SHORT COMMUNICATION

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# Phylogenetic analysis and microbiological findings of *Kosakonia cowanii* in *Sporophila angolensis* associated with fatal hepatitis

## *Análise filogenética e achados microbiológicos de* Kosakonia cowanii *em* Sporophila angolensis *associados à hepatite fatal*

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**ABSTRACT:** The Gram-negative *Kosakonia cowanii*, previously classified as *Enterobacter cowanii* (Enterobacteriaceae) is an environmental organism and recently identified in birds and humans. We report the identification of *K.cowanii,* a small, motile and Gram-negative rod-shaped bacterium in the liver of a hatchling *Sporophila angolensis* (Chestnut-bellied Seed-Finch) which died of hepatic disease diagnosed by histopathology and bacterial culture. The agent was identified by culture and confirmed by MALDI-TOF and PCR. The amplified samples were sent to sequencing and an antibiogram was performed to assess the sensitivity to antimicrobials. The relevance of *K. cowanii* as an opportunistic pathogen in birds is considered within the discussion of an environmental organism potentially emerging as an animal pathogen, possibly associated with human activity and climatic change. The description of disease by *K. cowanii* in a native passerine may worth further epidemiological studies regarding the potential risk to avian, as well as other animals, public health and conservation.

**KEYWORDS:** avian medicine; emerging bacteria; one health; wildlife; zoonosis.

**RESUMO:** *Kosakonia cowanii*, uma bactéria Gram-negativa anteriormente classificada como *Enterobacter cowanii* (Enterobacteriaceae), é um organismo ambiental recentemente identificado em aves e humanos. Relatamos a identificação de *K.cowanii*, uma pequena bactéria em forma de bastonete, móvel e Gram-negativa, no fígado de um filhote de *Sporophila angolensis* (curió), que morreu devido a uma doença hepática diagnosticada por histopatologia e cultura bacteriana. O agente foi identificado por cultura e confirmado por MALDI-TOF e PCR. As amostras amplificadas foram enviadas para sequenciamento e um antibiograma foi realizado para avaliar a sensibilidade a antimicrobianos. A relevância de *K. cowanii* como patógeno oportunista em aves é discutida no contexto de um organismo ambiental que pode emergir como patógeno animal, possivelmente associado à atividade humana e mudanças climáticas. A descrição da doença causada por *K. cowanii* em um passeriforme nativo pode justificar futuros estudos epidemiológicos sobre o risco potencial para aves, outros animais, saúde pública e conservação.

**PALAVRAS-CHAVE:** animais silvestres; bactérias emergentes; medicina de aves; saúde única; zoonose.

## INTRODUCTION

*Kosakonia cowanii* is a small, motile, Gram-negative, rod-shaped, facultative anaerobic bacterium of Enterobacteriaceae family, previously classified as *Enterobacter cowanii*, present in soil, plants, birds and humans (Petrzik; Brázdová; Krawczyk, 2021). The *Enterobacter* sp. genus was reclassified into Kosakonia through genetic analysis, which now has nine species into the genus (Brady *et al.*, 2013). Due to the high metabolic rate, competitive strategy and possibility of rapid adaptation to the changing environment, the bacterium has been considered an emerging opportunistic pathogen but also, has an effect promoting plant growth by fixing atmospheric nitrogen and secreting hormones (Beatrice; Susanne; Silke, 2017). The *Enterobacter* sp. FY-97 reclassified as *Kosakonia oryzendophytica* has been proved to help plants resist infection and accelerate their growth, also solubilize phosphate in rice roots and able to obtain carbon sources from the plant to promote their own growth and metabolism (Gao *et al.*, 2022).

