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Original Article

# Isolation, Molecular Detection, and Sequence Analysis of Pathogenic *Leptospira* spp. in Paddy Field Water Samples in Amol, Northern Iran

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**Abstract**

**Background:** Leptospirosis constitutes a zoonotic affliction prevalent in tropical and subtropical locales, instigated by the pathogenic strains of *Leptospira*. The objective of the present study was to isolate and detect *Leptospira* from water samples sourced from paddy fields located in Amol, northern Iran.

**Methods:** A total of 108 samples were procured from rice fields during the spring and summer of 2023. Cultivation, microscopy, and molecular analyses were employed to accurately identify *L. interrogans*. Both conventional and real-time polymerase chain reactions (RT-PCR) targeting the *lipL32* gene were executed for the detection of *L. interrogans*. In addition, the sequence analysis of the *sucA* and *gluM* genes was performed after PCR.

**Results:** The findings revealed that 6/108 (5.55%) of the samples were culture-positive, with all cases corroborated through both RT-PCR and traditional PCR methods. A simple PCR assay showed positive results for 17/108 (15.74%) water samples. RT-PCR successfully identified 17/108 (15.74%) samples as positive for *L. interrogans*. The sequence analysis of the *sucA* gene from the water sample demonstrated high similarity to *Leptospira weilii*. Eventually, the sequence analysis of the *gluM* gene from two water samples displayed high similarity to *Leptospira borgpetersenii*.

**Conclusion:** This investigation highlights the efficacy of synergizing molecular techniques with traditional culture methodologies for the surveillance of *Leptospira* in areas characterized by an elevated risk. The current research serves as the inaugural report, delineating the emergence of leptospires from rice field samples, alongside the characterization of isolates obtained from culture.

**Keywords:** *Leptospira*, Cultivation, Real-time PCR, Water samples

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## Introduction

Leptospirosis is a significant zoonotic disease globally, primarily caused by the pathogenic *Leptospira* species. It is a severe health risk, particularly in the tropics and subtropics, where environmental and occupational exposure to contamination is high (1). This disease is characterized by a wide spectrum of clinical manifestations, ranging from nonspecific flu-like illness to life-threatening conditions, such as Weil's disease and pulmonary hemorrhage syndrome (2). The economic burden of the disease, in addition to its association with floods, agriculture, and poor sanitation, underscores its status as a neglected tropical disease (3).

Water is one of the most significant environmental reservoirs of *Leptospira*, especially in agricultural settings, such as rice paddies, which consist of stagnant water and appropriate climatic conditions for the survival and propagation of these bacteria (4). Rice fields are more relevant in northern Iran, where rice cultivation is one of the most prevalent agricultural activities. Farmers and workers are frequently exposed to water contaminated by infected animal urine, increasing their risk of leptospirosis

(5). Monitoring such water sources is critical not only for understanding the local epidemiology of the disease but also for designing effective preventive strategies.

Despite the importance of surveillance, the diagnostic methods for *Leptospira* have some limitations. The standard method for the clinical identification of leptospirosis is the microscopic agglutination test; however, it is less ideal for environmental samples due to the complexity of water contaminants and the need for live cultures. Traditional culture methods, while required for the isolation of viable organisms, are time-consuming with low sensitivity. Dark-field microscopy is a quicker, though non-specific, alternative (6). Molecular methods, in particular polymerase chain reaction (PCR)-based assays, revolutionized *Leptospira* detection. Real-time PCR (RT-PCR) targeting the *lipL32* gene, a conserved target within pathogenic species, provides sensitive and specific *Leptospira interrogans* detection directly from environmental samples (7). As Amol is an agriculturally significant region and there is a great possibility of leptospirosis, the current research attempted to isolate and identify *L. interrogans* in the water samples of paddy fields in the region. Through the combination of culture, molecular, and genotyping methods, the research proposes to provide detailed knowledge on the prevalence, distribution, and genetic diversity of *Leptospira* in the high-risk area. The findings will not only contribute to what is known about leptospirosis in northern Iran but also to the global effort in the control of this neglected yet significant zoonotic disease.

