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Clinical and environmental investigation of six cases of *Chryseobacterium arthrosphaerae* infections in a Southern Italian hospital

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Chryseobacterium spp. are Gram-negative, opportunistic pathogens antibiotic-resistant commonly found in the environment. The aim of this study was to investigate potential sources of *Chryseobacterium* infections in healthcare settings by comparing clinical and environmental isolates using phenotypic and genotypic analyses. Between June and July 2023, six cases of infection with *Chryseobacterium* spp. were identified in a hospital in Apulia, southern Italy. Environmental sampling (air, surfaces and water) was performed in parallel with routine clinical investigations. Isolates were subjected to antibiotic susceptibility testing and genotypic analysis using Sanger and Next-Generation Sequencing. Five cases of *Chryseobacterium* spp. infection were recorded in the Gastroenterology Department (Pavilion A) and one in the Vertebral Surgery Department (Pavilion B). *C. indologenes* was identified in blood and tracheal aspirate samples using MALDI-TOF MS. Environmental analysis carried out in the pavilions A and B isolated *C. indologenes* from sink tap in Pavilion B. Subsequently, genome sequencing revealed that *Chryseobacterium* strains misidentified as *C. indologenes* were more closely related to *C. arthrosphaerae*. Genetic analysis confirmed the cluster hypothesis involving four patients from the pavilion A, while no genetic link was found between the environmental and clinical strains. Innovative molecular methods in clinical and environmental investigations have allowed more accurate identification of the etiologic agent and possibly tracing the source of infection in the nosocomial setting. Control measures, such as patient isolation and room disinfection, have prevented the spread of infection.

Keywords *Chryseobacterium* spp., *C. indologenes*, Hospital infections, Bacteremia, Genome sequencing, Environmental surveillance

Until a few years ago, the genus *Chryseobacterium* belonged to the family *Flavobacteriaceae* on the basis of phylogenetic and phenotypic characteristics^{1,2}. However, according to a recent taxonomic classification,

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the genus *Chryseobacterium* spp belongs to the new family *Weeksellaceae* and includes 184 species and two subspecies^{3,4}. These bacteria are widespread in nature and have been isolated from a variety of environmental sources, including freshwater, soil, and plants. Additionally, they have been detected in food products such as raw milk, poultry, and fish, indicating their ecological versatility and ability to survive in diverse habitats^{2,5,6}. *Chryseobacterium* spp. are Gram-negative, rod-shaped, non-motile, non-spore-forming bacilli that typically produce yellow-pigmented colonies. They are oxidase- and catalase-positive, and their phenotypic characteristics facilitate preliminary identification. Initial identification generally relies on culture-based methods, followed by phenotypic characterization through biochemical assays or automated systems (e.g. Phoenix and Vitek). More advanced techniques, such as Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), offer faster and more accurate identification, while species-level resolution is commonly achieved using molecular methods, including genetic sequencing^{2,7,8}.

Clinically, *Chryseobacterium* spp. are part of the group of less common non-fermenting Gram-negative bacilli (NFGNB), alongside species such as *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex, *Alcaligenes xylosoxidans*, and *Elizabethkingia* spp. These microorganisms are increasingly recognized as opportunistic pathogens responsible for healthcare-associated infections, particularly in immunocompromised patients^{9,10}. Emergent data suggest that *Chryseobacterium* spp. may represent an increasing threat to patient health, particularly in the hospital setting. They are capable of colonizing immunocompromised patients through contaminated medical devices containing fluids, such as respirators, intubation tubes, humidifiers, catheters, and syringes^{11,12}.

Overall, infections caused by *Chryseobacterium* spp. pose a significant public health concern, with a reported lethality rate of 54.8%, largely due to their resistance to multiple antibiotic classes (cephalosporins, carbapenems, aminoglycosides, and polymyxins)¹³. Conversely, they may remain susceptible to fluoroquinolones, macrolides, and rifampicin. Notably, there are currently no standardized guidelines for antimicrobial susceptibility testing of the *Chryseobacterium* genus¹⁴.

Among different species of the genus *Chryseobacterium*, *C. indologenes* and *C. gleum* are the most frequently associated with human infections, particularly catheter-related bloodstream infections and ventilator-associated pneumonia, while other species are only rarely implicated in human pathology^{2,4,15–17}.

C. indologenes has increasingly been reported in recent years as an emerging nosocomial pathogen, particularly associated with cases of bacteremia and lower respiratory tract infections in immunocompromised patients and neonates undergoing mechanical ventilation^{18–22}.

Among the species that rarely cause disease in humans is *C. arthrosphaerae*, first identified in 2010 after being isolated from the faeces of *Arthrosphaera magna* (pill millipede)⁵. Recently, it was isolated from the blood of a patient with liver cirrhosis resistant to all antibiotics tested and from the cerebrospinal fluid of a patient with meningitis^{2,14}.

Between June and July 2023, six cases of infection or colonization by *Chryseobacterium* spp. occurred in patients hospitalized in two separate pavilions of a large hospital located in the Apulia region (Southern Italy). Because nosocomial infection was suspected, environmental investigations on air, surfaces, and water sample were conducted beyond the routine investigations on clinical samples.

The aim of this study was to identify the potential source and transmission pathway of infection by comparing clinical and environmental isolates of *Chryseobacterium* spp. through both phenotypic and genotypic characterization.

Materials and methods

Study design

The case series under investigation occurred between June and July 2023 in the largest hospital in Southern Italy, characterized by a pavilion structure comprising 1,550 beds in 33 separate buildings. The cases specifically involved two distinct Pavilions (A and B), located approximately 300 m apart, each supplied by separate water sub-centers.

