Future MICROBIOLOGY

CASE REPORT

Asaia lannensis bacteremia in a 'needle freak' patient

Edoardo Carretto*[,1], Rosa Visiello[1], Marcellino Bardaro[1], Simona Schivazappa[2], Francesca Vailati[3], Claudio Farina[3] & Daniela Barbarini[4]

The genus *Asaia* has gained much interest lately owing to constant new species discoveries and its role as a potential opportunistic pathogen to humans. Here we describe a transient bacteremia due to *Asaia lannensis* in a patient with a psychiatric disorder (compulsive self-injection of different substances). Common phenotypic methods of identification failed to identify this organism, and only restriction fragment lenght polymorphism of PCR-amplified 16S rRNA gene allowed for proper identification. The isolate was highly resistant to most antibiotics. The paper also discusses the currently available medical literature, acknowledges the potential problems linked to the isolation of these strains and proposes an approach to species identification that can be applied in a clinical microbiology laboratory.

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KEYWORDS
• *Asaia* • *Asaia lannensis* • bacteremia • clinical significance • molecular identification

A 28-year-old female patient, known to have psychiatric problems (compulsive self-injection of different substances), was admitted by the Emergency Department of the IRCCS Arcispedale Santa Maria Nuova of Reggio Emilia, Italy, due to pyrexia, with a temperature exceeding 40°C the night before the admission. As self-medication the patient took paracetamol early in the morning, and at the moment of admission her temperature was 37.8°C, with a blood pressure of 100/60 mmHg. The patient declared that during the night she had taken lorazepam for insomnia, dissolving the 2.5 mg capsule in water of unknown origin before self-injecting it intravenously. On physical examination, the patient appeared alert and somewhat orientated, with 98% oxygen saturation. No pathological signs were observed; cardiovascular, neurological and pulmonary systems were normal. Bilateral inguinal venepuncture lesions were noted. From the biochemical point of view, she showed only a mild leucocytosis with 13,050 cells/mm^3 with a slight normochromic normocytic anemia and other parameters within the corresponding normal range. Procalcitonin was 0.34 ng/ml and C-reactive protein was 2.5 mg/dl. Two different blood cultures (aerobic and anaerobic bottles) were collected and the patient was discharged with a course of amoxicillin-clavulanate and referred to the Infectious Disease Department for follow-up, however, she disappeared for long-term controls.

Blood culture samples were analyzed using the BACTEC FX system (Beckton Dickinson, USA). Three days and 22 h after the beginning of the analysis, a single aerobic bottle of the two sets collected was reported as positive by the instrument. The sample was plated on common agar media. After 48 h short Gram-negative aerobic bacilli grew on Columbia blood agar and chocolate agar, incubated at 36°C in air and 5%CO_2, respectively. Two days after small colonies also appeared

[1]Clinical Microbiology Laboratory – IRCCS Arcispedale Santa Maria Nuova, Reggio Emilia, Italy
[2]Infectious Diseases – IRCCS Arcispedale Santa Maria Nuova, Reggio Emilia, Italy
[3]Microbiology & Virology Laboratory – Azienda Ospedaliera 'Papa Giovanni XXIII,' Bergamo, Italy
[4]Virology & Microbiology Laboratory – Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
*Author for correspondence: Tel.: +39 052 229 6363; Fax: +39 052 229 6750; edoardo.carretto@asmn.re.it