## Methods

### *Study Area and Sample Collection*

Overall, 108 samples were collected during summer and autumn of 2023. In Amol, Mazandaran province, Iran, which is an area well-known for large rice paddies and cattle rearing, sampling was performed from paddy fields with the following sampling conditions:

Water was collected in 108 rice fields in the study area. Approximately 500 mL of surface water was pulled in sterile bottles at multiple locations in each field to collect a representative sample. The sample was cooled at 4˚C and sent to the laboratory immediately.

### *Culture and Microscopy*

An approximate volume of 1 mL from each water sample was inoculated into the modified Ellinghausen McCullough Johnson and Harris (EMJH) medium, which was fortified with 5-fluorouracil, rifampicin, and neomycin. The remaining urine and water specimens underwent centrifugation at a force of 3500 g for 15 minutes. Approximately 1 mL of the resultant pellet was subsequently employed for a 10-fold serial dilution within the modified liquid EMJH medium. The inoculated EMJH media were incubated at temperatures ranging from 28 ˚C to 30 ˚C over a span of 28 days, with observations for *Leptospira*-like microorganisms conducted on a weekly basis utilizing a dark field microscope. Positive samples underwent filtration through a 0.2-µM membrane filter, and 100 µL of the filtrate was aliquoted onto EMJH solid medium *Leptospira* Vanaporn Wuthiekanun agar plates for the purpose of isolation, in accordance with previously established methodologies (8-10). Successfully isolated leptospiral cultures were subsequently transferred into sterile cryovials containing a glycerol solution specifically designed to mitigate freeze damage. These cryovials were then stored in liquid nitrogen tanks at -80˚C for extended preservation.

### *Deoxyribonucleic Acid Extraction*

DNA was purified from water employing the QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions with accuracy. Purified DNA was then preserved at –20 ˚C until subsequently evaluated by PCR. All isolated DNA specimens were adjusted to a standardized concentration of 10–20 ng/µL.

### *Molecular Detection by Real-Time Polymerase Chain Reaction*

To support the amplification of the DNA, an RT-PCR was performed with primers that were specifically created for amplifying the *lipL32* gene that is associated with pathogenic *Leptospira* (1). The assay was further optimized through the incorporation of reference samples (*L. interrogans* serovar Icterohaemorrhagiae) that had been previously stored at the Pasteur Institute of Iran (North branch). The PCR amplification procedure was executed within a total reaction volume of 25 µL, which comprised 1 × Maxima® SYBR Green I/ROX qPCR master mix (Thermo Scientific, Germany), inclusive of Maxima® Hot Start Taq DNA polymerase, and deoxynucleotide triphosphates within a meticulously optimized PCR buffer that attained a final concentration of 2.5 mM $MgCl_2$. Both forward and reverse primers were utilized to reach the ultimate concentration of 0.4 µM each, succeeded by the addition of 10 µL of extracted DNA (the sample). Each PCR run incorporated negative controls, wherein 10 µL of nuclease-free distilled water was employed in place of the template DNA. *L. interrogans* serovar Icterohaemorrhagiae DNA ( ≤ 100 ng, 10 µL) served as the positive control. The PCR was performed on the Rotor Gene Q 2plex HRM system (Qiagen, Germany) with an initial denaturation at 95 ˚C for 10 minutes, followed by 40 cycles of denaturation at 95 ˚C for 15 seconds, annealing at 53 ˚C for 30 seconds, extension at 72 ˚C for 30 seconds, and an ending incubation step at 72 ˚C for 7 minutes. Melting temperature analysis (70–94˚C with readings every 0.5 ˚C) was performed following a cooling step of 30 ˚C for 1 minute, according to the manufacturer's instructions. The cutoff threshold was determined at a threshold cycle of 35, which, based on our empirical observations, represented the final cycle completely devoid of background interference. All experimental protocols were replicated a minimum of two times to ensure the reliability of the results (9,11).