Pavilion A consisted of five floors, including the ground floor and basement. The first floor accommodated the Gastroenterology Department, which consisted of five rooms, each equipped with two beds and one bathroom with a sink and a shower.

Pavilion B comprised three floors, including the ground floor and basement. The second floor accommodated the Vertebral Surgery Department, which consisted of five rooms, each with one/four beds. These rooms do not have dedicated bathrooms, but a single shared bathroom available in the corridor for patient use.

In accordance with the clinical and environmental monitoring protocols agreed with the health management of the hospital, the Microbiology and Virology Laboratory analyzed clinical specimens, while the Environmental and Food Hygiene Laboratory examined environmental matrices (air, water and surfaces). Since 2020, the hospital organized “Control Room Unit” focused on infection prevention and control. In particular, it managed the surveillance of healthcare-associated infections (HAI) and epidemiological investigations. Following episodes of suspect HAI, the Control Room Unit collaborated with the Environmental and Food Hygiene Laboratory to perform environmental investigations with the aim of identifying potential sources of infection.

When the possible outbreak of *Chryseobacterium* was suspected in the Gastroenterology Department, the Control Room Unit designed and implemented a specific case-control study. Cases were defined as patients with a positive blood culture for *Chryseobacterium* spp. detected during hospitalization. Controls were defined as patients hospitalized during the same period as the cases, without laboratory evidence of infection by *Chryseobacterium* spp., and present at the same time of at least one of the cases.

Data were collected from medical records for both cases and controls, including the following variables:

- Age.

- Sex.
- Condition of immunodeficiency.
- Invasive procedure during hospitalization.
- Use of urinary catheter during hospitalization.
- Use of intravascular catheter during hospitalization.

Data were entered into a database created by Ms Excel and analyzed by Stata MP18; Odds Ratios for each determinant, with 95% Confidence Intervals, were calculated, and significance was set at $p < 0.05$.

All protocols and experimental methods were approved by the Ethics Committee “Gabriella Serio” of Cancer Institute “Giovanni Paolo II” at IRCCS of Bari in Italy. The requirement for informed consent from the study subjects was waived by the Ethics Committee of IRCCS in Bari since the study sample was collected during routine clinical practices, and all personal data were anonymized prior the study. All methods were carried out in accordance with relevant guidelines and regulations of University of Bari in Italy.

In vitro clinical investigation

For each patient, two blood culture flasks (for aerobic and anaerobic microorganisms) were filled and incubated using the BacT/ALERT[®] 3D automated microbial detection system (bioMérieux, Marcy-l'Étoile, France). The system detects any growth (bacteria and fungi) in the blood culture bottle. After the bottles tested positive, Gram staining was performed to assess the morphology and coloring of the microorganisms.

To isolate bacteria, an aliquot of blood was sterilely withdrawn from the flagged positive flask and inoculated onto various culture media, including Chocolate Agar with PolyViteX[™] (PVX), Colistin-Nalidixic Acid Agar (CNA), and MacConkey Agar (McK) (bioMérieux, Marcy-l'Étoile, France). These were incubated at 36 ± 1 °C for 24–48 h, with PVX plates kept in a 5% CO₂ atmosphere.

To isolate fungi, Sabouraud Dextrose Agar medium with 0.5% chloramphenicol (bioMérieux, Marcy-l'Étoile, France), was used and incubated at 36 ± 1 °C for five days.

A tracheal aspirate sample was also processed using the same culture media and incubation conditions. The microorganisms grown on the culture plates were identified using MALDI-TOF MS (bioMérieux, Marcy-l'Étoile, France).

Routine antibacterial susceptibility to amikacin, cefepime, cefotaxime, ceftazidime, ceftazidime/avibactam, ceftalozane/tazobactam, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillina/tazobactam, tobramycin was performed using the VITEK 2 AST-N397 card (bioMérieux, Marcy-l'Étoile, France).

Minimum inhibitory concentrations (MICs) were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints, version 13.0, 2023 (<http://www.eucast.org/>).

Environmental investigations

In order to identify the source of the infection, environmental analyses were promptly conducted after the cases were reported to detect the presence of *Chryseobacterium* spp. Samples of water, air and surface were collected from the rooms of the patients who tested positive for *Chryseobacterium* spp., as well as from common areas (staff changing rooms, taps water used for patient hygiene), both before and after water supply and environmental sanitation procedures.

One liter of cold water was collected in sterile bottles from the taps of the sinks and showers taps in the hospital rooms occupied by *Chryseobacterium*-positive patients, as well as in adjacent rooms. The samples were then transported to the laboratory at a controlled temperature (+4 °C). For each sample, 500 ml of water was filtered through a cellulose ester membrane (47 mm diameter, 0.45 µm pore size; Millipore, Milan, Italy) and each membrane was then placed on PVX agar plates (bioMérieux, Marcy-l'Étoile, France) and incubated at 36 ± 1 °C for 48 h.

To detect microorganisms on flat, large surfaces (e.g. regular surfaces such as bedside tables and desks), a defined 10 × 10 cm area was sampled using a sterile delimeter. For irregular, small, or curved surfaces (e.g. infusion pumps, bed rail), the available area was swabbed. Swabs were placed in 4 °C refrigerated containers for immediate transport to the laboratory. Upon arrival, the samples were shaken for 20 s at room temperature. A 500 µL aliquot of the transport medium was inoculated onto PVX agar plates (both, bioMérieux, Marcy-l'Étoile, France) and incubated at 36 ± 1 °C for 48 h.

