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Case series study of nosocomial Legionnaires' disease in Apulia region (southern Italy): The role of different molecular methods in identifying the infection source

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Abstract. *Background and aim:* Legionnaires' disease is a severe form of pneumonia caused by the inhalation or aspiration of water droplets contaminated with *Legionella pneumophila* and other *Legionella* species. These bacteria are commonly found in natural habitats and man-made water systems. Legionnaires' disease is a significant public health problem, especially in healthcare settings where patients may be exposed to contaminated environmental sources. Nosocomial outbreaks have been reported worldwide, leading to high morbidity and mortality rates, and increased healthcare costs. This study aimed to compare, the clonal relationship of clinical *L. pneumophila* strains from two different hospitals with *L. pneumophila* strains isolated from the water supply. *Research design and methods:* In the period from 2019 to 2021, clinical and environmental strains involved in three cases of legionellosis were compared by means of pulsed field gel electrophoresis and sequence based typing techniques. *Results:* Our findings highlight the persistence of clonally distinct strains within each hospital examined. Furthermore, the *L. pneumophila* strains detected from hospital environmental sources were related to the clinical strains isolated, demonstrating the nosocomial origin of these cases. *Conclusions:* Therefore, it is important to implement more accurate surveillance systems both for epidemiological studies and to check the effectiveness of remediation procedures. (www.actabiomedica.it)

Key words: *Legionella*, Legionnaires' disease, nosocomial infection, case series study, molecular typing, PFGE, SBT

Introduction

Legionnaires' disease (LD) is a severe form of pneumonia subject to mandatory notification in class II. It is caused by the inhalation or aspiration of small water droplets contaminated with *Legionella pneumophila* and other *Legionella* spp., which are commonly found in natural habitats (e.g., rivers, lakes, and wet soil) or artificial water systems (e.g., cooling towers, hot tubs, and plumbing systems) (1-5).

Legionella can exist in different forms within water systems, such as free-floating, residing within ciliated protozoa such as *Tetrahymena*, or within amoebas such as *Acanthamoeba*, *Naegleria*, and *Hartmannella*, or attached to biofilm (6). The presence of biofilm and protozoa can provide *Legionella* with a supply of nutrients and protection against unfavorable conditions.

Based on advances in molecular techniques and genome sequencing technology, the genus *Legionella* has been shown to comprise 66 different species to date (7). Although *L. pneumophila* serogroup (sg) 1 is considered the main causative agent of disease, being responsible for 82% of reported cases, 3% of cases are attributed to other species, some of which are more prevalent in water supplies (8).

LD is a significant public health concern particularly in healthcare settings, where patients are more vulnerable to infection because of a compromised immune system, underlying medical conditions, or exposure to medical equipment (e.g., endoscopes, devices for artificial respiration and oxygen therapy, dental devices) (9-11).

Outbreaks of nosocomial LD have been reported in hospitals and healthcare settings worldwide, leading to significant morbidity and mortality, especially among immunocompromised patients, and increasing healthcare costs (12). In Europe, healthcare-associated infection was identified as the source of infection in 5% of all cases of LD reported in 2021, which was similar to previous years (8).

In Italy, 2.9% of the total notified cases in 2022 was of nosocomial origin. In particular, in Apulia (southeastern Italy) the incidence of legionellosis was 27.1 cases per 1,000,000 inhabitants, of which 7.5% were of nosocomial origin (13).

Recently, the legislative decree 18/23 (14) was implemented by the European directive 2020/2184. This included the search for *Legionella* among the risk assessment parameters in the water network of priority structures (hospitals and hotels), and set a limit of <1,000 colony-forming units (CFU)/L. The same decree cites guidelines (15), which recommend a limit for *L. pneumophila* of <100 CFU/L.

Among the techniques for microbiological investigation, the culture-based method on clinical samples of the respiratory tract remains the gold standard test because it allows for the isolation and identification of microorganisms at the species and serogroup levels. In parallel, numerous studies highlight the role of molecular investigations as an indispensable test for tracing the circulation of *Legionella* and identifying the source of infection through comparison between clinical and environmental strains (16-18). The current gold standard for typing *L. pneumophila* is sequence-based typing (SBT), which involves analyzing portions of seven gene targets and defining an allelic profile on the basis of polymorphic variations (19,20). Some studies have compared the results obtained by SBT with pulsed field gel electrophoresis (PFGE) (21-23) in outbreak investigations and in the development of targeted interventions (2).

