In vivo selection of a multidrug-resistant *Aeromonas salmonicida* during medicinal leech therapy

E. Ruppé[1], A. Cherkaoui[2], N. Wagner[3], G. C. La Scala[4], J.-Y. Beaulieu[5], M. Girard[1], J. Frey[6], V. Lazarevic[1] and J. Schrenzel[1,2]

1) *Genomic Research Laboratory, Division of Infectious Diseases,* 2) *Bacteriology Laboratory, Division of Laboratory Medicine, Department of Genetics and Laboratory Medicine,* 3) *Pediatric Infectious Diseases, Department of Pediatrics,* 4) *Division of Pediatric Surgery, Department of Pediatrics,* 5) *Hand Surgery Unit, Division of Orthopedic Surgery, Department of Surgery, Geneva University Hospitals, Geneva and* 6) *Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland*

Abstract

We report the selection in a 15-year-old boy of a multidrug-resistant, extended-spectrum β-lactamase (ESBL)-producing *Aeromonas salmonicida* after medicinal leech therapy that required an antibiotic prophylaxis based on piperacillin/tazobactam and cotrimoxazole. Whole genome sequencing of the strain indeed revealed 13 antibiotic resistance genes, including the ESBL CTX-M-3 and the unusual β-lactamase SCO-1.

Keywords: *Aeromonas salmonicida*, medicinal leech therapy, multidrug resistance, whole genome sequencing
Original Submission: 24 May 2017; **Revised Submission:** 15 September 2017; **Accepted:** 4 October 2017
Article published online: 10 October 2017

Corresponding author. E. Ruppe, Genomic Research Laboratory, Division of Infectious Diseases, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, CH-1205 Geneva, Switzerland.
E-mail: etienne.ruppe@gmail.com

Introduction

Our patient was a healthy 15-year-old boy who experienced traumatic proximal phalange avulsion of his left (nondominant) thumb. The finger was immediately reimplanted with success. Six days later, however, he sought care for acute ischaemia and underwent repeat exploration that identified complete arterial and venous thrombosis. For the venous congestion, medicinal leech therapy (MLT, also referred as hirudotherapy; Fig. 1) was initiated (9 February 2016, referred as day 0; Table 1). Concomitantly, because of surgery and the infectious risk of MLT, piperacillin/tazobactam-based prophylaxis was initiated, and a sample of the leech conservation medium (tap water) was sent at day 8 for culture to the bacteriology laboratory. It yielded *Acinetobacter johnsonii, Aeromonas veronii* and *Moraxella osloensis,* all identified by matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (MALDI Biotyper; Bruker Daltonics, Bremen, Germany; database version 3.1) and displaying a wild-type susceptibility to antibiotics (Table 1). MLT was successfully conducted for 14 days while the patient received piperacillin/tazobactam prophylaxis. Various wound swabs were sent for culture, which yielded *Stenotrophomonas maltophilia* and *Aeromonas* sp. that remained susceptible to β-lactams. No infectious complication was seen. Prophylaxis with cotrimoxazole was provided for 3 days after MLT ended (from day 13 to day 15). At day 26, a multidrug-resistant *Aeromonas salmonicida* (named ASG1) was recovered from the wound after culture on blood-supplemented agar plate under aerobic conditions at 35°C and identification by MALDI-TOF MS. The phenotypic traits of ASG1 are shown in the Supplementary Table S1. Of note, no sign of surgical site infection was observed, and the presence of ASG1 was considered to be colonization. Accordingly, no antibiotic treatment was initiated. Another sample of the wound taken at day 35 yielded *S. maltophilia* but not *A. salmonicida* (Table 1).

The strain ASG1 displayed a phenotype compatible with the production of an extended-spectrum β-lactamase (ESBL) according to the double-disk synergy test and was resistant to all the tested β-lactams except imipenem (minimal inhibitory concentration (MIC) 0.38 mg/L) and meropenem (MIC 0.5 mg/L). Moreover, the strain was resistant to ciprofloxacin, gentamicin and cotrimoxazole, while it was susceptible to amikacin, fosfomycin, tigecycline and colistin (MIC 0.25 mg/L). Because of this atypical resistance profile, the genome of the strain was sequenced. Patient consent for reporting the case was obtained on 2 November 2016.