*Kosakonia cowanii* used to be identified as *Enterobacter agglomerans* and *Enterobacter cowanii* when it was reclassified

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(Brady *et al.*, 2013). In humans, its association to disease was reported in rhabdomyolysis and bacteremia in human accidents involving rose thorns (Washio *et al.*, 2018) and in 53 pediatric cases of trauma with plants thorn by *Pantoea agglomerans*, which is the old classification of the *K. cowanii* (Cruz; Cazacu; Allen, 2007). Also, cholecystitis was reported in an immunocompromised human associated with *K. cowanii* and an retarded uterine growth and cardiac arrhythmia in a premature neonatal infected by *K. cowanii* (Berinson *et al.*, 2020; Duployez *et al.*, 2021). Still, even though *K. cowanii* has been isolated from humans before, it has only recently been associated with exogenous sources of infection.

However, human infection has been more commonly reported after traumatic inoculation through thorn, or by ingestion in contaminated food and subsequent intestinal colonization. In fish, *K. cowanii* was responsible for the production of biofilm in the intestine, associated with *Micrococcus* spp. (Washio *et al.*, 2018). The formation of biofilms occurs through cell appendages or flagella organized into aggregated multicellular structures adhered to each other (Burtseva *et al.*, 2021). *K. cowanii* has mostly been described as an important plant pathogen, for instance, causing infection in soybean plantation (Krawczyk; Borodynko-Filas, 2020). *K. cowanii* could have an beneficial or adverse effect in humans, being able to compete with pathogenic bacteria (Petrzik; Brázdová; Krawczyk, 2021). Also, it could be a possible strategy in controlling trypanosomes by being capable of colonizing its vector midgut and prevent trypanosomiases by the acidification of the environment (Weiss *et al.*, 2019).

No previous description was found in the literature regarding *K. cowanii* in association with avian disease, only the infections caused by its precedent classification as the genus *Enterobacter* sp. By saying that, we describe and characterize *K. cowanii* associated with hepatic disease in hatchling *Sporophila angolensis* and consider the potential significance of an environmental bacterium in opportunistic infections and conservation.

## MATERIAL AND METHODS

### Microbiology

The study described in the manuscript entitled above was retrospective and developed in a pre-available diagnostic sample. A sample was collected from the liver of a *Sporophila angolensis* during necropsy. The bird was an infant from a commercial breeder experiencing higher mortality rates among young birds and hatchlings. The collected liver material was inoculated into Brain and Heart Infusion (BHI) broth and aerobically incubated (24 hours/37 °C). The growth was subsequently subcultured on BHI, MacConkey (MC), Hektoen (HE), Brilliant Green (BG) and Salmonella-Shigella (SS) agar plates, incubated (37 °C/24 hours) and colonies evaluated for tinctorial properties. The biochemical tests performed included catalase, oxidase, Rugai-Araujo (Rugai; Araujo, 1968) and citrate, in order to determine the genus and species (GAO *et al.*, 2022). The confirmation was performed by MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry). The obtained pure culture was subjected to 24h antibiotic susceptibility test (antibiogram) in Mueller-Hinton (MH), with amikacin, tobramycin, ceftazidime, meropenem, clindamycin, penicillin, gentamicin, tetracycline, ciprofloxacin, norfloxacin, azithromycin. The histopathology results for the liver sample revealed bacterial hepatitis.

### Polymerase Chain Reaction

The total DNA was purified by thermal extraction from the identified colonies and used as template for the PCR reactions. Primers for specific genes for *Kosakonia cowanii* were designed based on sequences of primer pair 2 (Table 1).

### Sequencing and phylogenetic analysis

PCR products were purified and sent to sequencing (Sanger). Sequences were analysed for quality, aligned and the consensus sequences obtained. The consensus sequences of *Kosakonia cowanni* were phylogenetically compared with sequences available at the GenBank.