In addition, active air sampling on solid substrate was performed using the Surface Air System (SAS Super ISO 180; PBI International, Milan, Italy). For each sampling, 1000 L of air were drawn from the SAS, which was placed approximately 1 m above floor level. PVX 90 mm plates were used for microbial recovery and incubated at 36 ± 1 °C in a 5% CO₂ atmosphere for 48 h.

Suspect colonies of *Chryseobacterium* grown on PVX from water, surfaces and air appearing smooth, circular, yellow-pigmented or white 1–2 mm in diameter were identified by MALDI-TOF MS (bioMérieux, Marcy-l'Étoile, France). The results were expressed as detected or not detected.

DNA extraction, Sanger sequencing, WGS, and sequence analysis

All isolates (clinical and environmental) identified as *Chryseobacterium* spp. by MALDI TOF were received at the Istituto Zooprofilattico Sperimentale of Apulia and Basilicata (Putignano, Bari, Italy) and subjected to diagnostic genotyping investigations in order to assess their possible genetic relationship and identify the potential source of environmental contamination.

Genomic DNA was extracted from isolated colonies using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. In order to assign a rapid taxonomic classification, PCR and Sanger sequencing of the partial 16 S rRNA gene, using 16 S rRNA universal primers 517 F and 1541R²³, was

first performed. The obtained amplicons were purified using ExoI/FAST AP enzyme systems (Thermo Fisher Scientific, Waltham, MA, USA). The purified PCR products were sequenced using BigDye 3.1 Ready (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and the 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) for electrophoresis. The sequences were imported and assembled using BioEdit software²⁴ v.7.0.5, and the assembled sequences were used as BLAST-nucleotide input in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast>)²⁵.

Once the outcome of Sanger sequencing was obtained, Whole-Genome Sequencing (WGS) was required to detect any genetic correlations among the isolated bacterial strains. Starting from the previously purified genomic DNA, an indexed genomic library for each isolate was prepared using the Illumina DNA Prep Sample Preparation Kit (Illumina, San Diego, CA), as described²⁶. A 2 × 250 paired-end sequencing run was performed on the Illumina MiSeq platform.

Primary bioinformatics analyses were performed following a customized Galaxy v23.0.2 workflow at Galaxy Europe platform (<https://usegalaxy.eu/>), that includes read quality control with fastp²⁷, de novo sequence assembly with SPAdes²⁸, gene prediction with Prokka²⁹, assembly quality and contamination check with QUAST and Checkm^{30,31}.

Species attribution was performed by means of ribosomal Multi-locus sequence typing (rMLST, <https://pubmlst.org/species-id>)³² and JSpecies (pairwise Average Nucleotide Identities ANI, calculated by BLAST + and MUMmer, <https://jspecies.ribohost.com/jspeciesws/#home>)³³. The 95% ANI cutoff was considered as the similarity threshold for attributing genome assemblies to species *C. arthrospiraerae* (NCBI reference genome: strain_LMY, NZ_CP073074.1). Furthermore, pairwise genetic comparison among the sequenced samples was carried out by pairwise ANI calculation (FastANI tool v1.34)³⁴. Given the scarcity of complete public *C. arthrospiraerae* genome sequences, a reliable Core Genome Multi-Locus Sequence Typing scheme cannot be generated. Thus, a reference-based genetic approach was considered: specifically, whole genome SNP analysis was conducted, by using Galaxy-Snippy variant caller v 4.6.0³⁵ on the investigated datasets and the above mentioned *C. arthrospiraerae* reference genome. The global SNP matrix table was polished by removing variant clusters (i.e., variants located ± 10 bp each other) and highly polymorphic sites (> 2 identified alleles per site). SNP differences among samples were evidenced through GrapeTree v1.5 software (implemented in March 2025)³⁶. Furthermore, FASTA-formatted SNP table was given as input to IQ-Tree webserver (<http://iqtree.cibiv.univie.ac.at/>)³⁷, that efficiently performs base composition check, nucleotide substitution model selection (model selected: TVM + F + ASC, as defined in: <https://iqtree.github.io/doc/Substitution-Models>), ultra-fast bootstrapping ($n = 1000$ bootstrap) and consensus Maximum-Likelihood phylogenetic tree construction.

Results

Outbreak description

On 3 July 2023, five cases of *Chryseobacterium* spp. infection were reported in patients admitted to the Gastroenterology Department (Pavilion A).

The first patient (M.L.E. Gast_1), a 65-year-old woman, was admitted on 31 May 2023 for acute liver failure after transfer from Infectious Diseases Ward of another hospital of Apulia region, Italy, and the first symptoms (fever) related to an infection with *Chryseobacterium* spp. appeared on 7 June 2023, with a positive blood culture on 9 June 2023. This was followed by another blood culture on 12 June that was positive for the same microorganism.

After treatment with cefepime 2 g × 3/day and vancomycin 1 g × 2/day, the patient had two negative blood cultures on 19 and 20 June 2023, respectively. The patient underwent gastroscopy on 16 June 2023 and colonoscopy on 13 June 2023. Due to the worsening clinical condition and positive blood culture (3 July 2023) for the same microorganism, the patient was transferred to the Intensive Care Unit on 4 July 2023.

The second patient (B.K. Gast_2), a 59-year-old woman, was admitted on 10 June 2023 for anemia. On 14 June 2023, she underwent gastroscopy; in the same day, she presented fever, for which blood culture was carried out, positive for *Chryseobacterium* spp. She received Ceftriaxon 2 g × 1/die for 6 days; after treatment, symptoms disappeared, and blood culture resulted negative. On 21 June she was discharged in good health condition.

