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Identification and Clinical Significance of *Helcococcus* species, with Description of *Helcococcus seattlensis* sp. nov. from a Patient with Urosepsis

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***Helcococcus* spp. are Gram-positive, catalase-negative, facultatively anaerobic cocci that are associated with wound and prosthetic joint infections as well bacteremia and empyema. Five *Helcococcus* spp. strains were isolated from our patient population, including 2 strains of *Helcococcus kunzii* from trauma-associated wounds, 2 *Helcococcus sueciensis* strains from blood and abscess, and a novel *Helcococcus* spp. strain from blood associated with urosepsis. Based on the phenotypic and phylogenetic evidence, we propose that the unknown bacterium be classified as *Helcococcus seattlensis* sp. nov. We found that all 5 tested *Helcococcus* strains grew as satellite colonies around *Staphylococcus aureus* and, interestingly, both *H. kunzii* strains were isolated together with *S. aureus*. In addition to 16S rRNA gene sequencing, conventional methods for leucine aminopeptidase (LAP) and pyrrolidonyl arylamidase (PYR) testing can be cost-effective and efficient for differentiation of *Helcococcus* spp. from *Abiotrophia* and *Granulicatella* species. Using nonstandard methods, we found that all tested *Helcococcus* spp. had high MICs of >4/76 μg/ml for trimethoprim-sulfamethoxazole, an antibiotic commonly used to treat urinary tract infections. High MICs for erythromycin, azithromycin, and clindamycin, and intermediate to high MICs for moxifloxacin, levofloxacin, and gentamicin were also observed among the *Helcococcus* strains.**

Three species of *Helcococcus* have been described by Collins et al.: *Helcococcus kunzii*, *Helcococcus sueciensis*, and *Helcococcus* ovis (1–3). *H. kunzii* has been isolated from blood, a prosthetic joint, wounds, abscesses, plantar phlegmon, and an implantable cardiac device (1, 4–9) and is thought to be a nonpathogenic skin colonizer (10). *H. sueciensis* has been isolated from a human wound (2), while *H.* ovis causes mastitis and urocystitis in animals (3). The susceptibility of *H. kunzii* to a few antibiotics has been described (11), but evaluation of a comprehensive antibiotics panel for *Helcococcus* spp. has been lacking. The identification of *Helcococcus* spp. remains challenging due to the slow growth of the organism and the inadequate databases for commercially available kits, such as API Rapid ID 32 Strep, API Rapid ID 32A, and API 20S Strep (bioMérieux) (1, 2, 8). The MicroSeq Full Gene database (v.0001a) and 500 database (v.0023b; Applied Biosystems) also lack any *Helcococcus* spp. Vitek 2 (bioMérieux) can only identify *H. kunzii* and no other *Helcococcus* spp. (8, 12). In the current study, we identified these isolates by 16S rRNA gene sequencing and aimed to develop a more efficient and affordable protocol to identify these bacteria of potential clinical significance. We also characterized a novel species of the genus *Helcococcus*, and we propose its classification as *Helcococcus seattlensis* sp. nov.

Case report of the novel *Helcococcus* sp. An 80-year-old male with a history of type 2 diabetes, obesity, coronary disease with bypass surgery, Reiter's disease, and hypertension presented with a fever of 101.6°F. Elevated cardiac enzymes (troponin at 0.34 μg/liter and creatine kinase MB isoenzyme at 7.5 μg/liter) with a brain natriuretic peptide level of >30,000 indicated heart failure. In addition, the patient experienced painful urination, and the urinalysis results suggested a urinary tract infection (UTI): leukocyte esterase was positive (3+), nitrite was negative, white blood cell (WBC) and red blood cell counts were high (>182/high-power field [HPF] and 40/HPF, respectively). Many WBC clumps were seen in the urine. His blood test revealed an elevated WBC level (15.3×10^9/liter), the hemoglobin level was slightly decreased (12.7 g/dl), and the platelet count was normal (270×10^9/liter). Blood and urine cultures were ordered. With the BacT/Alert 3D blood culture system (bioMérieux), the anaerobic but not the aerobic bottle turned positive on day 4. Gram staining showed Gram-positive cocci in clusters with variable cell sizes (0.5 to 1.8 μm in diameter). On subculture, the organism showed negligible growth on Columbia blood agar (BA) and slightly better growth on chocolate agar (CA). The colonies were nonhemolytic, gray, and pinpoint after 48 h of incubation at 35°C in air enriched with 6 to 7% CO_2. The results of 16S rRNA gene sequence analysis were compared to those for 4 closely related clinical strains that were previously recovered in our laboratory, as well as isolates from the GenBank database. The urine specimen collected at the same time as the blood was cultured on BA, CA, and MacConkey agar. Pinpoint colonies were observed on CA only, but they were not further identified because of a failure to appreciate the organism as a possible pathogen. However, Gram staining of the colonies on CA revealed Gram-positive cocci with the same morphology and sizes as the organism isolated from the blood culture. The fine lawn of colonies on CA resembled what was seen on the subculture of the blood isolate. The patient died the next day due to heart failure.

