CASE REPORT

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First detection of *Cupriavidus gilardii* in a bovine neonatal diarrhea outbreak

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Abstract

Background *Cupriavidus gilardii* is an aerobic, gram-negative, motile, glucose-nonfermenting bacillus, first described in 1999. Typically, it exhibits low pathogenicity in humans, causing opportunistic infections primarily in individuals with compromised immune systems. This bacterium has been also found in various environmental sources such as plants and contaminated soils. Notably, there have been no documented cases of *C. gilardii* infections in animals.

Case presentation This case report outlines a bovine neonatal diarrhea outbreak that occurred in Northern Greece, during which *C. gilardii* was isolated. Faecal samples from 5-day-old calves were collected and transported to the laboratory for further examination. Bacterial culture and next generation sequencing techniques were employed to confirm the presence of this bacterium in the samples. Following the isolation and identification of *C. gilardii* from the samples, an autogenous vaccine was produced and administered to the cows within the farm. Subsequent to vaccination, a progressive reduction in calf diarrhea and deaths was observed, leading to their eventual complete resolution. To the best of our knowledge, this represents the first documentation of *C. gilardii* isolation from cases of bovine neonatal diarrhea.

Conclusion This case report presents the first isolation case of *C. gilardii* from animal samples and more specifically from calf faecal samples. It represents an important observation, providing evidence that this opportunistic human pathogen could contribute to clinical symptoms in animals.

Keywords Calf diarrhea, *Cupriavidus gilardii*, Public health

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Background

Cupriavidus gilardii is an aerobic, gram-negative, motile bacillus which is characterized by its inability to ferment glucose. Originally identified in 1999 by Coenye et al. [1], this microorganism has been frequently found in diverse environmental sources, including plants and soils contaminated with heavy metals. Previous reports have characterized it as a microorganism with low pathogenicity that primarily causes opportunistic infections, mainly in immunocompromised individuals [2–4]. Reported cases involve patients with conditions such as acute lymphocytic leukemia [2], severe idiopathic aplastic anemia [3], or those who have undergone renal transplantation [4]. Kobayashi et al. [5] described the first case of an infection of *C. gilardii* in a patient without obvious immunodeficiency. Human clinical samples, such as those from cerebrospinal fluid, bone marrow, wounds, and the respiratory tract, have sporadically revealed the presence of *C. gilardii* [1, 6, 7]. To date, only six documented cases of clinical infections directly attributed to *C. gilardii* have been reported [8]. In one of these cases involving a 26-year-old woman undergoing chemotherapy, the patient, among other clinical signs, experienced abdominal pain and diarrhea. An important aspect to note is the intrinsic resistance of *C. gilardii* to various antimicrobial agents such as aztreonam, meropenem, gentamicin, and tobramycin, as documented in previous reports [3, 9]. So far, *C. gilardii* has not been associated with any animal diseases, nor has it been detected in clinical samples collected from animals.

Case presentation

In 2022, a severe neonatal diarrhea outbreak was reported at a Limousin-breed beef cattle farm situated in the Central Macedonia Region, near the city of Aridaia, Greece. The herd consisted of 65 animals. The syndrome exhibited clinical signs primarily in 5-day-old calves, characterized by severe diarrhea leading to dehydration, anorexia, and depression. Several of the affected calves succumbed to the illness within an average period of 5 days. Despite treatment attempts using antibiotic therapy (amoxicillin, colistin), the affected animals showed no signs of clinical improvement. During the outbreak the mortality rate was as high as 70% causing substantial economic losses to the farm owner.

Two fresh fecal samples were directly collected from the rectum of diarrheic calves to prevent environmental contamination. These samples were promptly transported under cooling to the laboratory of Microbiology and Infectious Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece. Upon arrival at the lab, the samples were diluted in decimal dilutions in Phosphate Buffer Saline (Thermo Fischer Scientific, Massachusetts, USA) and underwent immediate culture onto blood agar (Oxoid, Hampshire, UK) and MacConkey agar plates (Oxoid, Hampshire, UK), which subsequently were incubated at 37 °C overnight. For the isolation of anaerobic bacteria, the same procedure was conducted, and the agar plates were incubated under anaerobic conditions at 37 °C for 48 h.