The third patient (C.M.M.C. Gast_3), a 68-year-old woman, affected by diabetes, was admitted on 19 June 2023 for multifocal hepatocarcinoma and was evaluated for inclusion on the transplant list. After dyspnea and fever she underwent a blood culture on 28 June which was positive for *Chryseobacterium* spp. The patient received antibiotic therapy with piperacillin/tazobactam 4,5 g × 3/die with improvement in respiratory symptoms and subsequent blood culture on 5 and 8 July 2023 with negative result. The patient underwent gastroscopy with ligation of esophageal varices on 28 June 2023.

The fourth patient (V.M. Gast_4), a 68-year-old man, was admitted on 26 June 2023. She had undergone liver transplantation and was undergoing ligation of esophageal varices. Again, following the onset of fever, a blood culture was taken on 30 June, which was positive for *Chryseobacterium* spp. The patient received antibiotic therapy with ceftriaxon 2 g × 1/die from 30 June and repeated blood culture on 1 and 3 July 2023 with positive result. After, the patient received antibiotic therapy with piperacillin/tazobactam 4,5 g × 3/die and subsequent blood cultures on 5 July 2023 with negative result.

Finally, the fifth patient (T.V. Gast_5), a 61-year-old man, a kidney transplant recipient from the Surgery Unit and on drug treatment with mycophenolate and cyclosporine, was admitted on 27 June 2023 with abdominal pain of unspecified diagnosis. After the onset of fever, blood culture was performed on 28 June 2023, which was positive for *Chryseobacterium* spp. He received ceftriaxon 2 g × 1/die for 6 days. After clinical deterioration (septic shock), the patient was transferred and admitted to the Intensive Care Unit in isolation on 30 June 2023 and blood culture negative on 1 and 2 July 2023. Then he was intubated on 5 July 2023 for worsening of the respiratory situation. Figure 1 shows the incidence curve of *Chryseobacterium* infection cases in the Gastroenterology Department.

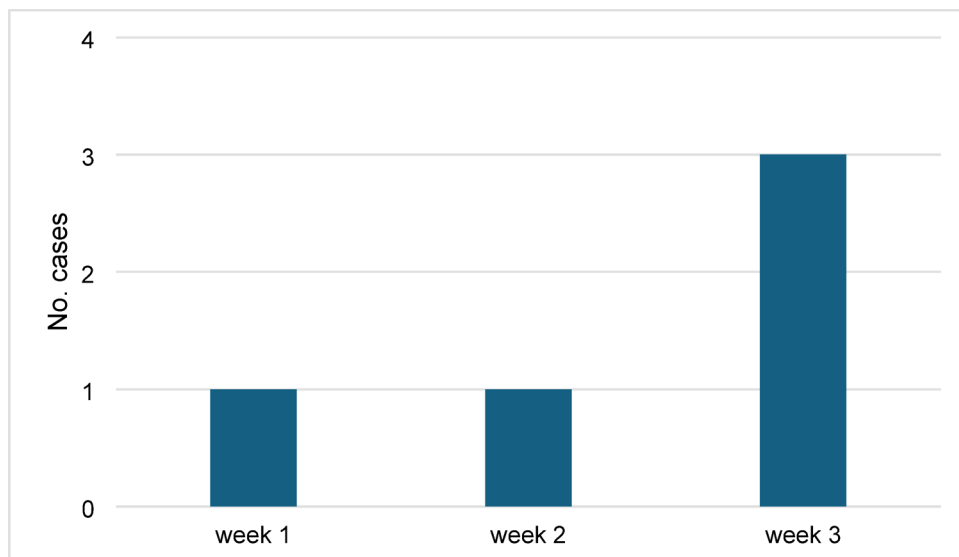


Fig. 1. Incidence curve of cases of *Chryseobacterium* spp. in Gastroenterology Department of Italian Southern hospital.

Patient	Gender	Age	Ward	Admission date	Positive sample (date first isolation)	Use of bathroom	Medical device	Underlying condition	Outcome
M.L.E. Gast_1	Female	65	Gastroenterology	31 May 2023	Blood (9 June 2023)	Yes	PIVC	Acute liver failure	Recovered
B.K. Gast_2	Female	59	Gastroenterology	10 June 2023	Blood (14 June 2023)	No	Urinary catheter and PIVC	Anemia	Discharged
C.M.M.C Gast_3	Female	68	Gastroenterology	19 June 2023	Blood (28 June 2023)	Yes	PIVC	Diabetes and multifocal hepatocarcinoma	Recovered
V.M. Gast_4	Male	68	Gastroenterology	26 June 2023	Blood (30 June 2023)	Yes	PIVC	Liver transplantation and ligation of esophageal varices	Discharged
T.V. Gast_5	Male	61	Gastroenterology	27 June 2023	Blood (28 June 2023)	No	Urinary catheter and PIVC	Kidney transplant recipient and abdominal pain of unspecified diagnosis	Recovered
A.A. Vert_1	Female	85	Vertebral Surgery	24 June 2023	Tracheal aspirate (3 July 2023)	No	Urinary catheter and PIVC	Sub capitate fracture of the right femur	Recovered

Table 1. Summary of six cases of *Chryseobacterium* spp. Infection in two wards in the largest hospital in Southern Italy. PIVC, Peripheral intra-venous catheter.