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TABLE 1 Summary of helcococcal infections at the Veterans Administration Puget Sound Health Care System

Case no.	Organism	Strain	Organism(s) co-recovered	Site of infection	Age (yrs)/ gender of patient	Underlying disease(s)	Treatment for current episode	History	WBC result based on:		
									Gram stain of specimen	Blood test (cells/liter)
1	*H. seattlensis* sp. nov.[a]	F5780	NA[b]	Blood	80/M	CAD,[c] type 2 diabetes	NA	NA	NA	15.3×10^9
2	*H. kunzii*	F7676	*S. aureus*	Toe abscess	25/M	PTSD[d]	SXT, cephalexin	NA	Negative	NA
3	*H. kunzii*	F5450	*S. aureus*	Inner thigh wound from trauma	68/M	CAD, colonic polyps	Vancomycin and piperacillin-tazobactam inpatient; cephalexin outpatient	Previous episode of wound infection at same site within past mo., treated with SXT without improvement	Negative	6.3×10^9
4	*H. sueciensis*	F6341	NA	Blood	64/M	Colon carcinoma, CAD	Vancomycin, piperacillin-tazobactam	NA	NA	8.7×10^9
5	*H. sueciensis*	F6503	*Corynebacterium* spp., two colony types	Surgical axillary wound	67/M	Axillary squamous cell carcinoma	Vancomycin, piperacillin-tazobactam inpatient; clindamycin outpatient	NA	Moderate	14×10^9

[a] The name *H. seattlensis* is proposed in the current study.
[b] NA, not available.
[c] CAD, coronary artery disease.
[d] PTSD, post-traumatic stress disorder.

MATERIALS AND METHODS

Bacterial strains. All 5 *Helcococcus* spp. strains were isolated during routine culture procedures at the VA Puget Sound Healthcare System. All isolates were stocked at −70°C using an alphanumeric system beginning with F for identification. Unless specified, all 5 clinical isolates, *H. kunzii* F5450, *H. kunzii* F7676, *H. sueciensis* F6341, *H. sueciensis* F6503, and the novel *Helcococcus* sp. strain F5780 were grown on CA (Remel, Lenexa, KS) at 35°C in air enriched with 6 to 7% CO_2.

Growth characteristics. The satellite test of all 5 strains was performed on BA (Remel, Lenexa, KS) with a streak of *Staphylococcus aureus* ATCC 25923 at 35°C in air enriched with 6 to 7% CO_2 and in ambient air. In addition, novel *Helcococcus* strain F5780, *H. kunzii* F5450, and *H. sueciensis* F6503 were tested for satellite colony formation on Mueller-Hinton agar (MHA) at 35°C in 5% CO_2–95% air. *H. seattlensis* F5780 was grown on CA at 3 temperatures under 4 atmospheric conditions: 30°C, 35°C, 42°C in ambient air, air enriched with 6 to 7% CO_2, a microaerophilic atmosphere generated by a Pouch-MicroAero apparatus (Mitsubishi Gas Chemical America, Inc., New York), and anaerobic conditions generated by a Pack-Anaero instrument (Mitsubishi Gas Chemical America, Inc., New York).