### Conventional Polymerase Chain Reaction

A set of primers was designed and utilized to selectively amplify the *lipL32* genes within pathogenic *Leptospira*. Positive control was *L. interrogans* serovar Icterohaemorrhagiae. The reaction mixtures of 25 µL consisted of 5 µL 5x PCR buffer, 2.0 mM $MgCl_2$, 0.4 µM primer each, 0.2 mM deoxynucleotide triphosphate mix, 1.25 units Taq DNA polymerase (SinaClone, Iran), and 5 µL DNA template. The PCR cycling conditions included primary denaturation at 95 ˚C for 2 minutes, and then 35 cycles of denaturation at 95 ˚C for 1 minute, primer annealing at 55 ˚C for 30 seconds, and extension at 72 ˚C for 1 minute, followed by a final extension at 72 ˚C for 5 minutes, and finally ending with an open holding time at 4 ˚C. Electrophoresis was subsequently run on a 1.5% agarose gel in 1x Tris-acetate-ethylenediaminetetraacetic acid buffer.

### Primer Design and Sequence Analysis of the gluM and sucA Genes

Two *Leptospira* isolates (W7 and W36, samples with good results in RT-PCR and culture) obtained from cultured water samples collected from rice paddies were selected for the molecular analysis of the *gluM* gene involved in the biosynthesis of extracellular polysaccharides and the *sucA* gene that encodes the E1 component of the 2-oxoglutarate dehydrogenase complex, which plays a key role in the tricarboxylic acid cycle and bacterial energy metabolism. DNA was extracted using a commercial extraction kit (Yekta Tajhiz, Iran), following the manufacturer's instructions. The PCR amplification of the *gluM* and *sucA* genes was performed using specific primers (Table 1) under standard cycling conditions. Primers for PCR were designed in the present study using Oligo primer analysis software (version 7). The amplified PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with safe stain. Positive PCR amplicons were purified and sent for sequencing (Pishgam Biotech Company, Iran). The recovered nucleotide sequences were compared with known sequences within the NCBI GenBank database using the BLAST algorithm to confirm identity and similarity. Multiple sequences were then aligned using ClustalW, and a phylogenetic tree was constructed from the neighbor-joining method using MEGA software (version X) to determine the evolutionary relationship of the isolates with reference strains (12).

## Results

### Results of the Culture and Isolation

Among 108 water samples taken from rice and paddy fields in Amol, 6 (5.55%) were detected as positive by the culture method and showed the presence of the spiral-shaped microorganism in a dark-field microscope image (Figure 1 and 2). All samples that were positive in the culture method were tested using confirmatory PCR, and the presence of the microorganism was confirmed accordingly. Based on the results, no colonies were observed on solid media.

### Molecular Detection Results

Among 108 water samples obtained from rice and paddy fields, 17 (15.74%) were detected positive for the presence of pathogenic *Leptospira* spp. (*L. interrogans*) by the PCR method targeting the *lipL32* gene (Figure 3). In addition, among all water samples taken from rice and paddy fields, 17 (15.74%) were positive for the presence of *Leptospira interrogans* by the RT-PCR method. The same samples that tested positive in the PCR method also tested positive in the RT-PCR assay. Further, all culture-positive samples demonstrated positive molecular detection in both direct PCR and direct RT-PCR assays.

### Sequencing Results

Figures 4 and 5 illustrate the electrophoresis results of the PCR product of genes *sucA* and *glmU*, respectively. The sequence results of the *sucA* gene (for one isolate: W7) and the *glmU* gene (for two isolates: W7 and W36) were registered to the NCBI, and the accession numbers PV613208 (W7-*sucA*), PV613209 (W36-*glmU*), and PV613210 (W7-*glmU*) were received accordingly. Figures 6 and 7 display the phylogenetic tree resulting from the analysis and the comparison of the obtained sequences with the GenBank similar sequences. The high similarity of the *sucA* gene sequence with *Leptospira weilii* and the similarity of the *gluM* gene sequence with *Leptospira borgpetersenii* were identified.

## Discussion

The present study investigated the prevalence and molecular detection of Leptospira spp. in water samples collected from Amol rice paddies, a region with favorable ecological conditions for the survival of leptospires. Through an integration of traditional culture and

**Table 1.** Nucleotide Sequences Used as Primers in PCR and Real-Time PCRs

| Target gene | Sequence (5' to 3') | Annealing (˚C) | Product size (bp) | Reference |
|---|---|---|---|---|
| *lipL32* (genus/PCR) | F: CCCAGGGACAAAAACCGTAA<br>R: ATTTGAGTGGATCAGCGGGC | 55 | 622 | This study |
| *lipL32* (genus/real-time PCR) | F: AAGCATTACCGCTTGTGGTG<br>R: GAACTCCCATTTCAGCGAT | 53 | 242 | (11) |
| *gluM* (sequencing) | F: TGTGGTATTAGCCGCAGGGAAG<br>R: GCAAATACAGCAATGTCTCCGTTC | 56 | 755 | This study |
| *sucA* (sequencing) | F: TCAGATGGACGGGCTCGTGATC<br>R: GCAGGCTCGTCCGTTTCATTATG | 58 | 657 | This study |

*Note*. PCR: Polymerase chain reaction; F: Forward; R: Reverse.