On 10 July 2023, a case of *Chryseobacterium* spp. infection was reported in a patient admitted to the Vertebral Surgery Department (Pavilion B).

The patient (A.A. Vert_1), a 85-year-old woman, was admitted on 24 June 2023 following a sub capitate fracture of the right femur. After three days the patient had a fever peak, and the culture test performed on 3 July 2023 on tracheal aspirate revealed the presence of *Chryseobacterium* spp. On 5 July 2023 chest x-rays showed pleural effusion, and the patient combined intravenous therapy with ceftriaxon 2 g \times 1/die + linezolid 600 mg \times 2/die, clarithromycin 500 mg \times 2/die. On 13 July 2023, the patient had negative tracheal aspirate for *Chryseobacterium* and positive for *Klebsiella pneumoniae*. Table 1 shows information relating to six cases of infection by *Chryseobacterium* spp.

Case-control study

A total of ten patients hospitalized in the same Gastroenterology Department were enrolled. Five patients with *Chryseobacterium* infection were classified as cases, while five patients without the infection served as controls. Among the ten subjects (six males and four females), the overall average age was 63 ± 8.4 years (range 55–82). Among the five cases (two males), the overall average age was 64.0 ± 3.9 years; among the five controls (four male), it was 62.0 ± 11.9 years. No association was found between *Chryseobacterium* infection and the determinants investigated (Table 2).

Determinants	Cases (n=5)	Controls (n=5)	Odds ratio	p-value
Urinary catheter	3	2	2.25 [0.17–28.25]	0.53
Intravascular catheter	5	4	0	–
Immunodeficiency	3	0	0	–
Invasive procedure during hospitalization	4	4	1 [0.04–22.17]	1

Table 2. Analysis of the determining factors associated with *Chryseobacterium* spp. infection.

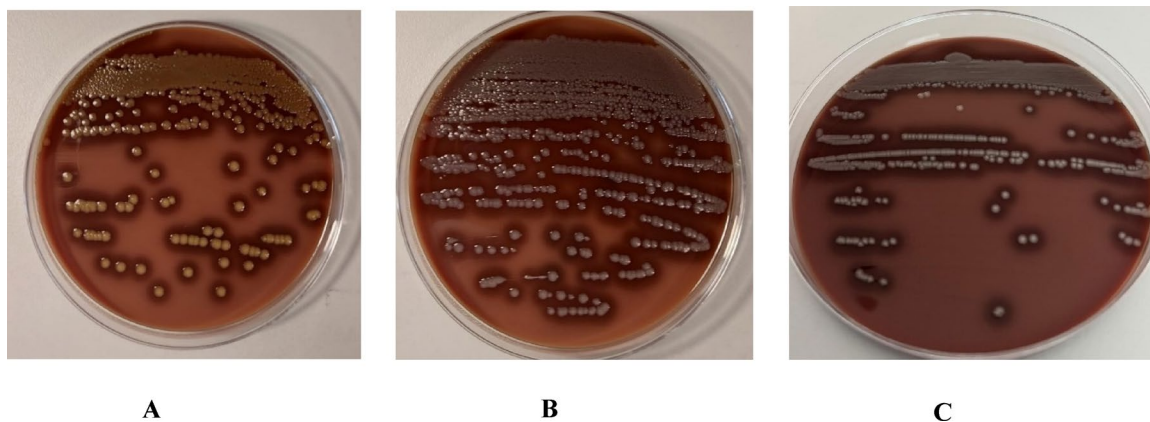


Fig. 2. Images of colonies of *Chryseobacterium* spp. from (A) blood (yellow colour), (B) tracheal aspirate (white colour) and (C) water (white colour) grown on PVX agar plates.

Containment measures

To prevent the spread of microorganism, a number of preventive measures were taken: reorganization of beds to spatially isolate patients with positive blood cultures; blocking admissions until the outbreak was resolved and performing blood cultures on other in-patients sharing the room to detect new positive cases; analysis for *Chryseobacterium* spp. in environmental matrices (water, air, surfaces); disinfection of the environment following environmental investigations.

Clinical investigations

Microscopic examination of the blood and tracheal aspirate samples revealed the presence of Gram-negative bacilli. Culture examination revealed the growth of yellowish colonies from the blood culture (Fig. 2A) and white colonies from the tracheal aspirate (Fig. 2B) and water sample (Fig. 2C) only on PVX plates. Species identification by MALDI-TOF was 99% for *C. indologenes*. Antimicrobial susceptibility patterns of blood culture and tracheal aspirate isolates showed that all clinical isolates were resistant to all antibiotics tested, with the exception of the strain from patient M.L.E. Gast_1, which showed sensitivity to the antibiotics cefepime and ceftolozane/tazobactam (Table 3).

Environmental investigations

On 4 July 2023, in the Gastroenterology Department (Pavilion A), ten water samples were collected from sinks and showers in inpatients bathrooms on the first floor, along with two additional samples from staff changing room sinks on the mezzanine floor. Furthermore, two water samples were taken from the Apulian Aqueduct adduction point, specifically from the reservoir and the water supply inlet. On the same date, 10 air samples and 48 surface samples were also taken from inpatient rooms and bathrooms.

Following environmental sanitation procedures conducted on 14 July 2023, the same types of samples (water, air, and surfaces) were collected again for verification.

In the Vertebral Surgery Department (Pavilion B), on 10 July 2023, one air sample and eight surface samples were collected in the room of a patient who tested positive for *Chryseobacterium* spp. On 11 July 2023, six water samples were collected from showers and sinks in the patient's room and the adjacent room. On 17 July 2023, an additional water sample was collected from a washbasin on the same floor, which was used for patient hygiene.