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on McConkey agar (incubated at 36°C in air ambient); no growth was observed on mannitol salt agar (MSA). On Columbia blood agar the colonies appeared pale rose, shiny, smooth and raised with an entire margin **(Figure 1A)**. After the passing of time, the colonies appeared enlarged and more pigmented **(Figure 1B)**. The isolate was oxidase, coagulase and urease negative, and catalase positive. Vitek2™ (bioMérieux, France) was initially used (GN card) to identify the strain and perform antimicrobial susceptibility testing. Contemporaneously, ASTs were also performed by using the disc diffusion method (Kirby Bauer) on Mueller Hinton agar and on Mueller Hinton agar with 5% sheep blood (MHSB). After 6.75 h, Vitek2™ identified the micro-organism as *Shigella* species (bionumber: 2005410520101210, good identification), but the system did not provide susceptibility test results. The micro-organism on MHSB was extremely resistant (since it was slow growing the results were evaluated after 48-h incubation at 36°C, air ambient). The growth characteristics, the negativity of the agglutination with *Shigella* antisera and the highly resistant susceptibility profile allowed us to exclude the isolate as belonging to the *Shigella* group. Thus, API-20E, API20-NE and API-32GN (bioMérieux, France), were set up according to the manufacturer's instructions and, contemporaneously E-test methodology (MIC test strip™, Liofilchem, Italy) was performed to obtain MICs to different antimicrobials for the isolate.

The isolate showed high MICs for most of the antibiotics tested. It was highly resistant to all the β-lactams and to chloramphenicol, fluoroquinolones, colistin, cotrimoxazole and vancomycin. Instead, it was susceptible to the aminoglycosides (amikacin, gentamycin and tobramycin), and to tetracycline. Since the strain presented with the peculiar characteristics mentioned above, we evaluated the antimicrobial susceptibilities using the breakpoints proposed by the Interpretive Standards for the Minimal Inhibitory Concentration (MIC) for Other Non-Enterobacteriaceae provided by CLSI [1]. All the resistant drugs showed a high-level resistance profile with confluent growth around the MIC test strips.

The API-20E generated a doubtful profile (code 0004252, T-index of less than 0.30 for different genera such as *Klebsiella* spp., *Shigella* spp., *Escherichia coli* and *Acinetobacter baumannii* complex). Also the API-20NE gave a doubtful profile (code 4040000, T-index of less than 0.33 for different genera such as *Pasteurella* spp. and *Acinetobacter lwoffii*). API-32GN provided a good identification of *Escherichia coli* (code 00003400000, T-index = 0.82). At this point, an API-50CH (bioMérieux, France) was performed, according to the manufacturer's instructions, to evaluate the ability of the strain to produce acid for different substrates.

The organism was also analyzed by using the matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) technology. Initially, the Bruker Biotyper Plus™ (Bruker, USA) instrument was used. The database of strains available for diagnostic purposes in clinical microbiology laboratories (i.e., the CE-IVD approved software) was unable to provide identification: different experiments performed after the procedure of strain extraction proposed a list of various micro-organisms with an identification score of less than 1.3 (no reliable identification). The strain was then referred to the Azienda Ospedaliera 'Papa Giovanni XXIII' in Bergamo, where the MALDI-TOF analysis was performed using the Vitek™ MS (BioMérieux, France). However, this system also was unable to identify the organism using the database approved for diagnostic purposes.

Since all the tests performed at this point were unable to provide correct identification of the strain, genotypical identification was performed by amplification and sequencing of a DNA fragment encoding for the 16S rRNA gene, according with the manufacturers' suggestions (Applied Biosystems, USA). The 1500 bp sequence obtained was compared with those present in GenBank using the BLASTn program and a similarity of 99.9% (1412 out of 1413 bases) was found with the genome of *Asaia lannensis* strain NBRC 102526 (GenBank accession number: NR_114144.1) and of *Asaia lannensis* strain AB92 (GenBank accession number: NR_041564.1, 1410 out of 1411 bases). The sequence of our isolate was then deposited in the GenBank database with the accession number KP208318.