Antibiotic susceptibility testing. The antibiotic susceptibility testing of all 5 *Helcococcus* isolates was performed according to the manufacturer's instructions by using the Sensititre system with the Streptococcus panel (STP6F; Trek Diagnostic Systems, Cleveland, OH), except that the results were read on day 3 due to the slow growth of the organisms. Strains for which the MICs were greater than the highest dilution on the Sensititre panel were further tested with the Etest to determine precise MICs, except for trimethoprim-sulfamethoxazole (SXT). All Etest experiments were performed on CA with incubation at 35°C in air enriched with 6 to 7% CO_2 for 2 days. Based on the facts that thymidine in sheep blood agar alters the action of sulfur drugs (13) and that *Helcococcus* strains showed negligible to no growth on MHA, an Etest of SXT could not be performed. The microbial resistance to SXT was subsequently confirmed by using the MicroScan system with a MICroSTREP plus panel (Siemens Healthcare Diagnostics, Deerfield, IL). Currently, no antimicrobial testing guideline from the Clinical and Laboratory Standards Institute (CLSI) is available for *Helcococcus*. The level of the MIC (high, intermediate, or low) determined in this study was in reference to that of *S. aureus* in CLSI guidelines (14).

Biochemical differentiation. Biochemical tests were performed with the API 20S Strep (bioMérieux) and Vitek 2 (bioMérieux) systems with 2-day-old *Helcococcus* cultures grown on CA. Leucine aminopeptidase (LAP) and pyrrolidonyl arylamidase (PYR) results were confirmed by using conventional LAP and PYR disks (Remel, Lenexa, KS).

16S rRNA gene sequencing. Strains were grown on CA for 48 h, and the DNA was extracted by using a Prepman apparatus (PE Applied Biosystems, Foster City, CA) following the manufacturer's instructions. 16S rRNA PCR and cycle sequencing of *Helcococcus* spp. were performed as previously described (15), with modifications. A 500-bp 16S rRNA gene sequence analysis was performed for *H. kunzii* F5450, *H. kunzii* F7676, *H. sueciensis* F6341, and *H. sueciensis* F6503, while 1,500-bp 16S 16S rRNA gene sequence analysis was performed for strain F5780. Three sets of primers (VB1 to VB6) were used to amplify the 1,500-bp 16S rRNA gene, and the first set (VB1 and VB2) was used for the 500-bp 16S rRNA sequencing. The primers were as follows: VB1 (TGGAGAGTTTGATCCTG GCTCAG), VB2 (TACCGCGGCTGCTGGCAC), VB3 (CCAGCAGCCG CGGTAATAC), VB4 (CGGGACTTAACCCAACATCTCAC), VB5 (GTG AGATGTTGGGTTAAGTCCCG), and VB6 (AAGGAGGTGATCCAGCC GCA) (15, 16). The sequences were analyzed and compared to information in the BIBI and GenBank databases (17). The phylogenetic tree was constructed with MUSCLE, a program for generating multiple alignments (18), with MEGA5.1 software (http://www.megasoftware.net/).

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA genes of the novel strain F5780, *H. kunzii* F7676, *H. kunzii* F5450, *H. sueciensis* F6341, and *H. sueciensis* F6503 have been deposited

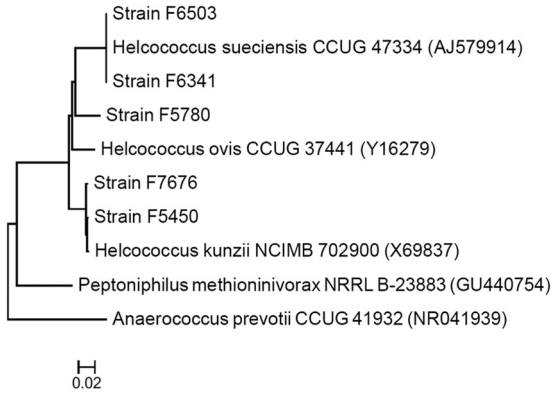

FIG 1 Unrooted tree showing the phylogenetic relationships of 5 *Helcococcus* spp. clinical strains to closely related species. The tree was inferred from 16S rRNA gene sequences (443 nucleotides) by using the neighbor-joining method, and the scale bar indicates the substitution rate per nucleotide. All names and accession numbers (in parentheses) are those cited in the GenBank database.