**Figure 1.** Dark Field Microscope Image of a *Leptospira* Isolate Obtained From a Paddy Field Water Sample

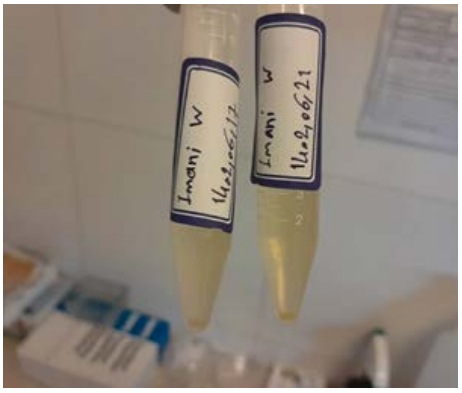

**Figure 2.** Growth of *Leptospira* spp. Isolated From Paddy Field Water Samples in the EMJH Semi-solid Medium. *Note*. EMJH: Ellinghausen McCullough Johnson and Harris

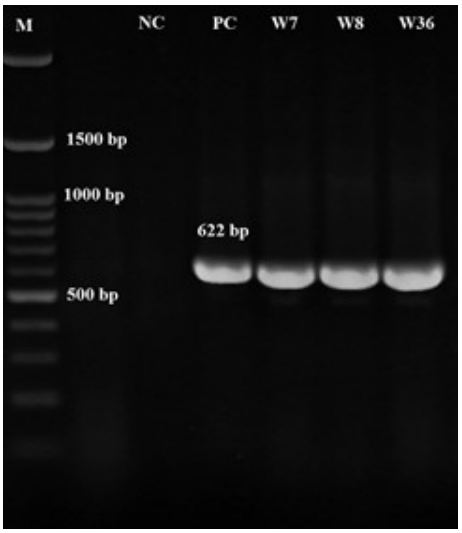


**Figure 3.** Agarose Gel Electrophoresis of PCR Products Amplified Using *lipL32* gene-Specific Primers From *Leptospira* spp. Isolated From Paddy Field Water Samples. *Note*. PCR: Polymerase chain reaction

molecular techniques, the important prevalence of pathogenic *Leptospira* species within this habitat was revealed, confirming the public health significance of

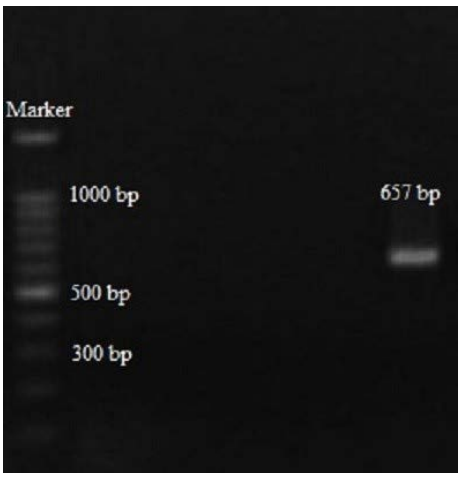


**Figure 4.** Agarose Gel Electrophoresis of PCR Products Amplified Using *sucA* gene-Specific Primers From *Leptospira* spp. Isolated From Paddy Field Water Samples. *Note*. PCR: Polymerase chain reaction