Genome Analysis

Genomic DNA was extracted using the MagCore Genomic DNA Tissue Kit (RBC Bioscience, New Taipei City, Taiwan) and was sent to Fasteris (Plan-les-Ouates, Switzerland) for sequencing. The library was prepared using the Nextera XT

https://doi.org/10.1016/j.nmni.2017.10.005

FIG. 1. Leeches on replanted left thumb. Note use of plastic cup to target treatment on distal phalanx and prevent leeches from escaping area requiring treatment. Perforated lid is used to close cup. Consent from patient's representative for using pictures was obtained on 2 November 2016.

DNA Sample Preparation Kit according to the Illumina (San Diego, CA, USA) instructions, and was sequenced on an Illumina MiSeq with 250 bp paired-end reads. The Trimmomatic package [1] was used to remove bases that corresponded to the standard Illumina adapters. A total of 208 982 cleaned reads were obtained (sequence read archive no. PRJNA377399). The reads were assembled with IDBA-UD [2], yielding 150 contigs (the largest contig being 215 626 bp, N50 74 kbp) for a total length of 4 971 056 bp. The median depth of sequencing was 16×. Using JSpeciesWS [3], the average nucleotidic identity (ANI) with *A. salmonicida* type strain ATCC 33658 was 97.0% (range of ANI with other *A. salmonicida* genomes 96.7–97.2%, Supplementary Table S2). The mean ANI with other *Aeromonas* species for which genomes are available was 83.8% (range 76.8–90.4%, Supplementary Table S2). The alignment of the 16S rRNA sequence of ASG1 together with those of other *Aeromonas* species showed that ASG1 clustered with *A. salmonicida* (Fig. 2). The genes were predicted and annotated with PROKKA [6]. We aligned the amino acid sequences of the topoisomerases (*GyrA, GyrB, ParC* and *ParE*) of ASG1 with those of the reference strain A449 and found the Ser83Ile (*GyrA*) and the Glu93Lys (*ParC*) mutations that likely conferred the ciprofloxacin resistance observed in ASG1. The antibiotic resistance determinants (ARDs) were searched using BLASTP [7] using the ResFinder [8] and ResFinderFG (https://cge.cbs.dtu.dk/services/ResFinderFG/) databases and a cutoff of 80% amino acid identity over 80% of the reference sequence. We found 13 ARDs. Aside from the intrinsic FOX, CphA and OXA-12-like β-lactamases found in *Aeromonas* spp. [9], we identified the CTX-M-3 ESBL, TEM-1 and—more surprisingly—the SCO-1 inhibitor–resistant class A β-lactamase, all of which, taken

TABLE 1. Sample and culture results

Day	Action	Sample	Bacteria	AMX	AMC	PIP	TZP	FOX	CXM	CAZ	CRO	FEP	IMP	MEM	ETP	ATM	AN	GEN	CIP	LVX	SXT	FOS	TGC	COL
0	MLTstarts																							
8		Leech conservation liquid	*Acinetobacter johnsonii*							S		S	S	S			S	S	S	S	S			
8			*Aeromonas veronii*		R		S			S	S	S							S	S	S			
8			*Moraxella osloensis*		S				S		S								S	S	S			
10		Thumb (wound)	*Stenotrophomonas maltophilia*							S														
10			*Myroides odoratus*																					
13	MLT stops																							
20		Thumb (wound)	*Stenotrophomonas maltophilia*							S										I	S			
20			*Aeromonas* sp.[a]		R						S		S						S		S			
26		Thumb (wound)	*Stenotrophomonas maltophilia*							S										I	S			
26			*Aeromonas salmonicida*[b]	R	R	R	R	R	R	R	R	R	S (0.38)	S (0.5)	R (2)	R	S	R	R		R	S	S	S (0.25)
35		Thumb (wound)	*Stenotrophomonas maltophilia*							S										S	S			

