Isolation of a novel *Streptococcus anginosus* subspecies, AW1 from wound infection in Al-Madinah Al-Munawarah province

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ABSTRACT

Objective: In this study, we report the isolation of a new bacterial strain from post-caesarean surgical site wound infection.

Methods: The strain, named AW1, was identified as a streptococcal species based on morphological and biochemical features. Interestingly, this species was not grouped using the Lancefield Grouping Assay. 16S rRNA gene sequence analysis was done.

Results: The bacterium was a facultative anaerobic, non-haemolytic, non-motile, non-spore-forming, Gram-positive coccus, arranged in chains. It was negative for catalase and oxidase activities. 16S rRNA gene sequence analysis indicated that there was 99 % concordance between the 16S rRNA of AW1 and that of the most closely related species such as *Streptococcus anginosus* F0211 and another species of streptococci in the neighbour-joining tree, *Streptococcus species* CM36.

Conclusions: Based on these interesting findings, we propose a new and novel subspecies, *Streptococcus anginosus* AW1.

KEY WORDS: *Streptococcus anginosus*, 16S rRNA, phylogenetic analysis, novel bacterial strain

Introduction

Streptococcus species cause an array of infections ranging from wound infection to abscess formation. Streptococci are classified according to their phenotypic, haemolytic characters and serotyping which rely on specific carbohydrates present on the bacterial cell wall [1]. *Streptococcus milleri* is a subgroup of non-hemolytic viridans streptococci, which includes three different streptococcal species: *Streptococcus intermedius*, *Streptococcus anginosus*, and *Streptococcus constellatus* [2]. Viridans streptococci in particular, *S. anginosus* is usually found as normal flora in the human oral cavity, skin and gastrointestinal tract but may cause abscesses and systemic infections in certain cases such as in patients with depressed immunity [3]. The unique feature of the *S. anginosus* group that makes it different from other groups of streptococci like Streptococcus pyogenes (also known as Streptococcus group A) and *Streptococcus agalactiae* (also known as Streptococcus group B) is its ability to cause abscesses in infected humans [4].

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Members of the *S. anginosus* group are part of the normal flora of the gastrointestinal tract in both adults and children; however, in certain cases, they may cause severe abdominal infections. These include liver abscess, cholangitis, peritonitis, appendicitis, subphrenic abscess, pelvic abscess, abdominal wound infections and postoperative infections following visceral trauma or surgery [5]. Study of bacterial phylogeny and taxonomy using 16S rRNA (ribosomal RNA) gene sequences has been widely used due to its presence in almost all the bacteria, the stability of its function over time and its large size that is suitable for informatics purposes [6,7]. Also, 16S rRNA gene sequencing is considered to be one of the most common tools that have been applied for classification of the genus Streptococcus [8]. Streptococcus has been grouped into six different groups, *S. anginosus, S. salivarius, S. bovis, S. mutans, S. pyogenes*

and *S. mitis*, based on their 16S rRNA gene sequences [9]. A subspecies are considered when minor phenotypic or genetic variations in species or their internal clusters of strains. It is genetically close to the species but phenotypically divergent [10]. However, it is sometimes not easy to differentiate to species level between isolates. Recently, other DNA targets have been proposed for subspecies identification such as the rpoB gene (a fragment of the gene encoding the-beta subunit of RNA polymerase) and sequencing of sodA gene (a fragment of the manganese dependent superoxide dismutase) [11,12]. Nonetheless, 16S rRNA gene sequence analysis has emerged as a preferred genetic technique for species identification of bacterial isolates [13,14]. This study reports the isolation of a novel bacterial-strain-from post caesarian section wound infection. The strain, named AW1, exhibited phenotypic features that do not completely match with the patterns of known *S. anginosus* subspecies. The 16S rRNA gene of the isolated AW1 strain was 99% concordant with the most closely related subspecies, *S. angi*nosus based on 16S rRNA gene sequencing. Based on this finding and subsequent studies on the novel gene, a new subspecies of S. *anginosus* named as AW1 is proposed.