The taxonomy and the lineage reports were consistent with the genus *Asaia*, but different matches within the species of the genus were possible. A similarity of 99.6% (1407 out of 1413 bases) was found with *A. bogorensis* NBRC 103511 (GenBank accession number: AB682098.1), 99.4% (1425 out of 1433 bases) with an uncultured *Asaia* species clone

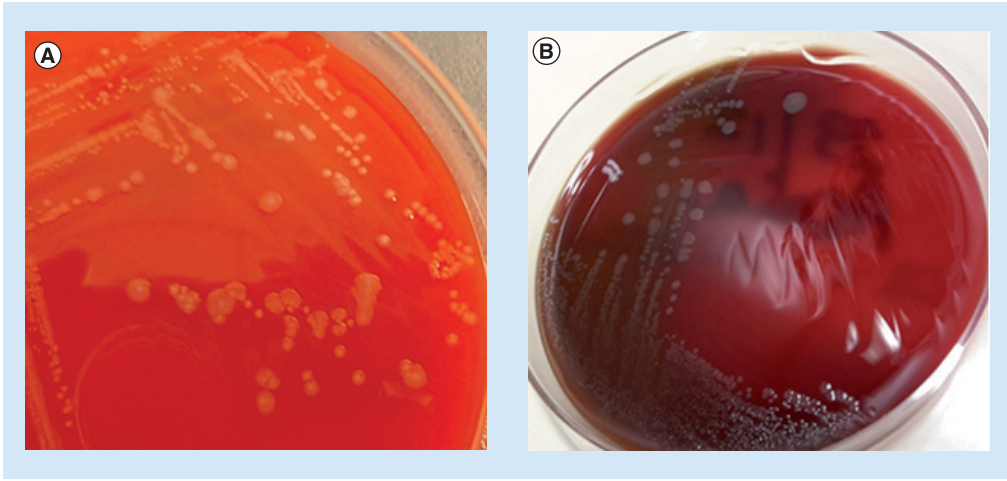

Figure 1. Strains growth on Columbia blood agar, incubation at 36°C air ambient, after 72 h (A) and 7 days (B).

(GenBank accession number: KF414320.1) and 99.4% (1425 out of 1434 bases) with *A. platycodi,* GenBank accession number JF514557.1.

At this point, the restriction fragment length polymorphism technique (RFLP) of PCR-amplified 16S rRNA gene was utilized, which was demonstrated to being able in distinguishing among the species belonging to the genus *Asaia* [2,3]. Briefly, the 16S rDNA amplification products obtained after a PCR with universal primers 27f-CM and 1492r, performed according to the protocol described by Franck *et al.* at maximum level of stringency (annealing temperature = 60°C) [4], were digested with four different restriction enzymes namely *Sna*BI, *Bsa*JI, *Sty*I and *Hpa*II (New England Biolabs, USA), as previously described [2,3]. The results of the digestion (shown in **Figure 2**) allowed us to definitively identify the micro-organism as *Asaia lannensis* after comparison with restriction patterns obtained for type strains of *Asaia* species in previous works.

Discussion & conclusion

Asaia species are Gram negative, belonging to the family Proteobacteria, class of Alpha-Proteobacteria, order of Rhodospirillales. Strains classified in this genus are characterized by weak or no oxidation of ethanol to acetic acid, hence they are also known as Acetic Acid Bacteria (AAB) or *Acetobacteraceae*, together with members of the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* [5,6]. At the point of writing this report, *Asaia* species comprise of *A. bogorensis, A. lannensis* (previously known as *A. lannaensis*), *A. siamensis, A. krugthepensis, A. astilbis, A. platycodi, A. prunellae* and *A. spathodeae* [2,3,5,7–9]. All of these species were originally isolated from tropical flowers, orchid trees or from fermented glutinous rice found in south-east Asia [10], even if they have been found worldwide, thus being considered nowadays as cosmopolitan bacteria [7].

According to a study by Patil in 2013, *Asaia bogorensis* was shown to enhance leaf and fruit growth of pineapple plants and to regulate root growth. It is recognized as a phosphate solubilizing micro-organism, aiding the growth and development of plants [11].

Asaia is also found in the gut and reproductive tract of mosquitos of the genera *Aedes* and *Anopheles* [12]. As the main component of the mosquito associated microbiota, it has a beneficial role in the normal development of the *Anopheles* larvae, and it also appears to fix nitrogen, providing the host insect with this organic compound [13,14]. The symbiotic *Asaia* species also represent an interesting experimental model, since they can be grown *in vitro* and thus can be engineered at the chromosomal level. This genus has been proposed as a potential candidate for malaria control through manipulation of the bacterium for anti-*Plasmodium* factors [15].