Numbers in parentheses refer to MIC, expressed in mg/L.
Antibiotic susceptibility testing was not performed for *Myroides odoratus*. Antibiotic susceptibility testing was performed by disk diffusion method following recommendations of European Committee on Antimicrobial Susceptibility Testing (EUCAST) except for *Aeromonas* species; in this case, CLSI (M45-A2) rules applied. MICs were measured by Etest (bioMérieux, Marcy-l'Etoile, France) using CLSI breakpoints.
AMC, amoxicillin/clavulanic acid; AMX, amoxicillin; AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CLSI, Clinical and Laboratory Standards Institute; COL, colistin; CRO, ceftriaxone; CXM, cefuroxime; ETP, ertapenem; FEP, cefepime; FOS, fosfomycin; FOX, cefoxitin; GEN, gentamicin; IMP, imipenem; LVX, levofloxacin; MEM, meropenem; MIC, minimum inhibitory concentration; MLT, medicinal leech therapy; PIP, piperacillin; SXT, cotrimoxazole; TGC, tigecycline; TZP, piperacillin/tazobactam.
[a]This strain was identified by MALDI TOF MS as *Aeromonas* sp. with a score value of 2.348.
[b]This strain (ASG1) was identified by MALDI TOF MS as *Aeromonas salmonicida* with a score value 2.154.

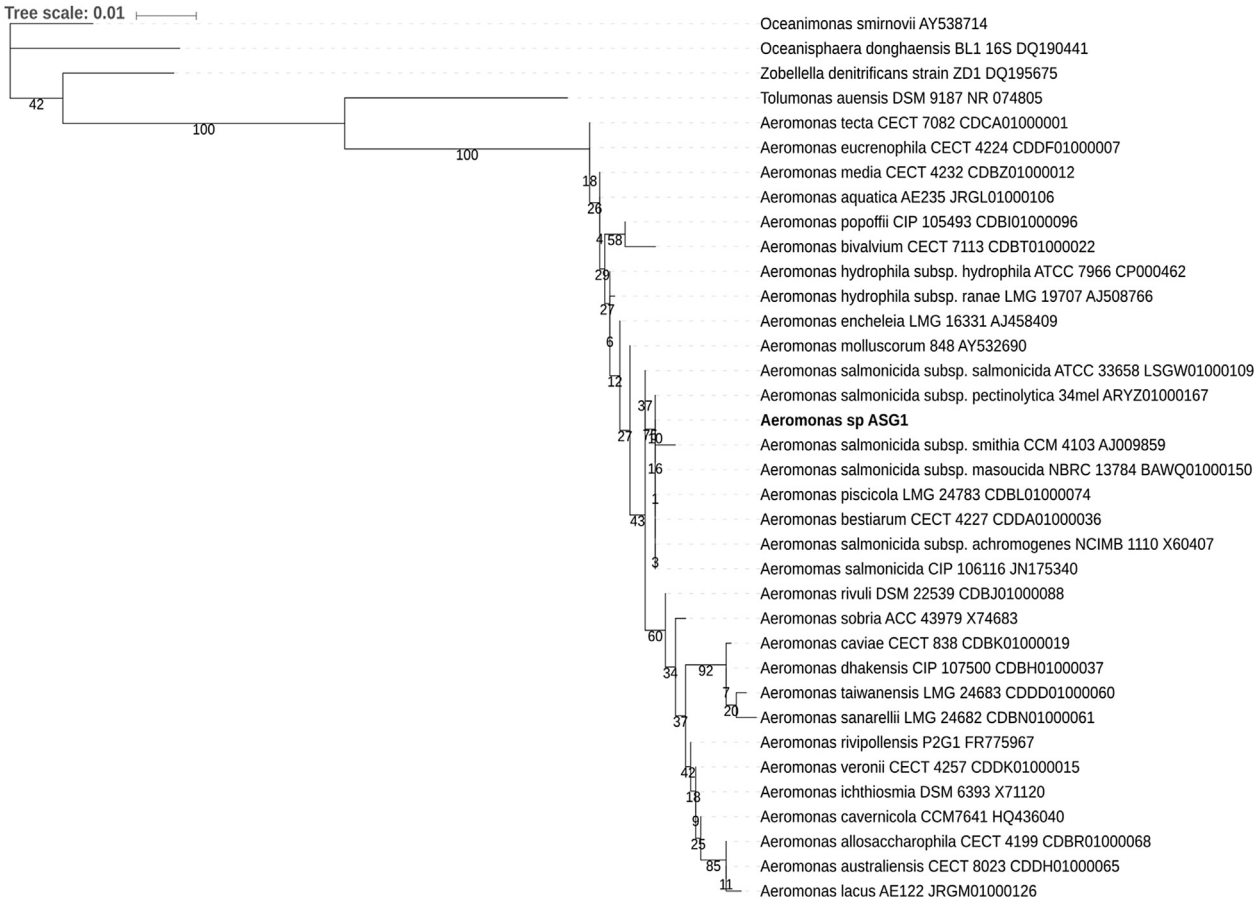

FIG. 2. Phylogenetic tree of 16S rRNA gene sequences encoding genes of *Aeromonas* species strain ASG1 and of *Tolumonas auensis* DSM_9187 (member of *Aeromonadaceae* family) to root tree. Tree was built using maximum likelihood (PhyML [4]) and iTOL online tool [5].