Materials and Methods

Blood agar base was procured from Oxoid, Basingstoke, United Kingdom. API strips (20 STREP) were supplied with database 4.0 from bioMe´rieux, Marcy l'Etoile, France. Wizard Genomic DNA Purification Kit was from Promega, USA, the primers were from HT Biotechnology, Cambridge, United Kingdom while the QIAquick PCR purification kit was obtained Qiagen, Hilden, Germany. All the other chemicals were from Sigma, St. Louis, Mo. unless otherwise stated.

Patients and ethical statement:

A prospective, cohort study was conducted from December, 2011 to December, 2013; at Madinah Maternity and Children Hospital (MMCH), Obstetrics and Gynecology department, Madinah, Saudi Arabia, where 276 infected wound samples were collected aseptically from patients. Demographic and clinical data for this prospective study were published in our previous study The study protocol was approved by the Studies and Research Committee, MMCH, General Directorate of Health, Madinah, Saudi Arabia [15]. The subjects gave informed consent to the work. The research has complied with all relevant international guidelines and institutional policies.

Bacterial isolates identification and antibiotic susceptibility assays

The bacterial species isolated in this study were identified using standard microbiological methods such as their growth pattern in culture media, Gram staining for study of their morphological characteristics under microscope, and motility test. Different biochemical reactions for the isolated bacteria were also reported using the API system (20 STREP) and an automated VITEK 2 SYSTEM (bioMerieux Vitek) [16.17]. Antibiotics susceptibility testing for bacteria grown overnight on culture media at suitable temperature was carried out using standard Kirby Bauer disk diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) criteria to detect antimicrobial resistance. During this study, an unidentified species of streptococci was isolated from patient wound swab using the automated identification methods (automated vitek 2 showed low level of discrimination). Phylogenetic analysis based on 16S rRNA gene sequence was then performed to identify this unidentified microorganism [14].

PCR amplification of 16S rRNA genes

Bacterial DNA was extracted from overnight grown culture on LB medium using Wizard Genomic DNA Purification Kit based on manufacturer's instructions. Purified DNA was used as template for PCR reaction using specific primers to amplify full length 16S rRNA gene .[18] Forward and reverse primers, used in this study were, 16S-1F (5'-AGAGTTT GATCCTGGCTCAG-3') and 16S-1500R (5'-ACGGCTACCTTGTTACGACT-3'), respectively [19].The PCR mixture for 25-µl reaction contained: 10x buffer (10µl), Mg^{2+} (2 µl), template DNA (1 µl), dNTPs (2 µl, 200 µM), primer F (2 µl, 10 µM), primer R (2 µl, 10 µM), *Taq* DNA polymerase (0.5 µl, 1 U), and H_2O (5.5 µl). PCR was performed in Thermal Cycler (Applied Biosystem 2720, USA). The PCR conditions were optimized as follows: denaturation at 95°C for 5 min, 30 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and finally 10 min at 72°C.

Sequencing analysis of 16S rRNA genes

The nucleotide sequence analysis was performed by automated florescent dye terminator sequencing method [20]. Unidirectional dye terminator-based sequencing was done with 16S 1F-PCR-amplified segments covering V3 region of 16S rRNA gene. 16S rRNA gene sequence was analyzed using Blast search facility on NCBI database (http//:www.ncbi.nlm.nih.gov) .[21] Sequence alignments were done using Clustal W1.83 XP software and phylogenetic trees were constructed using neighbor-joining method using MEGA3 software [22].

Phylogenetic characterization

The phylogenetic relationship between strain AW1 and reference strains from GenBank database was determined using the PileUp method with GrowTree (Genetics Computer Group, Inc.).

Nucleotide sequence accession number

The 16S rRNA gene sequence of AW1 has been deposited in GenBank database under accession number LC034920.