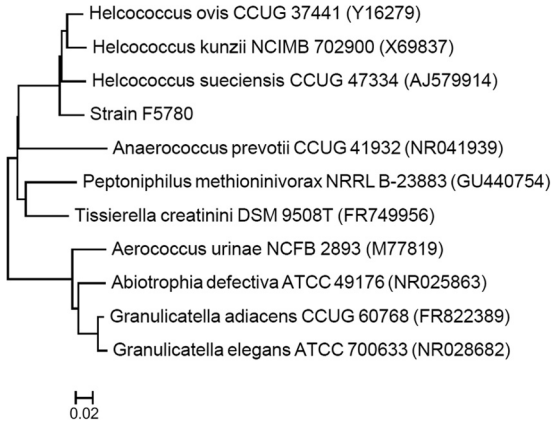

FIG 2 Unrooted tree showing the phylogenetic relationships of strain F5780 to closely related species and to more distant species that share similar morphological features and growth characteristics. The tree was inferred from 16S rRNA gene sequences (1,391 nucleotides) by using the neighbor-joining method, and the scale bar indicates the substitution rate per nucleotide. All names and accession numbers (in parentheses) are those cited in the GenBank database.

in the GenBank Data Library under accession numbers KF780874, KF780875, KF780876, KF780877, and KF780878, respectively.

RESULTS

The sites of isolation for all *Helcococcus* strains, patient demographics, and treatments are listed in Table 1. The phylogenetic and phenotypic characterizations of *H. kunzii* and *H. sueciensis* reported in previous studies (1, 2) matched our strains (Fig. 1; Table 2). The cell morphology of the novel strain F5780 is Gram-positive cocci in clusters of cells of variable sizes (0.5 to 1.8 μm in diameter). The organism grows best on CA (CA > BA) at 35°C (35°C > 30°C > 42°C) and equally well under different oxygen conditions (air, air enriched with 6 to 7% CO, microaerophilic, or anaerobic). The colonies are nonhemolytic, gray, and pinpoint on CA after 48 h of incubation at 35°C in air enriched with 6 to 7% CO_2. The organism is catalase, PYR, LAP, and motility negative when tested using conventional methods. The API 20S Strep test failed to identify the organism, but the results of negative PYR and LAP concurred with those demonstrated with Remel disks. The organism utilizes ribose to produce acid.

The comparison of sequences to the database shown in Fig. 1 and 2 revealed that strain F5780 is most closely related to species of the genus *Helcococcus*. The species with the highest percentages of similarity in sequence to this organism were *H. sueciensis* (94%),

TABLE 2 Important differences in phenotypic characteristics of *Helcococcus*, *Abiotrophia*, and *Granulicatella* isolates

Characteristic	*H. seattlensis* sp. nov. ($n = 1$)[a]	*H. kunzii*		*H. sueciensis*		*Abiotrophia defectiva*[d]	*Granulicatella elegans*[d]	*Granulicatella adiacens*[d]
		This study ($n = 2$)	Reported elsewhere[b]	This study ($n = 2$)	Reported elsewhere[c]			
Catalase	−	0/2	−	0/2	−	−	−	−
Leucine aminopeptidase	−	0/2	−	2/2	NA[e]	+	+	+
Pyrrolidonyl arylamidase	−	2/2	+	0/2	−	+	+	+
Growth at 42°C	+	1/2	v[f]	0/2	NA	+	−	+
Acid produced from:								
Trehalose	−	2/2	+	2/2	+	+	−	−
Lactose	−	2/2	+	2/2	+	+	−	−
Glycogen	−	2/2	v	1/2	−	−	−	−
Ribose	+	2/2	−	2/2	−	NA	NA	NA
Insulin	−	0/2	−	0/2	−	−	−	+
Starch	−	2/2	v	2/2	+	+	−	−
Raffinose	−	0/2	−	0/2	−	+	+	−
Production of:								
β-Glucuronidase	+	0/2	−	0/2	−	−	−	+
β-Galactosidase	+	2/2	−	2/2	+	+	−	−

[a] *n* is the number of isolates examined in the current study.
[b] See reference 1.
[c] See reference 2.
[d] See reference 20.
[e] NA, not available.
[f] v, variable.