The ability of AAB to adapt to different environmental conditions was recently pointed out in a paper of Chouaia *et al.*, which showed that most of AAB possess ubiquinol oxidases that allow their survival under micro-oxic conditions, such as those existing in the insect gut. Further

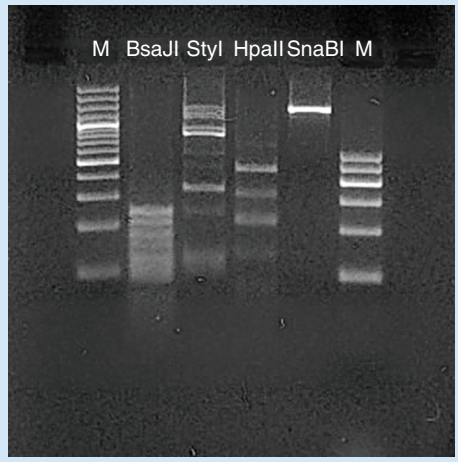

Figure 2. Restriction fragment length polymorphisms of the products of amplification of the 16S rRNA gene. Lane 1, molecular marker, 100–3000 bp; lanes 2–5, the restriction patterns obtained with the different endonucleases used; lane 6, molecular marker, 100–600 bp.

phylogenetic comparisons show that these terminal oxidases were present in the common ancestors of AAB, thereby constituting an ancestral character. These findings allow the Authors to postulate that AAB can express a constitutive propensity for thriving in micro-oxic environments including the insect gut, an environment with ample variation in its oxygen levels [16].

To our best knowledge, since 2004 *Asaia* spp. (mostly, *A. bogorensis* and *A. lannensis*) have hardly ever been reported to cause infections in humans. The three *A. bogorensis* cases include a patient undergoing peritoneal dialysis with peritonitis, and two episodes of bacteremias, both in intravenous drug abusers [17–19]. The three previously described cases of *A. lannensis* infections [10,20] are summarized in **Table 1**, together with the details of the present case. Isolation of another *Asaia* species was also described by Alauzet *et al.*; in that report, the isolate was collected from the sputum of a 2-year-old boy with cystic fibrosis, but the absence of species identification following several approaches with molecular techniques was considered with a cautious approach by the authors [6]. All the cases of bloodstream infections appeared to be transient bacteremias.

In trying to establish if *Asaia* spp. play a role as a human pathogen, Epis *et al.* carried out a study to evaluate the presence of both antibodies against *Asaia* (using an immunofluorescence assay with whole cell *Asaia* antigens) and *Asaia* DNA in blood by analyzing the whole blood and serum of 496 people, known to have been exposed to mosquito bites (*Anopheles gambiae* and *Anopheles arabiensis*) [15], given that *Asaia* spp. is demonstrated to be present in the salivary glands of mosquitoes [12]. All samples were found to be negative both for the presence of antibodies and bacterial DNA, showing that *Asaia* does not circulate in the blood of the general healthy population [15].

In the present report, a case of transient bacteremia due to *Asaia lannensis* with uncertain source is described. However, the patient's characteristics were really peculiar: her lifestyle, a young woman known to auto inject 'all sorts of substances', putting her at risk of bacteremias due to environmental micro-organisms. Although not clearly reported in the case history of this episode, she was known to use water from plants to prepare solutions for injecting herself. Unfortunately, this hypothesis could not be further investigated, since she disappeared for further controls. All the commonly used phenotypic identification systems failed to identify the micro-organism. Some phenotypical systems did not provide reliable identifications, whilst others suggested a misleading good identification (API-32GN). These findings are described in other papers regarding human infections due to *Asaia* species [10,18,20]. However, the susceptibility profile of the isolate (multidrug resistance, even to colistin), the growth characteristics and the morphology of the colonies allowed exclusion *per se* of all the identification proposed.