## RESULTS AND DISCUSSIONS

The necropsy was performed in a *Sporophila angolensis* and the swabs collected from the liver were initially cultured on BHI broth and subcultured. Gram's stain was performed and revealed a pure colonie of gram-negative rod-shaped bacteria. The broth was cultured in Brain Heart Infusion, MacConkey, Salmonella-Shigella and Brilliant Green agar. The colonies cultured in BG were characterized by shiny, smooth and mucoid

**Table 1.** Primer pair 2 for *Kosakonia kowanii*.

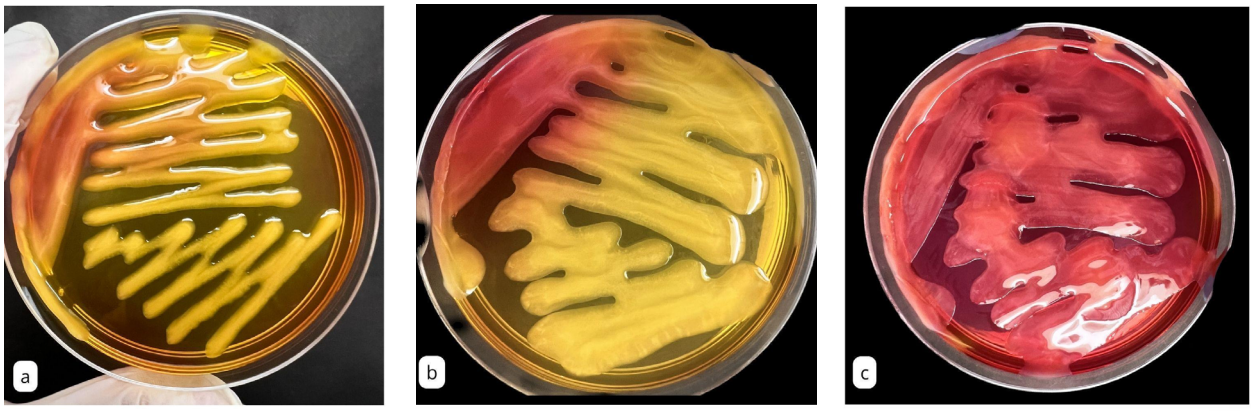
| Primer pair 2 for *Kosakonia kowanii* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC% | Self complementarity | Self 3' complementarity |
| Forward primer | TGCCTGATGGAGGGGGATAA | Plus | 20 | 95 | 114 | 60.03 | 55.00 | 3.00 | 1.00 |
| Reverse primer | TTTAACCTTGCGGCCGTACT | Minus | 20 | 869 | 850 | 59.96 | 50.00 | 6.00 | 2.00 |
| Product length | 775 | | | | | | | | |

yellow colonies surrounded by intense yellow zones at 24h by the fermentation of the lactose in the medium (Fig. 1a). In the advance of time, the medium became red as the phenol red served as a pH indicator of alkalinization (Fig. 1b-1c).

In MacConkey agar, the isolate was lactase positive in the first hours of growth presenting few non-confluent smooth round colonies of about 1-2 mm colored by pink. The isolated colony was inoculated in Rugai Araujo which had its indole and L-tryptophan negative, sucrose and glucose fermentations positive, production of gas, although $H_2S$ and Lysine were negative (Fig. 2a). Also, it presented positive catalase, as a drop of 3% hydrogen peroxide was added in a slide containing a small amount of bacteria resulting in oxygen bubbles (positive) and negative oxidase reactions. The Simmons citrate (citrate as carbon source and ammonium nitrate as nitrogen source) was positive (alkaline) (Fig. 2b).