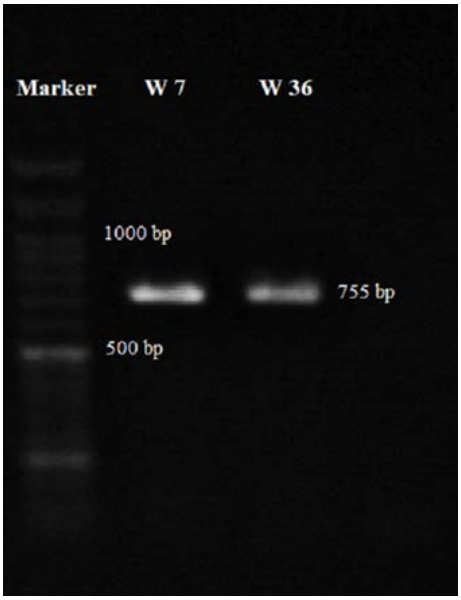


**Figure 5.** Agarose Gel Electrophoresis of PCR Products Amplified Using *glmU* Gene-Specific Primers From *Leptospira* spp. Isolated from paddy field water samples. *Note*. PCR: Polymerase chain reaction

waterborne leptospirosis in the north of Iran.

While conventional culture is considered the gold standard for *Leptospira* detection, its sensitivity is limited in environmental samples due to low bacterial load, long incubation time, and high contamination risk (13). In our study, a small subset of water samples (5.55%) yielded positive results through culture, confirmed by dark-field microscopy. These results align with the findings where small numbers of environmental samples were culture-positive despite being collected from high-risk areas (14,15). Ling et al reported that 4.8% of paddy field samples were positive for the presence of *Leptospira* by both culture and molecular methods (16), which is considerably consistent with the results of the present study. The sampling area in the current study is located in northern Iran and the southern coastal strip of the Caspian Sea, an area that has extensive similarities in

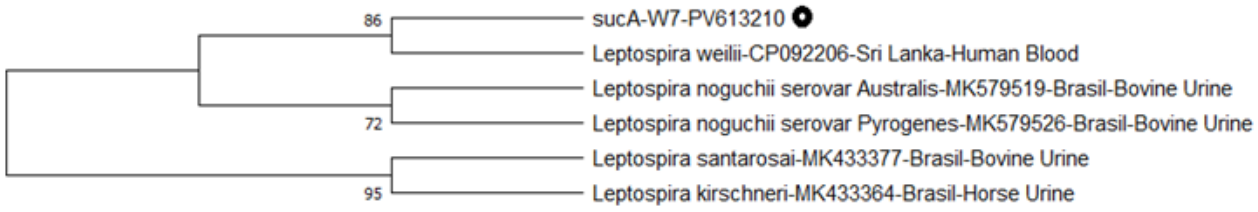


**Figure 6.** Phylogenetic Dendrogram Generated Using MEGA X Software Based on the *sucA* Gene Sequences of *Leptospira* spp. Isolated From Paddy Field Water Samples and Reference Sequences From the NCBI Database. *Note*. NCBI: The National Center for Biotechnology Information

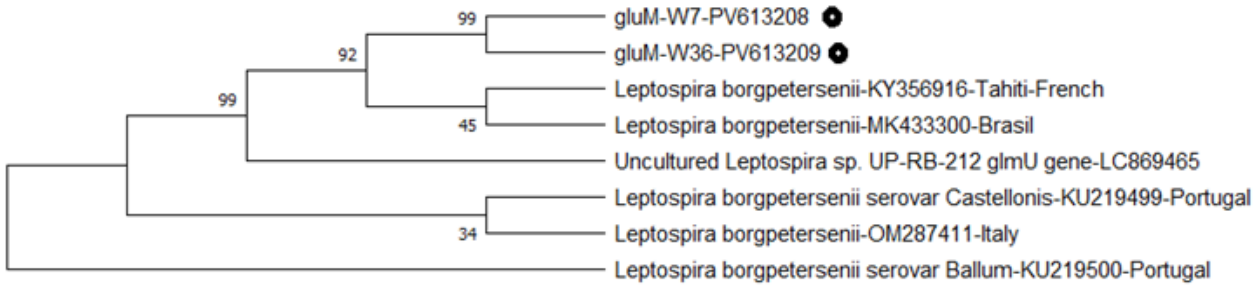


**Figure 7.** Phylogenetic Dendrogram Generated Using MEGA X Software Based on the *glmU* Gene Sequences of *Leptospira* spp. Isolated From Paddy Field Water Samples and Reference Sequences From the NCBI Database. *Note*. NCBI: The National Center for Biotechnology Information

terms of climate with Southeast Asia, where many studies show similar prevalence of *Leptospira* in these areas (17).