In Apulia (southern Italy), the Regional Reference Center for the surveillance of infectious diseases (OER) collects all the informations obtained from environmental and clinical investigations in a cloud database dedicated to legionellosis. In the case of clusters or in cases where it is possible to compare the clinical strain with the environmental strain, the OER is required to send the *Legionella* strains to the National Reference Laboratory.

In the period from 2019 to 2021, three cases of legionellosis occurred in patients hospitalized in two independent hospitals (A, B) in the Apulia region. Because nosocomial infection was suspected, in addition to the routine investigations on clinical samples, environmental investigations were carried out to verify the contamination of the water network by *Legionella*. The aim of this study was to isolate the environmental strains and compare them with the clinical strains using different molecular approaches to identify the source of infection.

Patients and methods

Legionellosis case description

Definition of a nosocomial legionellosis case

In accordance with the 2018 EU/EEA case definition (24), a case of LD is defined as nosocomial when the patient is hospitalized for 10 days before the onset of symptoms and presents an acute infection of the lower respiratory tract detectable by clinical and/

or radiological examination, supported by one or more of the following: isolation by culture of *Legionella* from respiratory specimens, detection of *L. pneumophila* antigen in urine, and/or a four-fold increase in specific antibody levels.

Case 1

In March 2019, a 77-year-old male was admitted to hospital A (nephrology ward, located on the ground floor) for ureteral carcinoma, having recently undergone surgery and chemotherapy. After 30 days of hospitalization, a fever, cough, and shortness of breath developed.

Initial investigations, including a chest X-ray, did not provide an accurate diagnosis and the patient's symptoms continued to worsen despite treatment with antibiotics. A *Legionella* urinary antigen test was performed and found to be negative. Culture of a bronchoalveolar lavage sample was requested. However, because of the severity of the disease, the patient died.

Environmental investigations were started in the two hospital rooms (room no. 5 and room no. 6) that the patient had occupied during hospitalization and in a room located remotely as a control (room no. 9). Three water samples (C1-SK5, C1-SK6, C1-SK9) were collected from the sink tap in each room.

Case 2

In March 2021, a 34-year-old female was admitted to hospital A (hematology ward, located on the third floor) for acute myeloid leukemia and underwent chemotherapy. After 16 days of hospitalization, she developed a fever, cough, and shortness of breath, which became progressively worse. She was initially treated for suspected bacterial pneumonia with broad-spectrum antibiotics, but her symptoms did not improve. A chest X-ray showed bilateral infiltrates in the lungs. The *Legionella* urine antigen test was negative. Culture of a bronchoalveolar lavage sample was requested.

Environmental investigations were immediately started: one water sample (C2-SK4) was taken from the sink tap in the room where the patient was hospitalized (room no. 4) and two samples (C2-SK1, C2-SK8) were taken from strategic points in the water network as controls.

Case 3

In March 2021, a 68-year-old female was admitted to hospital B (oncology ward, located on the third floor) for pancreatic cancer and concomitant rheumatoid arthritis. She had undergone chemotherapy for pancreatic cancer and was also on immunosuppressant drugs for rheumatoid arthritis.

Twelve days after admission, the patient developed a fever, cough, and shortness of breath, which gradually worsened. The patient was initially treated for suspected bacterial pneumonia with broad-spectrum antibiotics, but symptoms did not improve.

The *Legionella* urine antigen tested positive. The culture method was performed on bronchoalveolar lavage. Targeted antibiotic therapy was immediately started, and oxygen support was also provided. Despite her critical condition, the patient recovered and was discharged from the hospital.

A total of three water samples (SK19, SH19, SK30) were taken in the two rooms where the patient was hospitalized (sink tap and shower from room no. 19, sink tap only from room no. 30). In this case, a control room was not sampled.