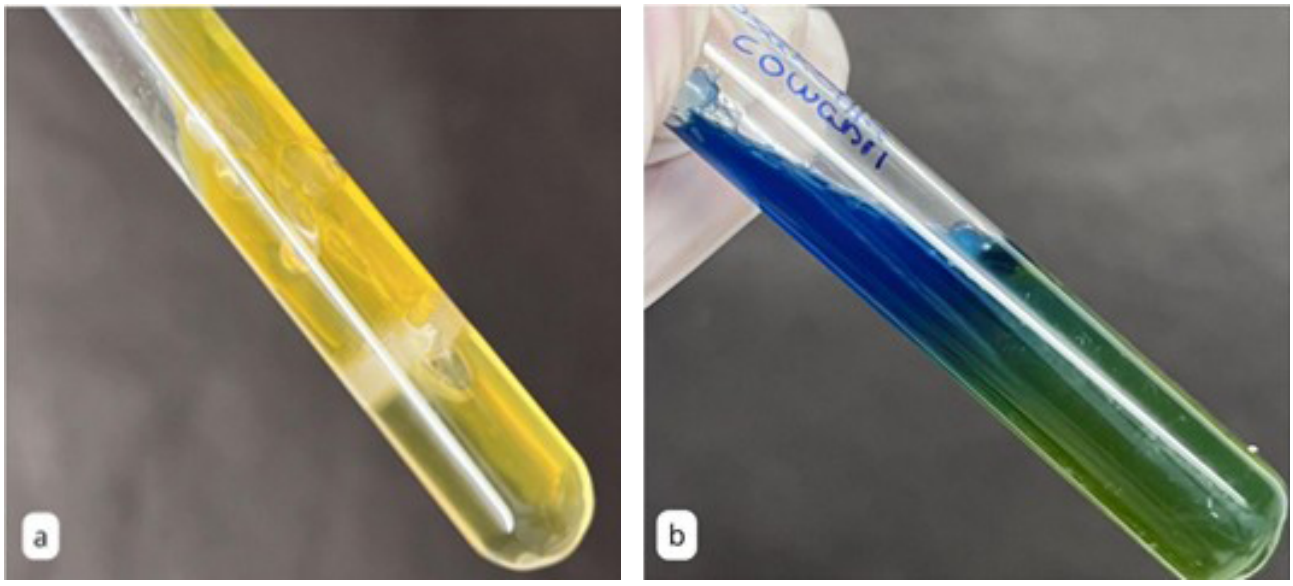
The biochemical tests identified the colony as part of the Enterobacteriaceae family, further identified as *Kosakonia cowanii* by Matrix-Assisted Laser Desorption Ionization mass spectrometry (MALDI-TOF) technique (Bruker Daltonics, Bremen, Germany), establishing a mass spectrogram. In mass spectrometry, samples are ionized by laser into charged molecules, the mass-to-charge ratio is measured and the molecular mass is analyzed according to the time-of-flight (TOF). This study was based on the morphological and biochemical characterization of this bacteria in order to identify its isolated colony and interpret the results. *K. cowanii* is a bacteria that uses sucrose, maltose and lactose and other sugars as their main carbon sources (Inoue *et al.*, 2000).

The DNA extracted from the purified colony was used as a template for the PCR reactions. Primers for specific genes for *Kosakonia cowanii* were designed based on sequences




Source: Laboratório de Doença das Aves - André Fernandes.

**Figure 1.** Cultures of *Kosakonia cowanii*. 1a. The 24h pure colonies were shiny, smooth and mucoid in appearance, with positive lactose utilization in the initial seeding areas. 1b. Confluent 48h mucoid colonies in Brilliant Green agar. 1c 72h mucoid colonies of *K.cowanii* in the alkalinized medium.