However, the isolation of *L. interrogans* in water samples from paddy fields in Amol highlights the potential public health risk associated with environmental exposure to contaminated surface water, as leptospirosis and rice field fever are commonly present in this region and affect many people every year (5,18). In this region of Iran, paddy fields are located in rural areas that are very close to cities. On the other hand, there are many domestic and wild animals, especially rodents, which are the main carriers of this bacterium and are the main way of transmitting contamination to these waters (19,20).

In this study, by PCR and RT-PCR, 15.74% of the water samples tested positive, indicating the remarkable presence of pathogenic *Leptospira* species in the rice cultivation areas. These findings conform to those of previous studies conducted in endemic regions, where paddy field environments were identified as major reservoirs for leptospires due to favorable conditions, such as high humidity, standing water, and rodent activity (16,21). The higher detection rate by RT-PCR in comparison to culture (15.74% vs. 5.55%) supports previous findings, indicating that molecular methods are more effective for the direct detection of Leptospira in environmental samples (16). In contrast to cultivation methods, molecular techniques, such as PCR and RT-PCR, offer higher sensitivity and specificity in *Leptospira* contamination detection and source identification, making them reliable tools for environmental surveillance (22,23). This is particularly important for rapid risk assessment in agricultural communities, where workers are frequently exposed to potentially contaminated water. In addition, many studies emphasize the effectiveness of the molecular identification of *Leptospira* using the *lipL32* gene targeting (24). In the present study, using both PCR and RT-PCR methods, the *lipL32* gene was targeted for molecular identification, and acceptable results were obtained accordingly. Given that *L. interrogans* is one of the most pathogenic species associated with severe human leptospirosis, its detection in agricultural water sources is alarming and warrants targeted interventions, including public health education, rodent control, and possibly vaccination strategies for at-risk populations.

The primary goal of sequencing the *Leptospira* genes in this study was to perform the multilocus sequence typing technique. Despite the culture and molecular confirmation of the studied samples, the other genes were not well sequenced in the present study. This method was performed in a parallel study on animal isolates and responded well (unpublished data). Therefore, only the sequences that were worth examining were evaluated in this study. Sequence comparison, similar to other methods, confirmed the presence of *Leptospira* strains, and a significant similarity was observed between *L. borgpetersenii* and *L. weilii* in the studied samples. *L. borgpetersenii* is most commonly associated with domestic animals, such as cattle, and other animals, including rodents (25,26), indicating the contamination of agricultural waters and paddy fields by these animals that roam freely in these areas.

In conclusion, the detection of pathogenic *Leptospira* in paddy field water through both conventional and molecular approaches highlights the potential public health risk in northern Iran. According to the sequencing results, the presence of genetically diverse species suggests complex transmission dynamics that warrant further investigation. Our findings emphasize the need

for environmental monitoring and preventive strategies, particularly for high-risk occupational groups, such as rice farmers.

## Conclusion

In conclusion, the detection of pathogenic Leptospira in paddy field water through both conventional and molecular approaches highlights the potential public health risk in northern Iran. According to the sequencing results, the presence of genetically diverse species suggests complex transmission dynamics that warrant further investigation. Our findings emphasize the need for environmental monitoring and preventive strategies, particularly for high-risk occupational groups, such as rice farmers.

### Authors' Contribution

**Conceptualization:** Esmail Fattahi, Rahem Khoshbakht.
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**Investigation:** Fereydoun Imani.
**Methodology:** Fereydoun Imani, Esmail Fattahi, Rahem Khoshbakht.
**Supervision:** Esmail Fattahi, Rahem Khoshbakht.
**Writing – original draft:** Fereydoun Imani.
**Writing – review & editing:** Esmail Fattahi, Rahem Khoshbakht, Sadegh Fattahi, Mohsen Asouri.

### Competing Interests

The authors declare that they have no conflict of interests.

### Ethical Approval

Essential ethical approval to conduct this study was obtained from the Ethics Committee of Islamic Azad University, Babol Branch, with the code of ethics: IR.IAU.BABOL.REC.1403.133.

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