Ethical consideration

The study protocol was approved by the studies and research committee, Maternity and Children Hospital, General Directorate of Health, Madinah, Saudi Arabia.

Statistical Analysis

Statistical Package for Social Sciences SPSS version 13 was used in this study. Frequencies, percentages were calculated. Chi-square test was used. *P* value <0.05 was considered significant.

Results

Identification of bacterial isolates and antibiotic susceptibility test

Demographic and clinical data for this prospective study were published previously in a study done by members in our group [15]. In brief, out of the 276 cases with surgical site wound infection (SSI) during 2012 - 2013, 189 (69%) of culture yielded no growth. Enterococcus species were isolated from 27 (10.5%) of cases. In 13 (4.5%) Pseudomonas species grew, while 12 (4%) produced E. coli and *Klebsiella species* and 2% each for coagulase-negative Staphylococcus, *Staphylococcus aureus* and MRSA. *Streptococcus species* were the least (1.8%) among isolates from SSI (Table 1).

Table 1. Isolated organisms from wound swabs.

Type of the organism	Number	Percentage (%)
Staphylococcus aureus	6	2
Methicillin-resistant *S. aureus* (MRSA)	6	2
Escherichia coli	12	4
Klebsiella species	12	4
Pseudomonas areoginosa	13	4.5
Enterococci	27	10.5
Coagulase negative staphylococci	6	2
Streptococcus species	5	1.8
No growth	189	69
Total	276	100%

Regarding antibiotic sensitivity pattern, out of 87 isolated organisms, 14 (16%) showed resistance to ampicillin, 10 (11.4%) were resistant to Trimethoprim/Sulphamethoxazole, 4 (4.5%) resistant to cephalothin, and 2 (2.2%) each for (aztereonam- cefoxotin – ceftazidim – ciprofloxacin - clindamycin). Erythromycin and piperacillin showed high sensitivity rates (only one resistant isolate for each). Furthermore, all isolated bacterial species were 100% sensitive to both gentamicin and vancomycin.

Isolation of *S. anginosus*

A 31-years-old woman was admitted to the Obstetrics and Gynecology department, Madinah, in 2013 to give birth by Cesarean section (CS). Seven days after caesarean section, surgical site infection was noted. Organism isolated from the wound was identified as Streptococcus according to the conventional bacteriological procedures including phenotypic characteristics on culture, negative catalase test and API 20 Strep strips while, automated vitek 2 system identified the organism as *Streptococcus gordonii* with low level of discrimination between *S. gordonii* and *S. anginosus*.

Table 2. Biochemical profile of strain AW1 by Vitek 2 system and API 20 STREP.

Vitek 2 system		API 20 STREP	
Catalase	-	Hippurate hydrolysis	-
Oxidase	-	Voges	+
Optochine resistance	+	Starch	-
Polymyxin B resistance	+	Glycogen	-
Bacitracin resistance	+	β-galactosidase	-
Novobiocin resistance	+	β-glucuronidase	-
D-Amygdalin	+	α-galactosidase	-
Phosphatidylinositol phos-	+	l-leucine arylamidase	-
Arginine dihydrolase	+		
Alanine	-		
Phenylalanine	-		
Proline arylamidase	+		
β-galactosidase	+		
Phosphatase	+		
l-leucine arylamidase	+		
Alanine arylamidase	+		
Tyrosine arylamidase	+		
Methyl-β-d-glucopyra-	+		
D-galactose, *N*-acetyl-glu-	+		
α-glucosidase	-		
l-aspartic acid arylamidase	-	21	
β-galactosidase	-	-22	
α-mannosidase	-	23	
Proline arylamidase	-	24	
β-glucuronidase	-	-25	
α-galactosidase	-	-26	
l-pyroglutamic acid arylami-	-	27	
Urease	-	28	
Maltose	+	29	
Mannose	+	30	
Salicin	+	31	
Sucrose	+	32	
Trehalose	+	33	
Xylose	-	34	
α-cyclodextrin	-	35	
Sorbitol	-	36	
Ribose	-	37	
Lactate	-	38	
Lactose	-	38	
Mannitol	-	40	
Pullulan	-	41	
Raffinose	-	41	
Hippurate hydrolysis	-	43	
Voges-Proskauer test	+	44	
Starch	-	45	
Glycogen	-	46	

