe by PCR-ELISA. The result of ELISA where equal amounts of nested PCR products (4 ng) were used. All CTW samples as well as the standard LP strain were positive in ELISA whereas all non-LP bacteria were negative (Table 3).

DISCUSSION

Water supply and CTW are the most common sources of LP contamination. Recently several approaches have been devised to detect these bacteria from water samples. Radioactively labeled nucleotide is hazardous and also requires the presence of relatively large numbers of cells in samples. Cultivation takes a long time (7-14 days) to respond (Jonas *et al.*, 1995; Aslani, 1997; Maiwald *et al.*, 1994). PCR, therefore, represents the method of choice and different target sequences have been proposed for amplification (Declerck *et al.*, 2006). The gene encoding macrophage infectivity potentiator of *L. pneumophila* (*mip*) and the *mip* like gene of other *Legionella* sp. and 5S rRNA gene have been used for PCR amplification and LP detection (Fiume *et al.*, 2005;

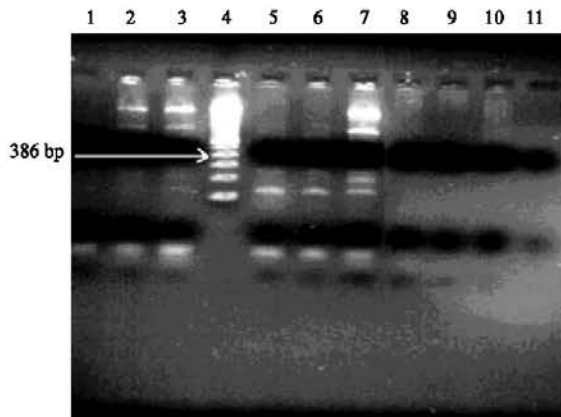


Fig. 3: Agarose gel electrophoresis of the LP first-step PCR products including along with water samples from cooling tower and 4 other bacteria. PCR products were separated on 2%-agarose gels and visualized by ethidium bromide staining. Lane 1: Negative control was from DD water. Lanes 2 and 3: Two cooling tower samples (Arak petrochemical company and Bandar Imam Petrochemical Company, respectively), Lane 4: size marker 8 (Roche), Lanes 5 and 6: Two other cooling tower samples (Behran oil refinery and Pars oil Company respectively) and Lane 7 positive control (standard LP). Lanes 8, 9, 10 and 11: include 4 non-LP bacteria (Streptococcus HubA, *Chlamydia pneumonia* SRB bacteria, Entrobacteriaceae, respectively)

Jonas *et al.*, 1995; Raggam and Leitner, 2002; Edelstein *et al.*, 1987; Wellinhouse and Cathrin, 2001; Michio *et al.*, 1993). Selection of 16S rRNA gene sequences, on the other hand, harbors many advantages (Joly *et al.*, 2006; Jonas *et al.*, 1995; Raggam and Leitner, 2002; Pasculle *et al.*, 1989; Michio *et al.*, 1993; Wellinhouse *et al.*, 2001). Large databases of 16S rRNA gene are available, facilitating selection of appropriate sequences and it has been used as a fundamental molecular marker in bacterial taxonomy. Large amplification products are generated that can easily be identified by gel electrophoresis. There are potential obstacles in PCR like presence of inhibitors, low levels of microbial contamination and the efficiency of the primers. To clarify the negative PCR resulted in amplification of CTW samples; we designed a nested PCR, which clearly showed positive amplification of a specific 301 bp PCR product. Such a strategy has also been used by others (Fiume *et al.*, 2005; Hiroshi and Hiroyuki, 1997; Jonas *et al.*, 1995). To verify the specificity of the amplified product, some researchers have used

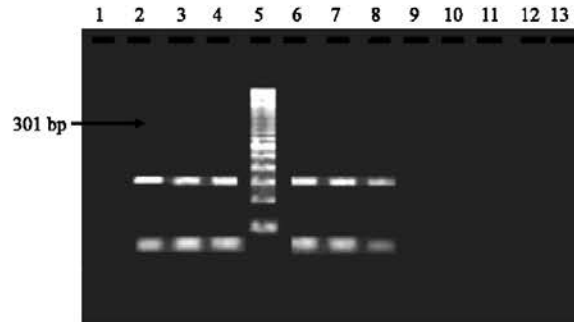


Fig. 4: Agarose gel electrophoresis of the LP specific nested PCR products of different samples as follows. PCR products were separated on % 2-agarose gels and visualized by ethidium bromide staining. Lane 1: Negative control was from DD water, Lane 2: Positive control (standard LP, ATCC33152), Lanes 3 and 4: 2 cooling tower water samples (Arak Petrochemical Company and Bandar Imam Petrochemical Company, respectively), Lane 5 size marker 100 bp (Fermentas) (V. Graiciuno, LITHUANTA), Lanes 6,7,8 and 3 cooling tower samples (Behran oil refinery, Pars oil refinery and Tehran oil refinery, respectively). Lanes 9, 10,11 and 12: 4 non-LP bacteria (Streptococcus H4bA, *Chlamydia pneumonia* SRB bacteria and Entrobacteriaceae culture, respectively)

Table 3: Detection of LP by PCR ELISA

Samples	OD ^a
Negative control	0.215
<i>L. pneumophila</i> (ATCC33152)	2.000
Arak petrochemical cooling tower	1.940
Behran oil refinery cooling tower	1.850
Pars oil refinery cooling tower	1.750
Bandar Imam petrochemical cooling tower	1.900
Tehran refinery cooling tower	1.600
SRB bacteria culture	0.270
Entrobacteriaceae culture	0.265
<i>Chlamydia pneumonia</i>	0.245
<i>Streptococcus pneumonia</i> H46A	0.235

^a: OD readings were performed at 440 nm

radioactive probes (Jonas *et al.*, 1995; Bej *et al.*, 1990; Michio *et al.*, 1993). However, nonradioactive techniques are often preferable and in this regard we chose to use biotin-avidin system to set up a specific probe-based ELISA technique for specific detection of the amplified product. All CTW samples were found to have LP contamination by this method. The presences of PCR inhibitors have also been documented (Horng *et al.*, 2006; Hiroshi and Hiroyuki, 1997). Such inhibitors may even be concentrated during DNA extraction, which may contribute to lowering of the PCR efficiency. The negative PCR results from CTW samples shown in Table 1 may

have resulted from such inhibitors. The second PCR (nested PCR) run on 2000 fold diluted primary PCR products may have effectively diluted the potential inhibitors yielding clear specific bands of 301 bp and providing a platform for increasing PCR efficiency. An important issue when PCR is used for detection of microorganisms is the potential power of PCR in amplifying DNA from dead bodies. (Hiroshi *et al.*, 1997; Maiwald *et al.*, 1994). To rule out such a possibility, all the CTW samples in our study were subjected to Lp specific and selective bacterial culture and all were found to contain live LP.

In summary, we have developed a PCR-ELISA technique to effectively amplify LP 16S rRNA from CTW samples. Present findings also prove the presence of Lp contamination in all the five oil industry plants raising the necessity of especial health care for their employees.

ACKNOWLEDGMENTS

We thank Dr. M. Akhondi and Dr. M.R. Sadeghi for their invaluable support. We are grateful to Roya Ghods and Pouneh Dokouhki for excellent technical assistance. Special thanks to Mr. A.A. Bayat for his kind cooperation in the experiments. This work was supported by grants from Avesina Research Institute.

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