TABLE 3 Antibiotic susceptibilities of *Helcococcus* species[a]

Antibiotic	MIC (μg/ml)				
	H. seattlensis sp. nov. F5780	*H. kunzii* F7676	*H. kunzii* F5450	*H. sueciensis* F6341	*H. sueciensis* F6503
Penicillin	0.06	0.25	<0.03	<0.03	0.06
Cefuroxime	<0.5	<0.5	<0.5	<0.5	<0.5
Ceftriaxone	<0.12	0.25	<0.12	<0.12	<0.12
Cefotaxime	<0.12	<0.12	<0.12	<0.12	<0.12
Cefepime	<0.5	<0.5	<0.5	<0.5	<0.5
Amoxicillin-clavulanic acid	<2/1	<2/1	<2/1	<2/1	<2/1
Gentamicin[b]	1	16	8	8	8
Vancomycin	1	<0.5	<0.5	4	1
Linezolid	2	4	2	2	2
Azithromycin[c]	<0.25	1	>2	<0.25	>2
Erythromycin	0.5	1	>256	<0.25	>256
Tetracycline	<1	<1	<1	<1	<1
Tigecycline	<0.015	0.03	<0.015	<0.015	0.03
Daptomycin	<0.06	0.5	0.5	0.12	0.12
Clindamycin	0.25	0.5	0.5	<0.12	>256
Moxifloxacin	4	<1	2	<1	8
Levofloxacin	16	1	1	<0.5	8
Trimethoprim-sulfamethoxazole	>4/76	>4/76	>4/76	>4/76	>4/76
Meropenem	0.5	<0.25	<0.25	<0.25	<0.25
Ertapenem	<0.5	<0.5	<0.5	<0.5	<0.5
Chloramphenicol	8	16	8	4	4

[a] An antibiotic susceptibility testing method for *Helcococcus* spp. has not been standardized by CLSI.
[b] The susceptibility to gentamicin was measured by Etest.
[c] Etest results for azithromycin were not available, and susceptibility was based solely on the result with the Sensititre system.

H. ovis (94%), and *H. kunzii* (92%). Species of other related genera, such as *Tissierella* and *Peptoniphilus*, were related more distantly (≤87%). Based on the phylogenetic (Fig. 1 and 2) and phenotypic (Table 2) evidence, we suggest that strain F5780 is a new species of the genus *Helcococcus*, for which the name *Helcococcus seattlensis* sp. nov. is proposed.

We performed a satellite test for all 5 clinical strains of *Helcococcus* on BA, and after 1 to 3 days of incubation at 35°C in air enriched with 6 to 7% CO_2, we found that the colonies adjacent to *S. aureus* ATCC 25923 showed better growth. For *Helcococcus seattlensis* sp. nov. F5780, the mean colony size adjacent to and distant from *S. aureus* was 197 ± 81 μm and 81 ± 26 μm, respectively (means ± standard deviations; $P < 0.001$ by *t* test; $n = 50$). The satelliting phenomenon of *H. kunzii* F5450, *H. sueciensis* F6503, and *H. seattlensis* F5780 was further tested on MHA. The results revealed a more distinct satellite pattern, such that *Helcococcus* strains grew only as satellite colonies proximal to the streaking of *S. aureus* on MHA but not away from it. Strains F5450, F6503, and F5780 showed positive colony satellite formation on days 2, 4, and 6, respectively. With regard to the stimulating effect of *S. aureus*, it is interesting that our 2 *H. kunzii* strains (F7676 and F5450) were isolated from wounds together with *S. aureus*. Also, the veterinary *H. ovis* was isolated with a *Staphylococcus* sp. (3). These observations suggested that the presence of *Staphylococcus* spp. may enhance the growth of *Helcococcus* spp.

Helcococcus spp. and other Gram-positive, slow-growing genera that have specific nutrient requirements, such as *Abiotrophia* and *Granulicatella*, can be difficult to identify (19–21). The positive satellite test as well as the rapid and inexpensive LAP and PYR tests can help distinguish the organisms (Table 2). Only a small amount of bacterial inoculum is needed to perform the tests, thus favoring the identification of slow growers.