This strain AW1 was susceptible to penicillin, ampicillin, erythromycin, and tetracycline. Novel subspecies of *Streptococcus anginosus: Streptococcus anginosus* AW1. The organism was a facultative anaerobe, non-motile, non-spore-forming, Gram-positive coccus arranged in chains or pairs. Optimum growth temperature was 37 °C. Colonies on sheep-blood agar after 48 h at 37 °C in CO2-enriched atmosphere were 1-2 um in diameter, circular, white to greyish, shiny, convex and non-hemolytic. They were negative for catalase and oxidase activities and resistant to optochine, polymyxin B, bacitracin and novobiocin. Enzymatic activities for D-Amygdalin, phosphatidylinositol phospholipase C, arginine dihydrolase, alanine-phenylalanine-proline arylamidase, β-galactosidase, phosphatase, l-leucine arylamidase, alanine arylamidase, tyrosine arylamidase, methyl-β-D-glucopyranoside, D-galactose, N-acetyl-glucosamine were detected. No activity was observed for α-glucosidase, l-aspartic acid arylamidase, β-galactosidase, α-mannosidase, proline arylamidase, β-glucuronidase, α-galactosidase, l-pyroglutamic acid arylamidase and urease. The bacteria also fermented maltose, mannose, salicin, sucrose, and trehalose, while, xylose, α-cyclodextrin, sorbitol, ribose, lactate, lactose, mannitol, pullulan, raffinose were non-fermentable (Table 2).

Sequence-based identification and phylogenetic affiliation of strain AW1

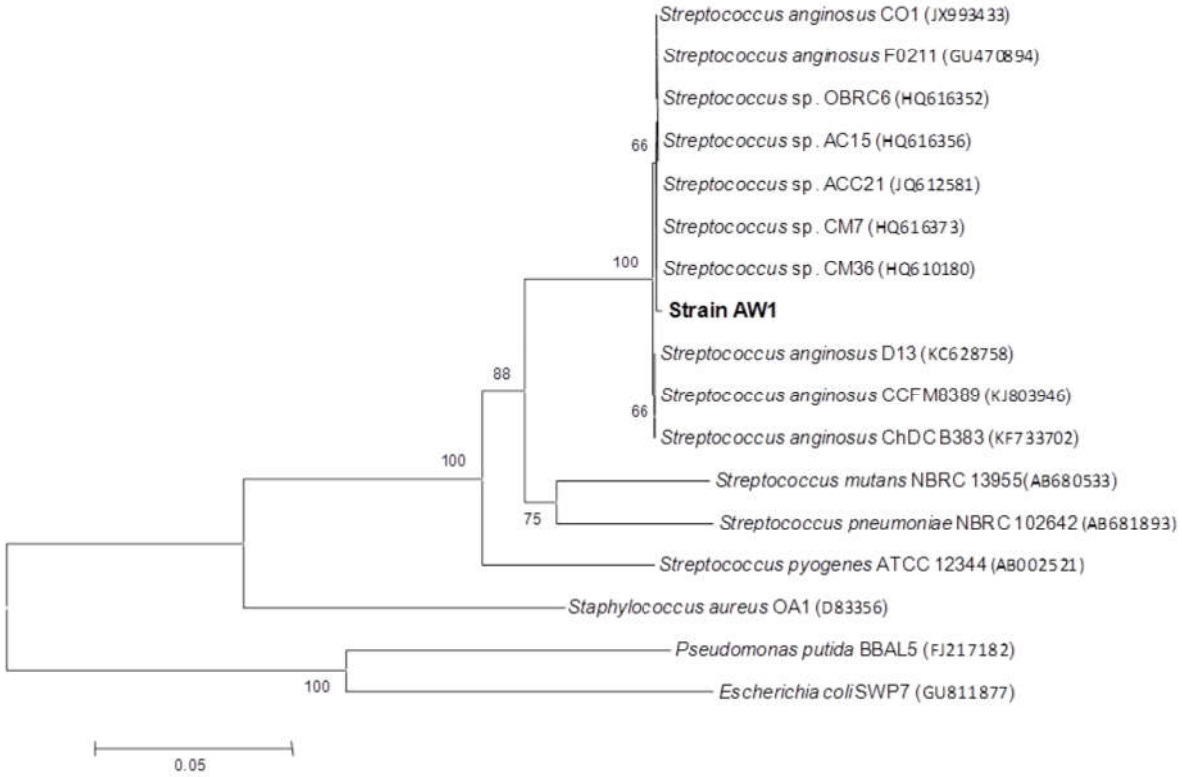
Strain AW1 was identified by partial sequencing of its 16S rRNA gene, which classify the isolate specifically into the genus, Streptococcus. According to Blast similarity matches, strain AW1 (LC 034920.1) showed 99% sequence homology to members of Streptococcus including *S. anginosus* in particular (Table 3).