This unusual isolate presented a challenge even when analyzed through the two MALDI-TOF systems commonly used in clinical microbiology laboratories, which utilize software approved for diagnostic purposes (CE-IVD marked). Different papers have suggested a possible role of this technology in *Asaia* identification [21–23]. In particular, in a study of Andrés-Barrao *et al.*, it was shown that MALDI-TOF MS is a suitable technique for the rapid identification of the most common AAB. However, in order to apply mass spectrometry for the unequivocal identification of these isolates, it is necessary to create libraries that are currently not available for instruments dedicated to clinical microbiology. Moreover, to implement the profiles of these micro-organisms in research use only (RUO)

Table 1. Synopsis of the literature data available for human infection due to *Asaia lannensis*.

Patients' characteristics	Source	Identification method	Drug susceptibility	Treatment	Ref.
Idiopathic dilated cardiomyopathy (possible nosocomial trasmission?)	PICC (likely)	16S rRNA gene sequencing followed by analysis of SNP	Extensively resistant (β-lactams except for meropenem – intermediate, ciprofloxacin, colistin, cotrimoxazole, vancomycin), susceptible to aminoglycosides and tetracyclines	Meropenem, PICC removal	[10]
Idiopathic dilated cardiomyopathy (possible nosocomial trasmission?)	PICC (likely)	16S rRNA gene sequencing followed by analysis of SNP	Extensively resistant (β-lactams except for meropenem – susceptible, ciprofloxacin, colistin, cotrimoxazole, vancomycin), susceptible to aminoglycosides and tetracyclines	Meropenem, gentamicin, PICC removal	[10]
Autologous hematopoietic stem cell transplant in cancer	CVC (likely)	16S rRNA gene sequencing	Extensively resistant (β-lactams, ciprofloxacin, colistin, cotrimoxazole, vancomycin), susceptible to aminoglycosides and tigecycline	Meropenem, tobramycin, lock therapy on CVC	[20]
Psychiatric disorder (compulsive self-injection of different substances)	Self-injection of water of unknown origin	16S rRNA gene sequencing followed by restriction fragment lenght polymorphism analysis	Extensively resistant (β-lactams, ciprofloxacin, colistin, cotrimoxazole, vancomycin), susceptible to aminoglycosides and tigecycline	Started amoxi-clav, lost on follow-up	Present study

CVC: Central venous catheter; PICC: Peripherally inserted central catheter; SNP: Single nucleotide polymorphism.

libraries, a large number of reference strains are required and such library creation necessitates skilled personnel (to avoid mistakes in the creation of bacterial spectra). It is also time consuming and not useful for single centers, due to the rarity of these isolates.

Regarding the use of AAB identification methods based on molecular biology, a very exhaustive review was recently published by Trček and Barja [23]. As described in this paper, 16S rRNA gene sequencing was not discriminative and allows *Asaia* genus but not species identification, which can be further performed using RFLP of 16S rRNA gene, an approach previously shown as valuable for discrimination of species with highly similar or identical 16S rRNA gene sequences [2,3,23].

The experiments were performed over a 2-day period, did not require any particular instruments and can be performed in clinical microbiology laboratories with a medium-level of skill in molecular biology. Regarding the interpretation of the results **(Figure 2)**: *Sna*BI allowed us to exclude *A. krungthepensis* (a single band was present instead of the two at 850 bp and 561 bp generated by this species); *Bsa*JI excluded both *A. siamensis* and *A. spathodeae* (absence of the 327 bp band); *Hpa*II permitted to differentiate the isolate from *A. bogorensis* for the presence of a band at 311 bp [2,3]. The remaining species were excluded because *A. astilbis* and *A. platycodi* are unable to grow at 37°C, whereas the strain we isolated did after 48 h **(Figure 1)** and *A. prunellae* grows on MSA and produces acid from trehalose with delayed fermentation, whereas the described isolate did not grow on MSA and the fermentation of trehalose (using API-50CH) was negative even after more than 72 h of incubation [7].