The antibiotic susceptibilities of *Helcococcus* species were studied by employing the Sensititre, Etest, and the MicroScan systems (Table 3). The MICs of *Helcococcus* species were interpreted based on the standard for *S. aureus* in the guidelines. All the tested *Helcococcus* species revealed a high MIC for SXT (>4/76 μg/ml), an indication of drug resistance. Intermediate to high MICs for gentamicin were observed for all tested *H. kunzii* and *H. sueciensis* isolates, but not with *H. seattlensis*. High MICs for erythromycin, azithromycin, clindamycin, and intermediate to high MICs for moxifloxacin and levofloxacin were also observed among the *Helcococcus* spp. strains. There are no CLSI guidelines for performing and interpreting antibiotic susceptibility tests for *Helcococcus*. The microdilution and Etest results were read on day 3, because the organisms grew slowly.

DISCUSSION

Strain F5780 resembled the species of the genus *Helcococcus* in cell morphology, microscopic appearance, growth rate (slow), and negative catalase reaction. 16S rRNA gene sequencing revealed that this organism is most closely related to *H. sueciensis* and *H. ovis*. The lack of PYR and LAP of F5780 was distinct from other medically significant bacteria that are similar based on Gram stain, catalase reaction and growth characteristics, such as other *Helcococcus* species, *Abiotrophia*, *Granulicatella*, and *Streptococcus*. Another important trait for all *Helcococcus* species was their high MIC for SXT, to which *Abiotrophia* and *Granulicatella* are also susceptible (19).

The facts that strain F5780 in our current study was recovered from a patient with urosepsis and that *H. kunzii* caused urocystitis in a sow suggest that *Helcococcus* species can cause UTI (12). Our finding that all tested *Helcococcus* species had high MICs (>4/76 μg/ml) for SXT is of medical importance. SXT is currently recommended by the Infectious Diseases Society of America (IDSA) to treat cystitis,

pyelonephritis, and catheter-associated UTI (22, 23), while our data showed that SXT may not be effective against *Helcococcus* spp.

H. kunzii was isolated together with *S. aureus* from wounds (Table 1, cases 2 and 3) (1, 6). We speculate that helcococci may occur more commonly in clinical specimens but are not detected unless *S. aureus* is present to enhance the growth or unless particular culture conditions are used. Lacking a good database and a clear algorithm, *Helcococcus* might be neglected due to its slow growth and the difficulty in its identification, resulting in an inappropriate choice of antibiotics for the patient.

Description of *Helcococcus seattlensis* sp. nov. *Helcococcus seattlensis*. se.at.tlen'sis. N.L. masc. adj. *seattlensis* of Seattle, the city in which the organism was isolated. Cells are Gram-positive cocci and occur singly or in clusters with variable cell sizes (0.5 to 1.8 μm in diameter). Cells are nonmotile and non-spore forming. The organism grows best on CA (CA > BA) at 35°C (35°C > 30°C > 42°C) and equally well under different oxygen conditions (air, air enriched with 6 to 7% CO_2, microaerophilic, or anaerobic). The colonies are nonhemolytic, gray, and pinpoint on CA after 48 h of incubation at 35°C in air enriched with 6 to 7% CO_2. It grows as satellite colonies adjacent to both clinical and ATCC 25923 strains of *S. aureus*, while the satellite phenomenon is more remarkable on Mueller-Hinton agar than on sheep blood agar. On blood agar, the mean colony sizes adjacent to and distant from *S. aureus* are 197 ± 81 μm and 81 ± 26 μm, respectively. On Mueller-Hinton agar, the organism only grows as satellite colonies proximal to the streaking of *S. aureus*. It takes 3 days and 6 days to grow as satellite colonies on blood agar and Mueller-Hinton agar, respectively. The organism is catalase, PYR, and LAP negative when tested by conventional methods. It produces β-glucuronidase and β-galactosidase. Acid is produced from ribose. The organism is resistant to trimethoprim-sulfamethoxazole.

The type strain of *H. seattlensis* sp. nov. is strain F5780, which was isolated as the sole organism from blood of a patient with urosepsis. Its 16S rRNA gene sequence has been deposited in the GenBank data library under accession number KF780874. The isolate has been accepted for deposition in the ATCC (ATCC number to be determined).

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