Moreover, on 6 July 2023, 18 samples of surface were collected in two boxes of the Intensive Care Unit II, where patients positive for *Chryseobacterium* spp. had been transferred.

Overall, *Chryseobacterium* spp. was not detected in any air or surface samples collected across all wards where infection cases had occurred. The bacterium was identified in only one water sample, taken from the tap of the washbasin used to clean patients in the Vertebral Surgery Department. After the installation of a point-of-use filter, a follow-up water sample from the same tap tested negative for *Chryseobacterium* spp.

	Clinical samples						Environmental sample
Patients	M.L.E. Gast_1	B.K. Gast_2	C.M.M.C Gast_3	V.M. Gast_4	T.V. Gast_5	A.A. Vert_1	Water Vert_1
Antibiotics	MIC	MIC	MIC	MIC	MIC	MIC	MIC
<i>amikacin</i>	> 32	> 32	> 32	> 32	> 32	> 32	≥ 64
<i>cefepime</i>	0.5*	16	> 16	16	> 16	16	1*
<i>cefotaxime</i>	32	32	32	32	32	32	32
<i>ceftazidime</i>	32	32	32	32	32	> 8	2*
<i>ceftazidime/avibactam</i>	> 8	> 8	> 8	> 8	> 8	> 8	8
<i>ceftazone/tazobactam</i>	0.5*	> 16	> 16	> 16	> 16	> 16	1*
<i>ciprofloxacin</i>	2	> 2	2	> 2	> 2	2	2
<i>gentamicin</i>	> 8	> 8	> 8	> 8	> 8	> 8	≥ 16
<i>imipenem</i>	> 8	> 8	> 8	> 8	> 8	> 8	> 8
<i>meropenem</i>	> 8	> 8	> 8	> 8	> 8	> 8	≥ 16
<i>piperacillin/tazobactam</i>	64	> 64	> 64	> 64	> 64	> 64	≤ 4*
<i>tobramycin</i>	> 8	> 8	> 8	> 8	> 8	> 8	> 8

Table 3. Minimal inhibitory concentration (MIC) values of clinical and environmental strains of *C. indologenes* for each antibiotic tested. All clinical and environmental strains of *C. indologenes* showed resistance to the molecules tested, with the exception of those marked with an asterisk (*) which were found to be sensitive. The MIC of the antibiotics is expressed in mg/L.

Sample	ANiB score (% aligned sites with reference genome)	ANIm score (% aligned sites with reference genome)
M.L.E. Gast_1	97.38 [87.30]	98.19 [88.07]
C.M.M.C. Gast_3	97.38 [87.18]	98.19 [88.08]
V.M. Gast_4	97.37 [87.16]	98.19 [88.07]
T.V. Gast_5	97.36 [87.09]	98.19 [88.07]
A.A. Vert_1	97.57 [89.87]	98.19 [91.16]
Water sample Vert_2	97.22 [86.97]	98.09 [88.08]

Table 4. Species attribution based on sequence similarity to the reference genome (NCBI identifier NZ_CP073074.1, *C. arthrosphaerae* strain LMY). Threshold for species attribution: score > 95%. Software: ANI-BLAST and ANI-Mummer.

Antimicrobial susceptibility pattern of the isolate from water showed that it was resistant to most antibiotics except some cephalosporins, penicillins and β -lactams (Table 3).

In silico analyses

Overall, five colonies of *Chryseobacterium* spp. isolated from clinical specimens (four from blood cultures and one from a tracheal aspirate), each corresponding to one of the five patients involved in the suspected outbreak were analyzed in silico. In addition, the strain isolated from the water sample positive for the microorganism was analyzed. Unfortunately, the *Chryseobacterium* spp. strain isolated from the blood culture of one of the five patients (B.K. Gast_2) admitted to the Gastroenterology Department was not preserved.

All clinical and environmental isolates identified by MALDI-TOF as *C. indologenes* were found to belong to the species *C. arthrosphaerae* (Table 4), by Sanger sequencing of a portion of the 16 S rRNA gene (approximately 960 bp long), with a query cover rate of 99% and an identity rate ranging from 97.54% to 99.48%. The five strains isolated from patients admitted to Pavilions A and B and the strain isolated from water in Pavilion B were then subjected to whole genome sequencing. Details for the sequenced genomes are reported in Supplementary (Supplementary Table 1): a 5.2 Mbp long genome was generally obtained, with a 38% GC content. Genomic pairwise comparison evidenced a very strong sequence similarity among M.L.E. Gast_1, C.M.M.C. Gast_3, V.M. Gast_4, and T.V. Gast_5 isolates (greater than 99.99% similar each other, Table 5).

A more detailed view was provided by wgSNP analysis. The four above-mentioned isolated have only < 5 SNP differences (out of 52590 variant sites), while the strains isolated from A.A. Vert_1 and the environment are very distant to the genetic cluster from Pavilion A Gastroenterology, (Fig. 3A). Maximum-likelihood tree (Fig. 3B) provides a consistent picture. ML-based phylogenesis on the 52,590 variant sites clearly supports the idea that the strains isolated from patients M.L.E. Gast_1, C.M.M.C. Gast_3, V.M. Gast_4, and T.V. Gast_5 have a very close common ancestor, while the isolate from patient A.A. Vert_1 and the isolate from water sample Vert_2 are very distant.