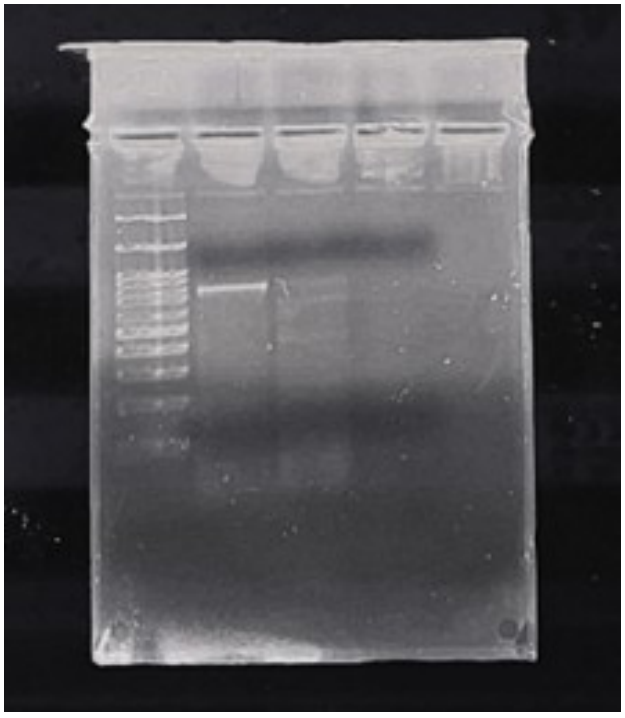



Source: Laboratório de Doença das Aves - André Fernandes.

**Figure 2.** Biochemical identification of *Kosakonia cowanii*. 2a. Identification of an Enterobacteriaceae in Rugai. 2b. Simmons citrate showing a positive result and color change to deep blue.

described by the 18146 strain 888-76 16S ribosomal RNA (NCBI Reference Sequence: NR_025566.1). The DNA amplification was performed using the following criterias. The electrophoresis, whose objective is to separate the amplified DNA based on its size and electrical charge, was performed on the same day on a 1,5% Dextrose agar and 5% ethidium bromide. The molecular identification was possible with the PCR, which was positive for *Kosakonia cowanii* (Fig. 3).