Culture-based method for clinical and environmental investigation

Clinical samples were seeded onto plates containing GVPC agar (Liofilchem Srl, Teramo, Italy) supplemented with glycine, vancomycin, and polymyxin B (to inhibit the growth of other bacteria) and cycloheximide (to inhibit the growth of yeasts and molds). The plates were incubated at 36°C ± 2°C for two weeks in a humid environment (to prevent desiccation of the plates). The colonies suspected to be *Legionella* spp. were subcultivated on sheep blood agar (bioMèrieux SA, Marcy l'Etoile, France) without L-cysteine and incubated at 36°C ± 2°C for another two weeks. Finally, the isolates for each case (classified as C1, C2, and C3, respectively) were identified at the serogroup level by latex agglutination tests with polyvalent (Biolife Italiana Srl, Milan, Italy) and monovalent antisera (Biogenetics Srl, Tokyo, Japan).

The search for *Legionella* in the water supply of the hospitals involved in the cases under study was

conducted according to ISO 11731:2017 (25) by sampling a liter of water from the water supply points present both in the rooms where the patients were hospitalized and in other rooms located on the same floor as a control. The samples were taken under the conditions of use, specifically without preliminary flushing and without flaming, then immediately transported to the Regional Reference Laboratory at room temperature and protected from light (26).

Each sample was filtered through a 0.22 µm isopore polycarbonate membrane (47 mm in diameter with a pore size of 0.22 µm) (Millipore Corporation, Bedford, MA, USA), resuspended in 10 ml of the same water sample, and vortexed vigorously using a vortex mixer for at least 2 min. Subsequently, 200 µl of sample was seeded onto GVPC medium (Biolife Italiana, Monza, MI, Italy) followed by incubation at 36°C ± 2°C for 7–10 days in a humid environment, and quantitative evaluation was expressed in colony-forming units/liter (CFU/L). Suspected colonies were subcultured on buffered charcoal yeast extract (BCYE) agar plates (BioMérieux, Marcy l'Etoile, France) with and without L-cysteine. Colonies that grew only on BCYE agar plates with cysteine added were identified as previously described for clinical investigations (25).

Molecular investigation of clinical and environmental strains

For each case of LD enrolled in the study, a molecular comparison was carried out between the clinical and environmental strains of *Legionella* isolated from the water network of rooms associated with patients and control rooms. To establish genomic matching between clinical and environmental isolates a comparison was performed using two molecular techniques: pulsed field gel electrophoresis (PFGE) and sequence-based typing (SBT).

Pulsed field gel electrophoresis (PFGE)

PFGE of the 12 strains was performed using a modification of the CDC PulseNet standardized PFGE protocol, as previously reported (27). All PFGE profiles were digitalized, and the phylogenetic relationship was assessed using the fingerprinting software GelJ (28). The phylogenetic relationship was depicted with a phylogenetic tree obtained using the unweighted pair-group method with the arithmetic mean (UPGMA). A 2.5% tolerance in band position differences was applied **(**29). Strains were assumed to be clonally related when the percentage of similarity was higher than 90%.

Sequence-based typing (SBT)

Genomic DNA was extracted using a QIAamp mini kit (QIAgen) by a QIAcube instrument (QIAgen). The SBT method was performed according to the standard protocol prepared by the ESCMID study group for *Legionella* infections (ESGLI) using seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) (30). For each gene sequence, a distinct allele number was assigned through the SBT database for *L. pneumophila* (https://webarchive.nationalarchives.gov.uk/ukgwa/20190501130700/http://bioinformatics.phe.org.uk/legionella/legionella_sbt/php/sbt_homepage.php.

The combination of these allele numbers defines the allelic profile to which a sequence type (ST) is attributed using the SBT database.

Results

Hospital A

Two cases of LD were diagnosed in hospital A: the first occurred on the nephrology ward, the second occurred on the hematology ward.

Culture-based method for clinical and environmental investigation

L. pneumophila sg 6 (Lpn6) was isolated from the bronchoalveolar lavage of case 1 and from the sputum of case 2 (hereafter referred to as strains C1 and C2, respectively). Regarding environmental investigations, Lpn6 was isolated from both water samples collected from the rooms occupied by the two patients and from the control rooms (Table 1).

Table 1. Results of *Legionella* spp. detection from water samples by culture-based methods.