Strain	Closest match Identity	Accession No.	Similarity (%)
AW1	*Streptococcus* sp. CM36	HQ610180	99
	Streptococcus sp. CM7	HQ616373	99
	Streptococcus sp. ACC21	JQ612581	99
	Streptococcus sp. AC15	HQ616356	99
	Streptococcus sp. OBRC6	HQ616352	99
	Streptococcus anginosus F0211	GU470894	99
	Streptococcus anginosus CO1	JX993433	99

Phylogenetic examinations verified the affiliation of the isolated strain AW1 into Streptococcus clusters as revealed by clustering of strain AW1 at the same phylogenetic

branch with members of Streptococcaceae. Figure 1 represents the phylogenetic tree based on 16S rRNA gene sequence analysis and shows the relationship between strain AW1 and its representative species along with other related genera. AW1 was regarded as a novel strain based on its relative low similarity and high bootstrap values expressed on the phylogenetic tree

Figure 1. Neighbour-joining tree showing the phylogenetic relationship between strain AW1 and closely related genera from GenBank database with accession numbers (in parentheses). The bar represents 0.05 substitutions per site, bootstrap values (n = 1000) are displayed.



Discussion

Streptococcus anginosus and closely related species constitute commensal flora of the human oral mucosa and urinary microbiome whose clinical significance remained inconspicuous due to problems encountered with its species identification. In 1991, Whiley and Beighton indicated that *S. anginosus* was the most common among the species of the anginosus' group (or '*S. milleri*') as it represented about 58–64% of all strains of anginosus group isolated from clinical specimens in many hospital population [23]. Furthermore, the most common species of 'anginosus' group that has been detected and identified from gastrointestinal and urogenital tracts was *S. anginosus*, which has also been isolated from most other anatomical sites [24,25].

In our study, *Enterococcus* species were the most prevalent organisms (10.5%) isolated from surgical wound infection (SSI) while, Streptococcus spp. were the least prevalent (1.8%). 189 cultures yielded no growth, and this could be explained by the fact that most of the wound swabs sent to our lab were usually taken under antibiotics cover. Another group reported that *S. aureus* was isolated in 31.8% of the cultures and was shown to be the predominant agent in post-cesarean wound infections .[26] While another study from Nigeria reported gram negative enteric bacilli as the predominant organisms (accounting for 29.5% of the cultures) [27]. Interestingly, in this work, most of the isolated organisms showed high resistance to penicillin, while gentamicin and vancomycin were the most sensitive drugs to all the isolates. Isolated *Streptococcus* strain in our study was non-group able with different groups of Lancefield antisera as well as non-haemolytic on culture media. Reissmann and co-workers recorded that, the organisms isolated from Vellore, a region in southern India, were a variant of different strains of anginosus species, which were significantly different from each other in terms of both their geographic origin and Lancefield type. The Lancefield grouping yielded the groups A, C, G, and F while some strains were non-identifiable. In contrast to our studies, anginosus