The antibiotic susceptibility test revealed resistance to clindamycin and penicillin, intermediary susceptibility to azithromycin, amikacin, tobramycin and susceptibility to ceftazidime, meropenem, gentamicin, tetracycline, ciprofloxacin and norfloxacin (Table 2). Even though *K. cowanii* is a known plant pathogenic bacteria, it is present in food, cereals and even in humans and animals, having the capability of initiating endogenous infection on people or animals that consume infected food or have traumatic experience with plants and thorns.



Source: Laboratório de Doença das Aves.

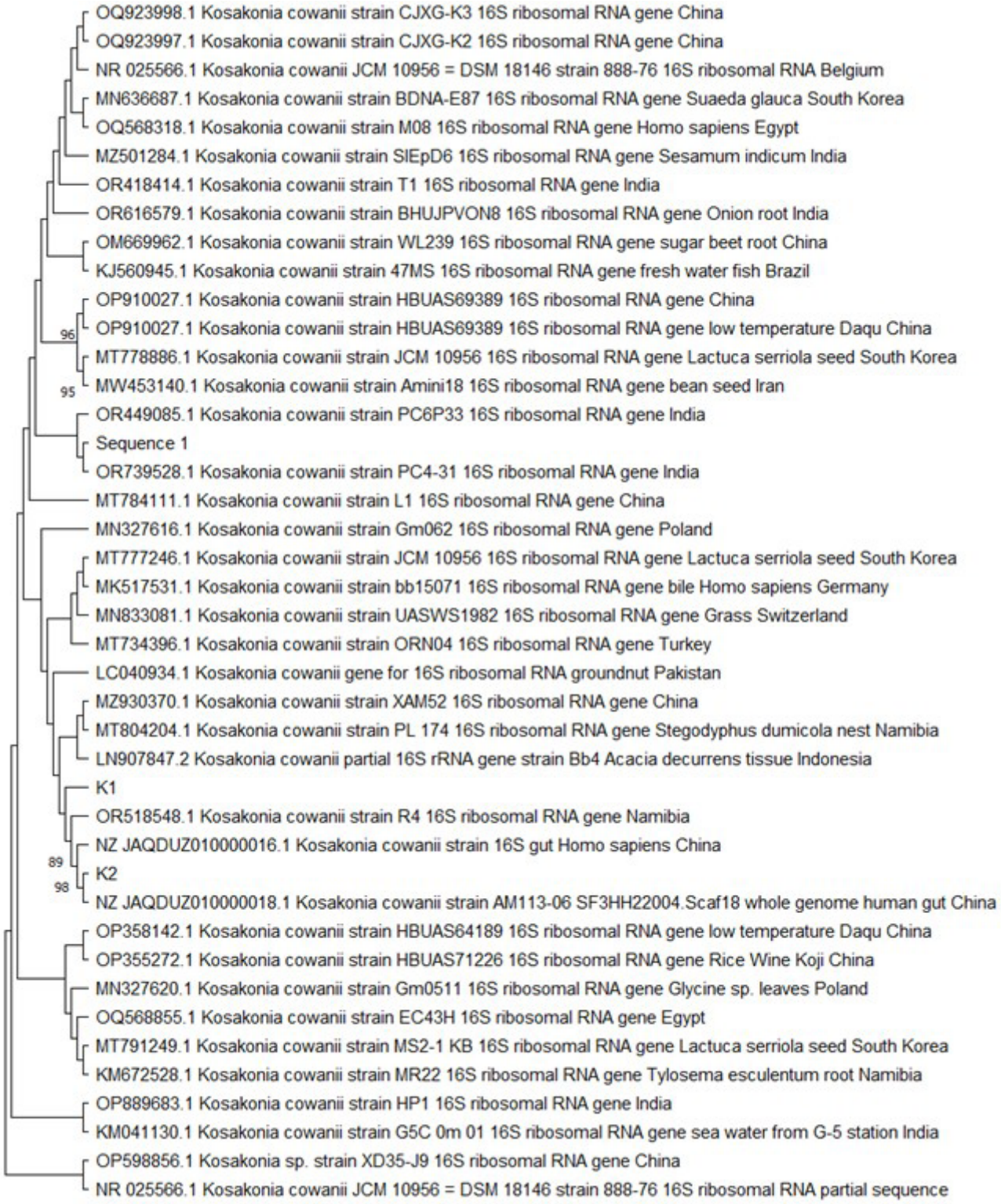
**Figure 3.** Electrophoresis of *Kosakonia cowanii* on a 1,5% Dextrose agar and 5% ethidium bromide on a 775 length.