Case	Environmental sample	ID	CFU/L	*L. pneumophila* serogroup
Case 1 -Hospital A	Sink tap (room no. 5)[a]	C1-SK5	11,000	Lpn sg6
	Sink tap (room no. 6)[a]	C1-SK6	5,000	Lpn sg6
	Sink tap (room no. 9)[b]	C1-SK9	15,000	Lpn sg6
Case 2 -Hospital A	Sink tap (room no. 4)[a]	C2-SK4	21,000	Lpn sg6
	Sink tap (room no. 1)[b]	C2-SK1	49,000	Lpn sg6
	Sink tap (room no. 8)[b]	C2-SK8	10,000	Lpn sg6
Case 3 -Hospital B	Sink tap (room no. 19)[a]	C3-SK19	16,000	Lpn sg1
	Shower tap (room no. 19)[a]	C3-SH19	7,400	Lpn sg1
	Sink tap (room no. 30)[a]	C3-SK30	960	Lpn sg1

[a] patient room; [b] control room.

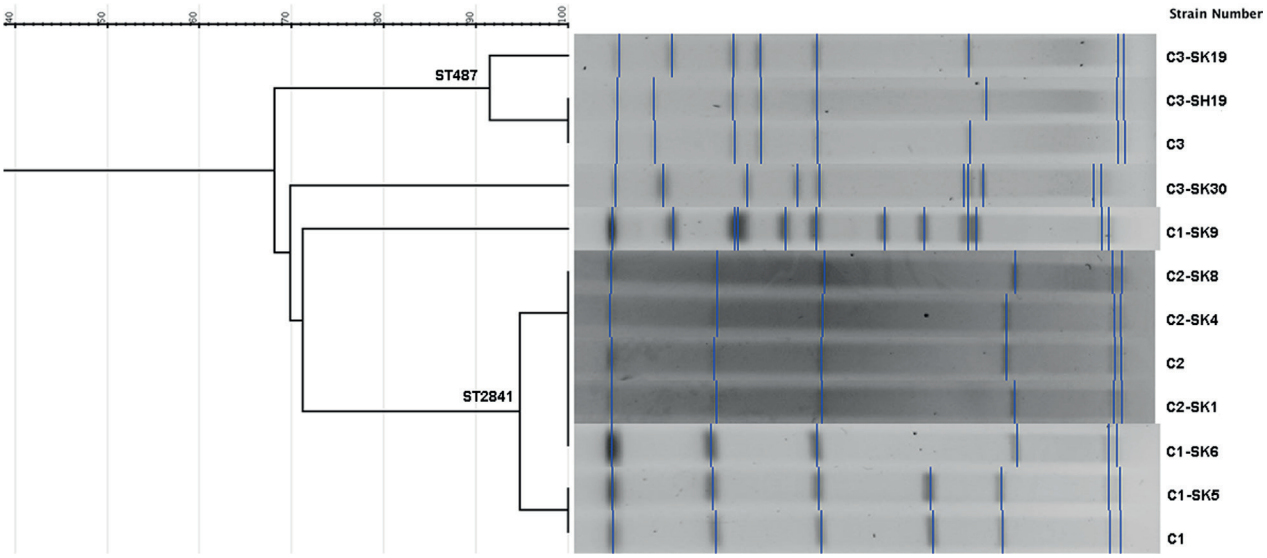

Figure 1. Dendrogram and PFGE patterns (generated by *Asc*I) of the clinical and environmental *L. pneumophila* strains.

Molecular investigation of environmental and clinical strains

In case 1, the PFGE-profile of C1 was assigned as pulsotype P1 (Figure 1). This pulsotype was found to be indistinguishable from that exhibited by strain C1-SK5 and closely related to that exhibited by C1-SK6 (pulsotype P2). Conversely, the pulsotype (named P3) exhibited by strain C1-SK9 differed from pulsotype 1 in at least eight *Asc*I fragments and was found to be distantly related to the latter. Strain characterization by SBT, similar to PFGE typing, clustered *L. pneumophila* strains into two types: C1, C1-SK5, and C1-SK6 as ST2841, and C1-SK9 as ST1223 (Table 2).

In **case 2,** the PFGE profile (pulsotype P2) exhibited by C2 was found to be indistinguishable from that exhibited by strain C1-SK6 detected in case 1 (Figure 1). As for C2, strains C2-SK4, C2-SK1, and C2-SK8 (from room nos. 4, 1, and 8, respectively) exhibited pulsotype P2. All *L. pneumophila* strains isolated from patients and from water samples were classified as ST2841 based on SBT (Table 2).