After the incubation, pale colonies (lactose-nonfermenting) of gram-negative bacilli were grown on MacConkey agar plates while small, white colonies were present on Blood agar plates (Fig. 1). Bacteria had been detected at high dilutions in both samples. More specifically, 1.9×10^9 cfu/ml and 7.8×10^8 cfu/ml of the isolated bacilli were detected in the 2 examined samples, respectively.

DNA extraction from a single isolated colony at the highest dilution, was performed for both samples using the Nucleospin Microbial DNA kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. The concentration and purity of the eluted DNA were determined in an Eppendorf Biospectrophotometer (Eppendorf, Wien, Austria). Subsequently, the extracted DNA underwent sequencing using Oxford Nanopore Technology (ONT) sequencing employing the Rapid Barcoding Kit SQK-RBK004 (Oxford Nanopore Technologies, Oxford, UK) for library preparation. Prepared libraries were loaded into a R9.4.1D Flow Cell (Oxford Nanopore Technologies, Oxford, UK) and sequenced using the MinION Mk1C device (Oxford Nanopore Technologies, Oxford, UK). Raw data in FAST5 format were basecalled using Guppy v6.5.7 (Oxford Nanopore Technologies, Oxford, UK) and subsequently demultiplexed into individual sample barcodes. Bioinformatic metagenomic analysis was carried out using the EPI2ME Desktop Agent v3.7.3 (Oxford Nanopore Technologies, Oxford, UK). According to the results of the read analysis, the DNA of *C. gilardii* was the main taxon in both samples.

Antimicrobial susceptibility testing of the isolated bacteria in both samples was conducted following the guidelines set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Given the absence of established veterinary breakpoints for *C. gilardii*, this study employed clinical breakpoints designated for *Pseudomonas aeruginosa*, a practice previously used by other researchers [5, 8], and/or *Burkholderia pseudomallei*, a species within the same family as *C. gillardi* (*Burkholderiaceae*). The susceptibility to specific antimicrobial agents was determined by disk diffusion on Mueller-Hinton agar (Oxoid, Hampshire, UK). The susceptibility of the isolated bacteria to various antimicrobial agents is shown in Table 1.

Following successful isolation and identification of *C. gilardii* from the samples, an autogenous inactivated vaccine was produced and administered once intramuscularly to the cows, approximately 1 month prior
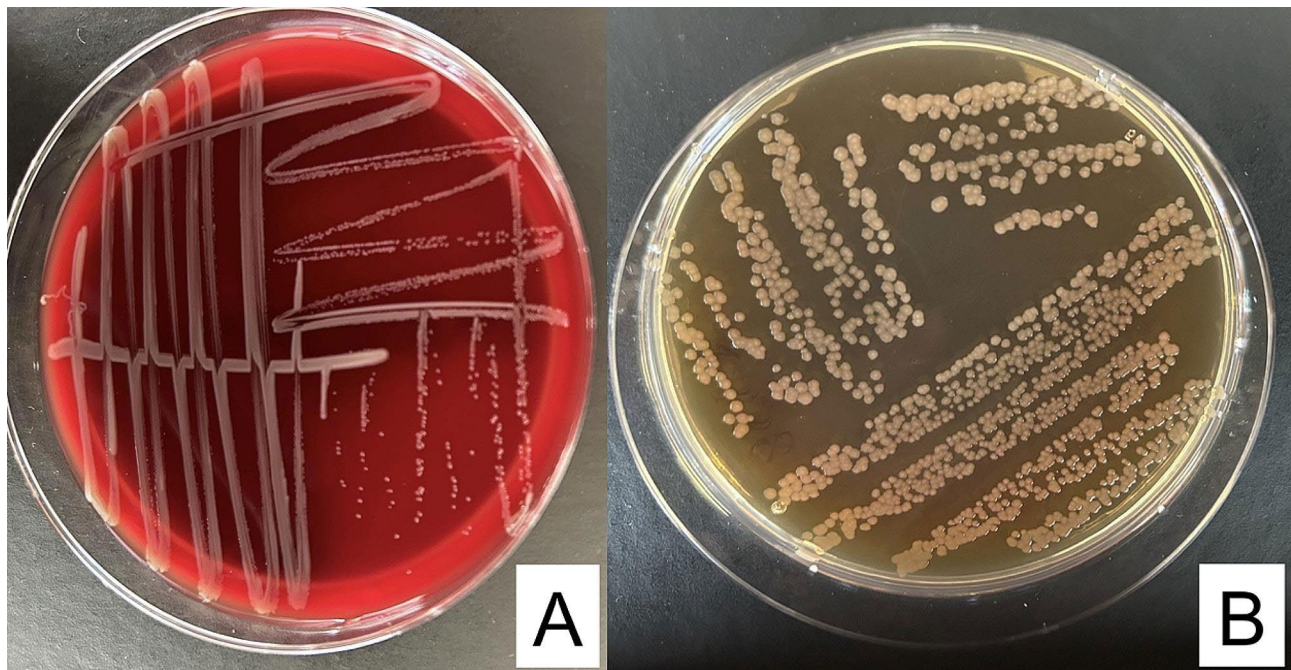
Fig. 1 Colonies of *C. gilardii* on Blood Agar **(A)** and Mc Conkey Agar **(B)**