strains isolated from Vellore showed all types of hemolysis.[28]. While in another study most of the strains of *S. anginosus* were found to be non-hemolytic as well as not highly associated with purulent infections when compared to the *S. intermedius* species [29]. Also, another study [reported the general features of culture and biochemical reactions for strains of *S. milleri* that were isolated from different human specimens30]. They showed that in terms of cultural characteristics, 56% were non-hemolytic, 25% were beta hemolytic, and only 19% were alpha hemolytic of all the examined isolates. In our study, a new bacterial strain was isolated from SSI, which is not known to be a common infection caused by *S. anginosus*. In a 2 years' study on the clinical and microbiological characteristics of 51 infections caused by *S. milleri*, Molina et al. reported bacteremia (6 cases), endocarditis (4 cases), 8 cases with cellulitis and subcutaneous abscesses, pleural empyema (8 cases), brain abscesses (5 cases), abdominal infections (5 cases), while 15 cases had other miscellaneous infections. They found *S. milleri* as the most common organism as it was isolated in 19 patients representing 44% of all the pathogens isolated [31]. Whiley et al. detected the variations in genotype of *S. anginosus* by studying 16s-23s rRNA intergenic spacer size polymorphisms by PCR amplification, ribotyping, and by performing DNA-DNA base pairing studies. They found that *S. anginosus* was heterogeneous at both the species and intraspecies (subspecies) levels [25]. Identification of the species within the *Streptococcus milleri* group was found to be difficult due to the overlapping phenotypic characteristics of individual members. Identification in this regard should be performed utilizing not only the biochemical characterization but also molecular based approaches like 16S rRNA gene sequence analysis. A previous study reclassified previously identified *Streptococcus milleri*" from clinical isolates to another streptococcus groups using 16S rRNA gene sequence analysis [2,32]. 16S rRNA gene sequences have also been explored for preliminary identification of novel clinically relevant isolates, including noncultured bacteria . Grinwis et al. identified a number of isolates from the sputum of adults with cystic fibrosis, for characterization of *S. milleri* group using 16S rRNA gene sequencing accurately utilizing only three phenotypic assays including, Lancefield typing, hyaluronidase production, and chondroitin sulfatase production[33,34]. Phylogenetic study based on 16S rRNA gene sequence analysis has also revealed a new species of the genus *Vibrio* isolated from human clinical specimens [35,36].

In this study, strain AW1 was tentatively identified by determining its phenotypic characteristics. However, accurate identification was performed based on its 16S rRNA gene partial sequence. Strain AW1 showed 99% sequence similarity to *S. anginosus*. Phylogenetic analysis performed on the basis of the partial 16S rRNA gene sequences confirmed the affiliation of strain AW1 to members of Streptococcaceae family as also revealed by its clustering with individual *Streptococcus* members at the same scale. A 99% sequence homology for partial 16S rRNA gene sequence along with high bootstrap value estimated by Neighbour-joining analysis clearly indicated the uniqueness of strain AW1 as a new member of the *S. anginosus*.

The high phenotypic and antigenic diversity within the *anginosus* group, and the circumstance that it is non-beta hemolytic species, is still not clear in samples containing normal flora that could cause infections, which are difficult to be diagnosed. So, contribution of the *anginosus* group as a whole to the epidemiology of infections caused by *Streptococcus species* is still not well understood and entails further studies in the future.

Conflict of Interest

We declare that we have no conflict of interest.

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