Hospital B

Culture-based method for clinical and environmental investigation

Regarding case 3, *L. pneumophila* sg 1 (Lpn1) was isolated from the bronchoalveolar lavage of a patient on the interventional oncology ward (hereafter referred to as strain C3). Environmental samples collected from rooms occupied by this patient tested positive for Lpn1.

Molecular investigation of environmental and clinical strains

The PFGE profile exhibited by C3 (strain isolated from the patient) was named pulsotype P4 (Figure 1). From samples taken from room 19, two *L. pneumophila* strains were found: one (named C3-SH19, isolated from the shower) showed the same pulsotype P4; the second (named C3-SK19, isolated from the sink tap) exhibited a pulsotype (named P5) that was phylogenetically related to P4 (it differed from P4 in only two *Asc*I fragments). Conversely, *L. pneumophila* strain C3-SK30, isolated from room 30, exhibited a PFGE profile (named pulsotype P6) that was phylogenetically unrelated to P4 (it differed from P4 in at least 12 *Asc*I fragments).

All Lpn1 strains isolated from patient and water samples belonged to ST487 (Table 2). Strain C3-SK30 belongs to ST2995 (Figure 1).

Clonal relationship

Molecular characterization of the *L. pneumophila* strains analyzed in this study highlighted the presence of two major clonally distinct groups (Figure 1): one comprising strains of ST2841 (detected in hospital A)

Table 2. Molecular typing of *L. pneumophila* strains by pulsed field gel electrophoresis (pulsotype) and sequence-based typing (allelic profile - ST).

Strain source	Clinical strains	Water network strains	Pulsotype	Allelic profile	Sequence type (ST)
Case 1 - Hospital A	C1		P1	3,10,1,28,14,4,3	ST2841
Sink tap (room no. 5)[a]		C1-SK5	P1	3,10,1,28,14,4,3	ST2841
Sink tap (room no. 6)[a]		C1-SK6	P2	3,10,1,28,14,4,3	ST2841
Sink tap (room no. 9)[b]		C1-SK9	P3	1,4,3,5,1,1,213	ST1223
Case 2 - Hospital A	C2		P2	3,10,1,28,14,4,3	ST2841
Sink tap (room no. 4)[a]		C2-SK4	P2	3,10,1,28,14,4,3	ST2841
Sink tap (room no. 1)[b]		C2-SK1	P2	3,10,1,28,14,4,3	ST2841
Sink tap (room no. 8)[b]		C2-SK8	P2	3,10,1,28,14,4,3	ST2841
Case 3 - Hospital B	C3		P4	3,6,1,28,14,11,11	ST487
Sink tap (room no. 19)[a]		C3-SK19	P5	3,6,1,28,14,11,11	ST487
Shower (room no. 19)[a]		C3-SH19	P4	3,6,1,28,14,11,11	ST487
Sink tap (room no. 30)[a]		C3-SK30	P6	1,4,3,1,1,1,1	ST2995

[a] patient room; [b] control room.

and one comprising strains of ST487 (detected in hospital B). Strains of ST2841 included two sub-groups (strains from case 1 and case 2) characterized by closely-related pulsotypes P1 and P2, respectively. Similarly, strains of ST487 included two sub-groups represented by the closely related pulsotypes P4 and P5.

Among the environmental samples collected at hospitals A and B, *L. pneumophila* strains with a distinguishing allelic type (ST) and PFGE profile of P3 and P6, respectively, were also detected. The distinguishing profiles, P3 and P6, were found to be distantly related to the clinical case profiles P1 or P2 and P4, respectively.

Discussion

The genomic correlations between clinical and environmental *Legionella* isolates enable identification of the source of infection and the subsequent prevention of new outbreaks by the implementation of appropriate control measures (23).

Molecular typing techniques are valuable tools for strain characterization and clinical and epidemiological studies (31). A combination of typing methods based on the detection of different genomic features might, especially for short-term studies, describe in more detail clonal and sub-clonal bacteria populations involved in clinical cases, outbreaks, and as sources of contamination (32).