Table 1 Antimicrobial agent susceptibilities of *C. Gilardii*

Antimicrobial agent	Zone diameter (mm)	EUCAST *P.* aeruginosa S ≥	EUCAST *P.* aeruginosa R <	Result	EUCAST B. pseudomallei S ≥	EUCAST B. pseudomallei R <	Result
AMC	38	-	-		50	22	I
PRL	38	50	18	I	-	-	
CAZ	16	50	17	R	50	18	R
FEP	33	50	21	I	-	-	
IPM	40	50	20	I	29	29	S
CIP	36	50	23	I	-	-	
TE	25	-	-		23	23	S
S	0	-	-		-	-	
CN	0	-	-		-	-	

* R=Resistant, I=Intermediate, S=Sensitive AMC Amoxicillin-Clavulanic Acid (30 µg), PRL Piperacillin (100 µg), CAZ Ceftazidime (10 µg), FEP Cefepime (30 µg), IPM Imipenem (10 µg), CIP Ciprofloxacin (5 µg), TET Tetracycline (30 µg), S Streptomycin (10 µg), CN Gentamicin (10 µg)

το the expected parturition (Clinical Trial Number 112/2004/18.12.2023 Faculty of Agricultural Sciences, University of Western Macedonia, Florina, Greece). Post-vaccination, a notable decline in incidence of neonatal calf diarrhea was consistently noted, with complete resolution of the symptoms and cessation of deaths over time.

Discussion

Neonatal calf diarrhea is a significant health challenge in both dairy and beef cattle herds worldwide, resulting in economic losses due to reduced calf growth and survival and increased cost of treatment [10]. Calves up to 30 days old is the primary age group.

susceptible to this condition [11]. The disease's etiology is complex and multifactorial including various infectious agents and predisposing factors (e.g. age, genetics, environment, management, nutrition, immune status, and concurrent illnesses) [11–14]. Among infectious agents, Enteropathogenic *Escherichia coli* (ETEC) and *Salmonella* spp. are the predominant bacterial pathogens, *Cryptosporidium* spp. and *Eimeria* spp. are the most common isolated protozoa, while bovine rotavirus (BRV), bovine coronavirus (BCoV), and bovine viral diarrhea virus are the most frequent viruses detected in outbreaks of the disease [15–18].

In the specific case of Aridaia beef cattle farm, and despite multiple treatment attempts using antibiotics, neonatal calf diarrhea did not respond. The identification of a specific colony type prevailing in higher dilutions within cultures had revealed the possible involvement of

those specific bacteria on the pathogenesis of this situation. The use of novel technologies e.g. next generation sequencing, proved at present to be a very useful tool in verifying a novel potential pathogen e.g. *C. gilardii* and further directing the next diagnostic and control steps.