	Water sample Vert_2	A.A. Vert_1	C.M.M.C. Gast_3	M.L.E. Gast_1	T.V. Gast_5	V.M. Gast_4
Water sample Vert_2	–	97.77	97.71	97.63	97.65	97.68
A.A. Vert_1	–	–	98.96	98.97	98.96	98.97
C.M.M.C. Gast_3	–	–	–	> 99.99	> 99.99	> 99.99
M.L.E. Gast_1	–	–	–	–	> 99.99	> 99.99
T.V. Gast_5	–	–	–	–	–	> 99.99
V.M. Gast_4	–	–	–	–	–	–

Table 5. Genome sequence comparison based on average nucleotide identity (percentage values), deriving from FastANI implementation on each genome assembly couple. Couples with high similarity values (> 99.99%) are consistent with WgSNP cluster analysis (Fig. 3A and B).

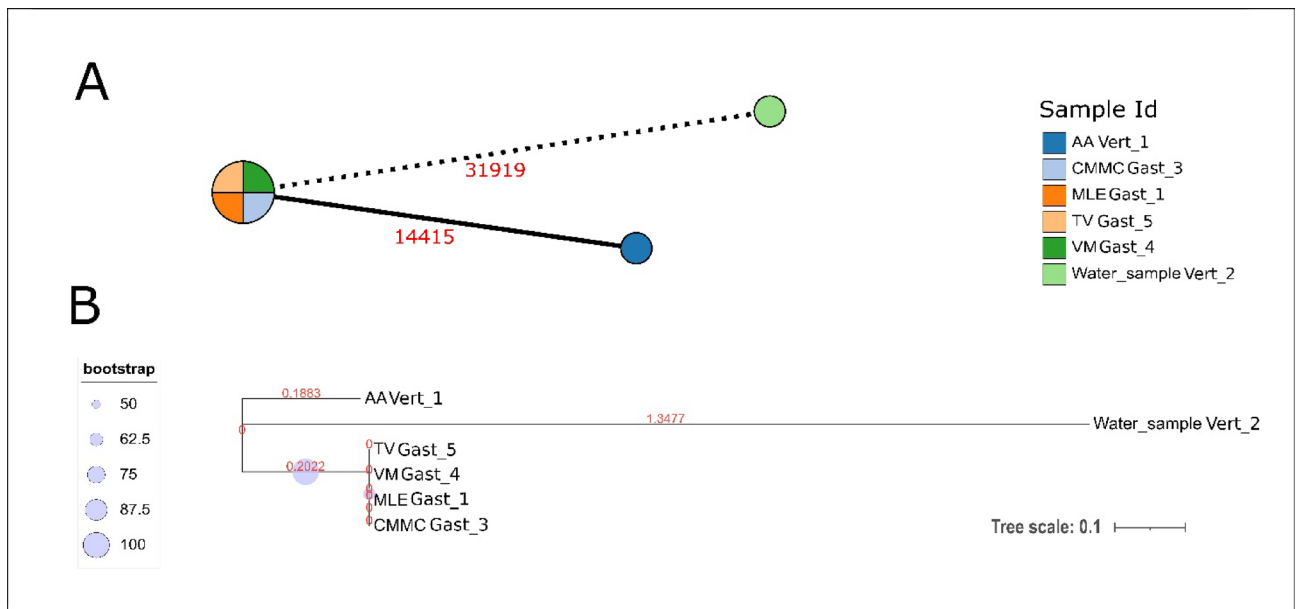


Fig. 3. (A) GrapeTree for SNP differences among investigated samples (colored according to Legend); red integer numbers: differences among nodes, with samples with < 5 differences clustered together; for visualization purposes, branch with > 20,000 differences has been shortened (dashed line); (B) Maximum Likelihood Tree: red floating numbers indicate branch lengths; violet bubbles, indicate branch support (according to bootstrap analysis).

Discussion

In our study, genetic analysis of the isolates supported the hypothesis of an outbreak infection involving four patients from the Gastroenterology Department. Although the analysis was based on a very limited number of genomes, two different molecular methods (Sanger sequencing of the 16 S rRNA gene and Whole Genome Sequencing) confirmed that these isolates were *C. arthrosphaerae* and supported the hypothesis that the bacterial isolates from patients M.L.E. Gast_1, C.M.M.C. Gast_3, V.M. Gast_4, and TV_Gast_5 are part of the same outbreak of infection. In contrast, in the Vertebral surgical Department, the analyses did not show any genetic relationship between the clinical strain and the only environmental strain isolated from water, despite both belonging to the same *C. arthrosphaerae* species. Moreover, the genotypic analyses revealed that the water sample strain and the clinical strain isolated from patient A.A. Vert_1 were genetically more distant from the other strains, suggesting no direct link.

Among the Chryseobacterium species, most human infections are caused by *C. indologenes*^{38,39}, while those by *C. arthrosphaerae* have rarely been reported. This is probably due to the misidentification of the microorganism. In accordance with previous studies, our results show that commercial phenotypic identification systems and traditional bacterial identification methods are not always reliable for the identification of Chryseobacterium species^{16,40}. Although MALDI-TOF is a state-of-the-art system for the rapid identification of microorganisms, it is necessary for the database to be constantly updated to obtain a correct identification of the microorganisms.