In this study, we assessed and compared the clonal relationships among *L. pneumophila* strains isolated from three clinical cases, which occurred in two different hospitals, with *L. pneumophila* strains detected from environmental sources possibly linked to the clinical cases.

This is the first study in Apulia comparing the genotypes of clinical strains and environmental strains isolated from water systems of *L. pneumophila* by SBT and PFGE. Previous studies conducted in the same region that involved SBT of clinical strains of *L. pneumophila* 1 (Lpn1) (33) and environmental strains of *L. pneumophila* 6 (Lpn6) (9) detected other STs, namely ST1, ST23, and ST42 for clinical strains and ST1989 for environmental strains.

In our case study, we isolated the same species and serogroup circulating as those detected in previous years (i.e., Lpn6 and Lpn1) but they were characterized by different sequences. In particular, Lpn sg 6 - ST 2841 (hospital A) was identified both from biological samples from two patients and from the water supply of their respective rooms, which may be consistent with a nosocomial origin for the clinical cases. To our knowledge, the ST2841 strain has never been documented in the literature as being responsible for legionellosis.

In the control room of case 1, the presence of Lpn6 - ST1223, isolated for the first time in Kuwait from a cooling tower and hot water building, was also detected (34). This demonstrates that strains apparently identical in terms of species and serogroup, but with distinct genomic features, can circulate in the same water network. Some authors have demonstrated that some STs are more virulent than others, especially those associated with nosocomial cases (35). Consequently, identifying the circulating STs in a water network can be useful in terms of devising interventions for prevention or more targeted therapeutic protocols (36). Furthermore, the fact that some water samples taken from the same hospital water network harbored genetically unrelated strains highlights the importance of multi-point sampling to gain the complete picture.

In hospital B, both the clinical and environmental strains were identified as Lpn1 - ST487, an ST identified in Belgium in a travel-related case (37). In the same hospital, a new strain was detected (strain C3-SK30) in one of the two rooms occupied by patient C3, later identified as ST2995.

In our opinion, this study highlights some useful considerations for LD control and prevention in healthcare settings, as follows:

a. molecular investigations can demonstrate that genetically unrelated strains of *Legionella*, even those belonging to the same species and serogroup, can coexist in the same water network;
b. in agreement with other authors (38), comparing the two molecular methods (SBT and PFGE), isolates with the same ST showed different pulsotypes. Specifically, Lpn6 - SBT 2841 (case 1) included two different pulsotypes: P1 (clinical and environmental strain) and P2 (environmental strain). Similarly,

Lpn1 - SBT 487 (case 3) included two different pulsotypes: P4 (clinical and environmental strains) and P5 (environmental strains);

c. the close clonal relationship between the *L. pneumophila* strains of case 1 and case 2, which occurred in the same hospital but a few years apart, underlines the importance of developing adequate corrective strategies to verify the effectiveness of the remediation procedures (10). In fact, just as it is important to study the antimicrobial resistance of *Legionella* against antibiotics commonly used in therapeutic treatments of the patient (39,40), so it is necessary to investigate the effectiveness of disinfection methods in the water network to avoid resistance to common remediation treatments that are not yet sufficiently documented (41-43).

In agreement with other authors (21-23), both techniques have advantages and disadvantages: PFGE seems to have a high discriminatory power, but requires time, specialized equipment for electrophoresis, computer-assisted image analysis, and high-proficiency operators (23); SBT is simple and quick to implement, but, in cases of outbreaks caused by multiple clones, was not able to provide sufficiently discriminatory results alone (44,45). Currently, the limitations of these two methods can be overcome by the use of new molecular approaches, such as whole-genome sequencing (WGS), analysis of single nucleotide polymorphisms (SNPs), or multilocus sequence typing (MLST), and such methods are mainly used to investigate LD outbreaks (46,47).

Conclusions

Legionellosis is an infectious disease that is not easy to manage. Various factors impact the characteristics of the causative agent and its capacity to adapt within water distribution systems. In addition to traditional culture-based investigations and accurate microbiological diagnoses, molecular investigations play a crucial role in understanding the local epidemiology of the disease, particularly in healthcare settings. By analyzing the genetic and molecular characteristics of strains found in the water distribution system, it becomes possible to track the source of infection. This knowledge provides valuable support for developing appropriate strategies to prevent and control the disease, with the ultimate goal of reducing its incidence.

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