together, likely explain the resistance to piperacillin/tazobactam. Using the plasmid-SPAdes assembler [24], we obtained a 21 555 bp contig containing the bla_{SCO-1}, $bla_{CTX-M-3}$, bla_{TEM-1} and *aac(3)-II* (conferring resistance to gentamicin) genes, together with genes associated with DNA mobility (Supplementary Fig. S1), supporting the location of bla_{SCO-1} on a mobile genetic element. We also found the aminoglycoside-modifying enzymes APH(3′)-Ia (resistance to kanamycin) and Aad(A2) (resistance to streptomycin). Eventually the Sul and DfrA12 encoding genes (which confer resistance to cotrimoxazole, which the patient received for 3 days after the cessation of MLT), the Tet(E) encoding gene (resistance to tetracyclines) and the CatA2 encoding gene (resistance to chloramphenicol) were identified. The search for plasmid replicons using PlasmidFinder [10] returned negative for contigs obtained with both IDBA-UD and plasmid-SPAdes.

Discussion

Aeromonas spp. are natural colonizers of the digestive tract of leeches. Hence, since MLT has been used, the risk of infections due to *Aeromonas* sp. has been raised [11]. Accordingly, antibiotic prophylaxis, supplementation of the leech conservation medium with antibiotics or both has been proposed [12]. While infection with ciprofloxacin-resistant *Aeromonas hydrophila* during MLT has been reported [13], most *Aeromonas* strains appear to be susceptible to third-generation cephalosporins and piperacillin/tazobactam in this context [14]. In addition, several cases of infections due to ESBL (including CTX-M)-producing *Aeromonas* have been reported, but not in connection with MLT [15,16].

In our case, ASG1 was likely selected by the exposure to piperacillin/tazobactam and cotrimoxazole. The origin of ASG1 and of its acquired ARDs remains unknown because no other resistant strain was recovered from wound cultures. One possibility is that the plasmid could have been transferred from a transient bacterium from blood. Arguments against this hypothesis are that a rectal swab performed in the patient at day 38 was negative for any ESBL-producing *Enterobacteriaceae,* and the patient did not show any clinical sign of bacteraemia. Another possibility is that the water in which the leeches were bathed could have been contaminated either by ASG1 or another strain that could have transferred the SCO-1 plasmid

to ASG1. Nonetheless, other *Aeromonas* strains have been recovered from the leech conservation medium and from the wound, but neither ASG1 nor another *A. salmonicida* strain was identified. Possibly they may have been outnumbered by other bacteria. However, we are confident that the *Aeromonas* sp. strain isolated at day 20 and ASG1 was distinct, firstly because of their high MALDI-TOF MS scores (2.348 for *Aeromonas* sp., 2.154 for *A. salmonicida* strain ASG1), and secondly because of the different antibiotic resistance profiles, especially for fluoroquinolones (which is driven by the two chromosomal mutations found in the topoisomerases of ASG1, while the *Aeromonas* sp. strain was susceptible to fluoroquinolones).

Even more surprising is the presence of SCO-1, a carbenicillinase that has rarely been reported [17–20]. The production of SCO-1 confers resistance to penicillins and to their combination with clavulanate but modestly decreases the susceptibility to piperacillin/tazobactam [18,19]. Of note, the genetic neighbourhood of bla_{SCO-1} was found to be similar to that of previous reports [17–20], being inconsistent with a new mobilization of bla_{SCO-1}. The original host of SCO-1 remains unknown. Interestingly, as of this writing, SCO-1 is not included in the popular ResFinder [8] and CARD [21] databases, while it is in ARG-ANNOT [22] and MEGARes [23].

In conclusion, our observation suggests that antibiotic exposure can select for multidrug-resistant *Aeromonas* spp. during MLT, which warrants surveillance of the emergence of resistance during MLT.