From a theoretical point of view, the evaluation of the colony pigmentation for the different strains may also be helpful, since they vary from light brown to rose and orange colonies. *Asaia lannensis* produced light rose colonies **(Figure 1)**, and thus is distinguished from *A. astilbis,* which produces dark rose colonies. *Asaia platycodi* and *A. prunellae* produce light brown to pinkish orange colonies [7], but are undistinguishable in their macroscopic appearance on Columbia blood agar with *Asaia spathodeae*. However, it is quite difficult to correctly define the strain species on this basis, since the evaluation of the colors is subject to personal interpretation. Further analysis of the digestion profiles generated with the same enzymes for *A. astilbis*, *A. platycodi* and *A. prunellae* may be useful, defining these techniques as the gold standard in the identification of *Asaia* species.

In other reported cases of infection, the portal of entry for these micro-organisms was postulated to be *in situ* indwelling devices, or undergoing procedures breaking the skin barrier [10,18,20].

A hypothetical risk factor linked to intravenous drug abuse is that substances such as lemon juice or vinegar are used to dilute heroin [6]. *Asaia* species thrives well in such acidic conditions and, therefore, these patients can be considered at increased risk for developing bacteremias. With particular mention to the above, the present case strengthens the question as to whether cases of *Asaia* species bacteremias can really be a rarity, since routine identification systems, including automated instruments, are unable to correctly identify this genus and clinical microbiologists may not be aware of these environmental micro-organisms. Moreover, molecular identification is not always possible in clinical laboratories and micro-organisms that only cause transient bacteremias are rarely referred to reference laboratories. On the other hand, to our knowledge, the only study on the evaluation of the prevalence of *Asaia* species in humans failed to demonstrate the possible circulation of these micro-organisms [15].

These micro-organisms appear to possess a very low pathogenic potential. In almost all cases described thus far, bacteremias due to *Asaia* species are self-limiting, and the low virulence of these micro-organisms is further highlighted by the fact that, when a known source such as catheter tip was removed, clinical improvement was quickly observed [10,18]. In this report, the pyrexia disappeared spontaneously in 24 h, since the antibiotic therapy administered empirically was ineffective *in vitro*.

This report confirms the high-level resistance of members of the genus *Asaia*. The strain was susceptible only to aminoglycosides and tetracyclines (similarly to previously described isolates [10,20], see also **Table 1**), and these drugs seem to be the best approach if therapy is required. Use of gradient MICs seems appropriate in evaluating the susceptibility pattern of these bacteria, whereas the automated instruments failed to provide a result. The proper incubation temperature remains to be defined: Alauzet *et al.* proposed 30°C, since this temperature allows the best growth of AAB [6]. In this case, since *Asaia lannensis* grows at 36°C, the ASTs were evaluated using gradient MICs on MHSB at this temperature, with evaluation after 48 h.

The multidrug resistance of these micro-organisms should be better investigated in order to establish if they possess mobile elements (plasmids, transposons) that can be transmitted to other bacteria.

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EXECUTIVE SUMMARY

- Bacteremia due to *Asaia lannensis* in a patient with known psychiatric disorders.
- Analysis of the identificative troubleshooting using conventional phenotypic methods and of the possible molecular approaches for the species identification: profiles for members of this genus should be included in the databases of matrix-assisted lase desorption/ionization – time of flight instruments used in clinical practice. The molecular approach depends on the laboratory setting. Based on our experience, it should at least include restriction fragment length polymorphism technique of polymerase chain reaction-amplified 16S rRNA gene.
- The description of the genus Asaia as environmental micro-organisms and its relationship as symbiont in mosquitos is discussed.
- The possible role of *Asaia* spp. as an opportunistic pathogen in humans: clinical microbiologists awareness about the possible isolation of these unusual micro-organisms.
- The problem of the multidrug resistance is evidenced, but requires further research.

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