In our study, MALDI-TOF identified clinical and environmental strains as *C. indologenes*, whereas sequencing of the 16 S rRNA gene confirmed them as *C. arthrosphaerae*. This challenge in species identification has been noted in previous studies², which reported the presence of *C. arthrosphaerae* in a patient's blood, although this isolate was initially identified as *C. indologenes* by MALDI-TOF. Liang et al.² showed that the sequencing of the

16 S rRNA gene revealed a 100% sequence identity of strain ED882-96, wrongly identified as *C. indologenes*, with the CC-VM-7 type strain of *C. arthrophaerae*. These findings underscore the importance of new technologies, such as NGS, for accurate microbial identification⁴¹. Moreover, NGS also plays a crucial role in tracking outbreaks and helps to determine whether patient isolates are linked to environmental strains.

A Spanish study⁴² shows that *C. indologenes* can be found in hospital environments. The authors reported the bacterium in the air and washbasins of an Intensive Care Unit following an outbreak involving 12 patients. Moreover, the same Pulsed-Field Gel Electrophoresis pattern was shared by patients and the air sample near the sink. Although the exact transmission route remained unclear, they highlighted that the washbasin could act as a reservoir for *C. indologenes* and other pathogenic microorganisms. In fact, after the washbasins were removed, the outbreak regressed.

In our study, unfortunately only one of the environmental samples was analyzed by WGS, so we could not definitively determine the source of the outbreak. However, we consider that the finding of *C. arthrophaerae* in the water of one of the two pavilions, although genotypically distinct from the clinical strain, demonstrates the possibility of nosocomial infection due to environmental contamination. Unfortunately, the number of public *C. arthrophaerae* complete genomes or associated raw sequences is very limited: thus, no reliable genetic comparisons (e.g., cgMLST or wgSNP analyses) can be carried out among our investigated strains and the few ones detected worldwide.

In our opinion, the finding of the microorganism in a single environmental sample out of all those examined (air, water and surfaces) was entirely fortuitous in the two pavilions where the infection occurred. Culture analysis for the detection of *Chryseobacterium* requires highly nutritious media such as blood agar and PVX, which may allow the overgrowth of other microorganisms present in environmental matrices⁴². This possibility could inhibit or mask the presence of suspect colonies that thus escape identification.

Given these challenges, it remains difficult to pinpoint the exact source of infection in both the Gastroenterology and Vertebral Surgery Departments. In particular, in the second Department, the patient was bedridden and therefore did not have access to the bathroom.

We hypothesize that healthcare workers' hands may have played a role in transmitting the microorganism to patients, either directly or indirectly after handwashing. Water taken from the taps in the toilets and used by healthcare workers to wash bedridden patients, could have contributed to the spread of microorganisms.

To ensure the safety of patients, healthcare workers, and visitors, it is crucial to implement a comprehensive approach to hospital hygiene. This includes essential measures such as hand hygiene training for healthcare workers, proper disposal of medical waste, isolating infected patients, and conducting regular environmental monitoring to prevent cross-transmission of pathogens. In our study, after analyzing the water samples, we implemented disinfection procedures on the water network, including shock hyperchlorination and the installation of absolute filters at all tap terminals. These measures were followed by microbiological control, which confirmed the absence of pathogens.

The new European Drinking Water Directive, implemented in Italy on 18 February 2023, highlights that water is not free from microorganisms and, if improperly managed, can serve as a vehicle for infectious diseases. In hospitals, particularly where immunocompromised patients are present, it is now mandatory to adopt the Water Safety Plan by 2029. Our hospital began implementing this plan in 2020, establishing routine and operational monitoring of the water supply. In addition, in order to reduce the spread of pathogenic microorganisms in the water, the hospital's technical management department implements a series of strategies: (i) replacement of absolute filters on faucets every 3 months, (ii) keep limescale-free or replace water faucet breakers, (iii) measurement of the concentration of residual disinfectant in the water supply, (iv) flushing of all water supply terminals (taps, showers) for 5 min for 5 days, alternating between cold and hot water⁴³.

Despite these efforts, our study demonstrated that compulsory microbiological parameters are not always sufficient to guarantee the safety of vulnerable patients. Therefore, in epidemic situations, a more extensive risk assessment is necessary to detect other microorganisms that may contribute to nosocomial infections.

Furthermore, our findings on antimicrobial susceptibility reveal resistance to several antibiotic classes (cephalosporins, carbapenems, and aminoglycosides) in all clinical strains and a single environmental strain of *Chryseobacterium* spp., as indicated in existing literature, although the data remains limited. Despite this resistance, the therapeutic approach we implemented led to a positive resolution of clinical cases. This highlights the importance of continuous vigilance and tailored interventions, particularly in preventing infections in high-risk hospital environments.

Conclusion

Following the spread of *C. indologenes* among patients admitted to two wards at a large hospital in Apulia, sequencing results of clinical and environmental strains revealed that the isolates belonged to the species *C. arthrophaerae*. This analysis confirmed the hypothesis of a cluster involving four patients from the Gastroenterology Department. Specifically, a genetic correlation was observed solely among the clinical isolates of patients in the Gastroenterology Department. No correlation was found between the environmental strain isolated from water and the clinical strains from the two involved wards.

Thanks to the timely analysis, patient isolation, and the implementation of water disinfection and environmental sanitation measures, the spread of the microorganism was halted and its transmission to other patients prevented. Therefore, it is crucial to ensure the proper implementation of routine cleaning procedures within the hospital environment to minimize the spread of microorganisms in nosocomial settings and prevent future infections.

Data availability

Sequence data that support the findings of this study have been deposited in the NCBI Genome (submission id: SUB15330113), within the NCBI BioProject PRJNA1264769.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

This study was approved (Code number: 1345/CE-14/09/2023) by Ethics Committee “Gabriella Serio”- Istituto Tumori “Giovanni Paolo II” IRCCS, Bari, Italy.

Additional information

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