The results presented in Table 1, indicate that the isolated bacteria were resistant to ceftazidime, according to the breakpoints of both *P. aeruginosa* and *B. pseudomallei*. Moreover, while there are no specific breakpoints defined for streptomycin and gentamicin, the absence of zone diameter in the disk diffusion test for these antibacterial agents (0 mm) signifies resistance against both compounds. Regarding the susceptibility of the isolates to the other tested antimicrobial agents, they demonstrated intermediate susceptibility to piperacillin, cefepime, and ciprofloxacin in line with the breakpoints defined for *P. aeruginosa*, and also to amoxicillin-clavulanic acid, according to the breakpoints for *B. pseudomallei.* In addition, both isolates exhibited susceptibility to tetracycline, as per the defined breakpoints for *B. pseudomallei.* It is noteworthy that although the isolated strain of *C. gilardii* exhibits susceptibility to imipenem, as per the breakpoints designated for *B. pseudomallei*, it demonstrates intermediate susceptibility based on the breakpoints specified for *P. aeruginosa*. To date, investigations on the susceptibility of *C. gilardii* to different antimicrobial agents in animal populations are missing. However, analogous studies in human contexts provide valuable insights. For instance, Kodayashi et al. [5] demonstrated resistance of the isolated *C. gilardii* strain to ceftazidime and gentamicin, a finding consistent with our research outcome. Similarly, Kweon et al. [19] reported gentamicin resistance in a *C. gilardii* strain. On the other hand, the strain was susceptible to ciprofloxacin and cefepime, a finding that contrasts with the results for the strain of the present study. Furthermore, Kweon et al. highlighted *C. gilardii's* susceptibility to imipenem and intermediate susceptibility to piperacillin, aligning with our study's results. In another study, Zhang et al. [8] showed that their isolated strain of *C. gilardii* was susceptible to ciprofloxacin, piperacillin, and ceftazidime, while our strain displayed intermediate susceptibility (susceptibility-increased exposure) to the first two agents and resistance to the latter. The differences observed in the antimicrobial susceptibility profiles underscore the pressing need to establish specific breakpoints for *C. gilardii*, both human and veterinary, particularly considering its emerging role as a causative agent in bovine neonatal diarrhea. Such breakpoints would facilitate precise and effective antimicrobial therapy for this bacterium in human and veterinary medicine.

Some important observations emerged during discussions with the farm owner were the following; Firstly, it was noted that the farm owner's parents, who had frequent contact with the animals, displayed symptoms such as weakness, malaise, and sporadic mild diarrhea. Upon seeking medical attention at the local hospital, physicians attributed their health issue to a bacterial infection; however, the precise etiological agent remained undetermined. Secondly, neighbouring cattle farms in the area were also experiencing similar challenges characterized by calf diarrhea and deaths. These additional observations raise the suspicion that the forementioned specific situations might be linked to the presence of *C. gilardii* in the farm and the area, reinforcing its substantial involvement in the recorded outbreak of neonatal calf diarrhea.

The fact that after the application of the autogenous vaccine on the cows of the farm, there was a noticeable improvement in the condition, ultimately leading to the cessation of deaths in the calves, confirms the hypothesis that the primary causative agent for this disease may be *C. gilardii*. This assumption is further supported by the identification of this particular bacterium using a high throughput technique such as ONT sequencing, which eventually supports "personalised" diagnosis and treatment/ control as well as the identification of means for the reduction of antibiotic resistance.

Conclusion

C. gilardii is an opportunistic pathogen that mainly affects the immunocompromised individuals. The present study describes the first case of *C. gilardii* isolation from a bovine neonatal diarrhea outbreak. The isolation and identification of this specific bacterium was conducted by using a combination of classical microbiology methods and high-throughput sequencing technology. Conducting antimicrobial susceptibility tests on the isolated strain revealed resistance or intermediate susceptibility to various antimicrobial agents. Subsequently, and keeping in mind to reduce antibiotic resistance development, an autogenous vaccine was developed and administered to the cows, leading to a marked enhancement in the farm's condition regarding the disease in calves. Given the status of *C. gilardii* as an opportunistic pathogen in humans and its potential implications for public health, the urgency for further investigation of its significance in animal health becomes important. As this being the first reported case of *C. gilardii* in its involvement in animal disease, the role of novel techniques in directing diagnosis and treatment by specialists in the future for defending unknown or potential pathogens is highlightened.

Abbreviations
C. gilardii   *Cupriavidus gilardii*
P. aeruginosa   *Pseudomonas aeruginosa*
B. pseudomallei   *Burkholderia pseudomallei*

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Supplementary Information

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Supplementary Material 1

Declarations

Ethics approval and consent to participate
The present study had been approved by decision 112/2004/18.12.2023 Faculty of Agricultural Sciences, University of Western Macedonia, Florina, Greece. Informed consent was obtained from the owner of the animals to collect the samples.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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