The interpretation of the values found in the antibiogram were carried out as based on references for the family Enterobacteriaceae. The analysis demonstrated the susceptibility to most (54,5%) antibiotics but total or partial resistance to relevant usually recommended principles. Even though this bacteria is a plant pathogen, it's resistance of antibiotics revels the necessity of more discussion about it's therapeutic in humans and animals as no natural antagonist is known, only viruses that infect strains of *K. cowanii* in humans and plants such as kayfunavirus and cronosvirus could be a potential tool to promote one health practices (Petrzik; Brázdová; Krawczyk, 2021).

The 16S rRNA gene amplicon sequences K1 and K2 were successfully grouped close to strains obtained from humans in China (NZ_JAQDUZ010000018.1) *Kosakonia cowanii* strain AM113-06 SF3HH22004.Scaf18, from the human intestinal microbiome, and a human strain from Namibia (OR518548.1) (Fig. 4). The "Sequence 1" is the product of a reference isolate of *Kosakonia cowanii*. Most of the sequences included in the tree are from strains obtained from a diversity of plant infections and seemed to be distantly related. The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 2063 is shown. The consistency index is 0.907371 (0.797240), the retention index is 0.714072 (0.714072), and the composite index is 0.647928 (0.569287) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985). The MP tree was obtained

**Table 2.** *Kosakonia cowanii* antibiogram.

| Antibiotics | Zone diameter breakpoints (mm) (EUCAST, 2022) | | | Result |
|---|---|---|---|---|
| | Sensitivity | Intermediary | Resistance | |
| **AMI (30μg)[1]** | ≥ 18 | 16-17 | < 15 | 17 (I) |
| **TOB (10 μg)[1]** | ≥ 17 | 13-14 | <14 | 15 (I) |
| **CAZ (30μg)[1]** | ≥ 22 | 20-21 | < 19 | 24 (S) |
| **MPM (10μg)[1]** | ≥ 22 | 17-21 | < 16 | 25 (S) |
| **CLI (μg)** | - | - | - | 0 (R) |
| **PEN (μg)** | - | - | - | 0 (R) |
| **GEN (10μg)[1]** | ≥ 17 | 15-16 | ≤ 14 | 19 (S) |
| **TET (30μg)[2]** | ≥ 15 | 12-14 | ≤ 11 | 21(S) |
| **CIP (05μg)[1]** | ≥ 25 | 23-24 | ≤ 22 | 26 (S) |
| **NOR (10μg)[1]** | ≥ 22 | 20-21 | ≤ 19 | 33 (S) |
| **AZI (15μg)[2]** | ≥ 16 | 11-15 | ≤ 10 | 13 (I) |




Source: Subtree-Pruning-Regrafting (SPR) algorithm.
**Figure 4.** Maximum Parsim analysis of *Kosakonia cowanii* 16S rRNA gene.

using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei; Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 42 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2154 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura; Stecher; Kumar, 2021).

The 16S rRNA gene amplicon sequences of *K. cowanii* K1 and K2 strains were successfully grouped (Fig. 3) close to strains obtained from humans in China (NZ_JAQDUZ010000018.1)

*Kosakonia cowanii* strain AM113-06 SF3HH22004.Scaf18 from the human intestinal microbiome, and a human strain from Namibia (OR518548.1). The closer identity to human sequences might suggest a more direct epidemiological relationship. The "Sequence 1" is the product of a reference isolate of *Kosakonia cowanii*.

In humans, a rare infection with *K. cowanii* was associated to acute cholecystitis (gall bladder), with the accumulation of lithiasis and partial necrosis, in a 61-year old immunocompromised man, with the obtained pure isolate whole sequence determined. The antimicrobial susceptibility testing Ampicillin, Ampicillin/sulbactam; Cefoxitin, Piperacillin; Piperacillin/Tazobactam; Fosfomycin; Meropenem; Ciprofloxacin and Colistin revealed two resistant antibiotics: Ampicillin and Fosfomycin (Berinson *et al.*, 2020).

Most of the sequences included in the tree (Fig. 4) are strains obtained from a diversity of plant infections, such as *Tyloserna esculetum, Lactuca serriola, Gycine* sp., *Arachis hypogaea*, *Sesamum indicum* and *Suseda glaucum*, grass, bean, sugar beet, and seemed to be distantly related. Also, from the environment, such as from the sea water, *Stegodyphus dumicola* (spider) nest and fresh water fish, but also included strains found in the production of rice wine or daqu (traditional Chinese liquor). Therefore, it seems that epidemiological and host conditions are required for successful host jumps. Seed endophytes include bacteria, fungi and viruses which may be vertically transmitted with the seed and play evolutive roles in the nutrient intake and reduction of abiotic and biotic stress in plants. For instance, *K. cowanii* stimulated *Arabidopsis thaliana* (Vandenkoornhuyse *et al.*, 2015), an edible mustard and *Lactuca serriola* (prickly lettuce) (Jeong *et al.*, 2021) growth and resilience during droughts.

The occurrence of multi-resistant bacteria is an usual find these days especially associated with domestic animals and the abuse of antimicrobial drugs. Besides that, the isolation of multi-resistant bacteria in wild animals stands as an important factor of its pathogen because it reveals the ecosystem's health and the anthropic factor associated with this find. The importance of *K. cowanii* as plant pathogen, although with normal occurrence in plant evolution and the adjustment to environmental change, may suggest that human activity, such as through agriculture and farming, and others which have been affecting the natural equilibrium, may have been a force, possibly more recent, for the spillover to humans and other animals.

## CONCLUSIONS

*K. cowanii* was found in pure infection in the liver of *Sporophila angolensis* with severe liver disease. Human activity, such as through agriculture and farming, including others may affect the natural equilibrium, and cause spillover to humans and other animals. The pathogenesis is not yet defined and the comorbidities may play an important role.

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