Conflict of Interest

None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.nmni.2017.10.005.

References

[1] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30:2114–20.
[2] Peng Y, Leung HCM, Yiu SM, Chin FYL. IDBA-UD: a *de novo* assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics 2012;28:1420–8.
[3] Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 2016;32:929–31.
[4] Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 2010;59:307–21.
[5] Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res 2016;44:W242–5.
[6] Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014;30:2068–9.
[7] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
[8] Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 2012;67:2640–4.
[9] Iaconis JP, Sanders CC. Purification and characterization of inducible beta-lactamases in *Aeromonas* spp. Antimicrob Agents Chemother 1990;34:44–51.
[10] Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 2014;58:3895–903.
[11] Whitlock MR, O'Hare PM, Sanders R, Morrow NC. The medicinal leech and its use in plastic surgery: a possible cause for infection. Br J Plast Surg 1983;36:240–4.
[12] Mumcuoglu KY, Huberman L, Cohen R, Temper V, Adler A, Galun R, et al. Elimination of symbiotic *Aeromonas* spp. from the intestinal tract of the medicinal leech, *Hirudo medicinalis*, using ciprofloxacin feeding. Clin Microbiol Infect 2010;16:563–7.
[13] Giltner CL, Bobenchik AM, Uslan DZ, Deville JG, Humphries RM. Ciprofloxacin-resistant *Aeromonas hydrophila* cellulitis following leech therapy. J Clin Microbiol 2013;51:1324–6.
[14] Verriere B, Sabatier B, Carbonnelle E, Mainardi JL, Prognon P, Whitaker I, et al. Medicinal leech therapy and *Aeromonas* spp. infection. Eur J Clin Microbiol Infect Dis 2016;35:1001–6.
[15] Wu CJ, Chuang YC, Lee MF, Lee CC, Lee HC, Lee NY, et al. Bacteremia due to extended-spectrum-β-lactamase-producing *Aeromonas* spp. at a medical center in Southern Taiwan. Antimicrob Agents Chemother 2011;55:5813–8.
[16] Ye Y, Xu XH, Li JB. Emergence of CTX-M-3, TEM-1 and a new plasmid-mediated MOX-4 AmpC in a multiresistant *Aeromonas* caviae isolate from a patient with pneumonia. J Med Microbiol 2010;59:843–7.
[17] Jin W, Wachino JI, Kimura K, Yamada K, Arakawa Y. New plasmid-mediated aminoglycoside 6′-*N*-acetyltransferase, AAC(6′)-Ian, and ESBL, TLA-3, from a *Serratia marcescens* clinical isolate. J Antimicrob Chemother 2015;70:1331–7.
[18] Poirel L, Corvec S, Rapoport M, Mugnier P, Petroni A, Pasteran F, et al. Identification of the novel narrow-spectrum beta-lactamase SCO-1 in *Acinetobacter* spp. from Argentina. Antimicrob Agents Chemother 2007;51:2179–84.
[19] Papagiannitsis CC, Loli A, Tzouvelekis LS, Tzelepi E, Arlet G, Miriagou V. SCO-1, a novel plasmid-mediated class A beta-lactamase with carbenicillinase characteristics from *Escherichia coli*. Antimicrob Agents Chemother 2007; 2007;51:2185–8.
[20] Papagiannitsis CC, Tzouvelekis LS, Kotsakis SD, Tzelepi E, Miriagou V. Sequence of pR3521, an IncB plasmid from *Escherichia coli* encoding ACC-4, SCO-1, and TEM-1 beta-lactamases. Antimicrob Agents Chemother 2011;55:376–81.
[21] McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, et al. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother 2013;57:3348–57.
[22] Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, et al. ARG-ANNOT, a new bioinformatic tool to discover

antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother 2014;58:212–20.

[23] Lakin SM, Dean C, Noyes NR, Dettenwanger A, Ross AS, Doster E, et al. MEGARes: an antimicrobial resistance database for high throughput sequencing. Nucleic Acids Res 2017;45:D574–80.

[24] Antipov D, Hartwick N, Shen M, Raiko M, Lapidus A, Pevzner PA. plasmidSPAdes: assembling plasmids from whole genome sequencing data. Bioinformatics 2016;32:3380–7. https://doi.org/10.1093/bioinformatics/btw493.