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Abstract

We report a nosocomial transmission of *Streptococcus gallolyticus* subsp. *pasteurianus* among three neonates, one of whom died. Genome analysis of the strains showed a specific pattern of metabolic and regulatory functions as well as of expressed antigens and antibiotic resistance genes that might have contributed to their specific virulence.

Streptococcoccus (S.) gallolyticus subsp. *pasteurianus* is a Lancefield group D streptococcus, formerly known as *Streptococcus bovis* biotype II.[1] By means of 16S rRNA gene sequencing, the *Streptococcus bovis* conglomerate could be differentiated into the genomospecies *S. gallolyticus* subsp. *gallolyticus* (former biotype I), *Streptococcus infantarius* subsp. *coli* (former biotype II/1), and *S. gallolyticus* subsp. *pasteurianus* (former biotype II/2).[2] *S. gallolyticus* subsp. *pasteurianus* specifically is associated with meningitis[3] while *S. gallolyticus* subsp. *gallolyticus* is linked to endocarditis and colon cancer.[4] We present a cluster of three neonatal cases with *S. gallolyticus* subsp. *pasteurianus* and we determined the strains' virulence characteristics with genome sequencing.

Patient 1. A preterm male neonate was born at 30 weeks postmenstrual age, after premature rupture of membranes and preterm labor. On day 7, the boy became cardiorespiratory instable. Vancomycin and amikacin were initiated for suspected late-onset sepsis. Cerebrospinal fluid showed protidorachia and leucorachia but culture remained sterile. Blood culture grew *S. gallolyticus* subsp. *pasteurianus* susceptible to penicillin. The boy recovered and was discharged at a postnatal age of 57 days.

Patient 2. A second preterm male neonate was born at postmenstrual age of 32 weeks because of maternal HELLP syndrome and oligohydramnios. On day 34, he suddenly deteriorated, developed a septic shock, diffuse intravascular coagulation, progressive metabolic acidosis, respiratory failure and pulmonary hemorrhage requiring mechanical ventilation. He deceased within two hours. From two blood cultures *S. gallolyticus* subsp. *pasteurianus* was grown, susceptible to penicillin, amoxicillin, gentamicin, vancomycin and resistant to erythromycin.

Because patients 1 and 2 were roommates on the Neonatal Intensive Care Unit (NICU), nosocomial transmission was suspected. Therefore, contact isolation was installed for patient

1, the patients' rooms were additionally cleaned and disinfected, extra focus on hand hygiene compliance and rectal screening on the ward were initiated. Accordingly, patient 3 was detected.

Patient 3. A boy with intrauterine growth restriction in monochorionic twins was born at 30-weeks postmenstrual age. During his stay in the NICU, he developed two episodes of necrotizing enterocolitis. On day 64, rectal screening culture was positive for *S. gallolyticus* subsp. *pasteurianus*. At that moment, the boy was asymptomatic. The boy was nursed in isolation up to discharge.

Methods

The *S. gallollyticus* subsp. *pasteurianus* strains were identified by means of MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen, Germany).

They were typed with pulsed-field-gel electrophoresis (PFGE) adapted after Descheemaker et al[5] at the Belgian national reference laboratory for *Enterococcus* spp. UZA Antwerp.

To confirm the identification, to definitely demonstrate the clonality of the three isolates and to search for possible virulence factors explaining the invasiveness of the strains in patients 1 and 2, the genome sequences of the three strains were determined by using the Illumina technology. Libraries were constructed by using the Illumina Nextera XT kit. Sequencing was performed on a Illumina MiSeq, 300 bases single read run. Sequences were assembled with Velvet.[6] The complete 16S rRNA gene sequences were extracted from the genome sequence assembly of the three clinical isolates. The genomic data of the second patient's strain (HC-2909-2) were deposited at EMBL (genome project ERS578714). Illumina reads alignments were conducted with BWA.[7] Single nucleotide polymorphism (SNP) calling was done using GATK.[8] To identify specific features of these strains, we compared their genomes to that of strain ATCC 43144 (genbank accession number AP012054).[9] Regions specific to the three strains were extracted by assembly of the BWA unmapped reads. Contigs larger than 300 bp

were considered and automatically annotated by RAST.[10] The 266 protein encoding genes annotated in 46 contigs were searched for genes of interest: antibiotic resistance and putative virulence associated functions. SNPs with strain ATCC 43144 were visualized by using SyntView. Differences between the three isolates were determined by aligning reads on the contigs of strain HC-2909-2 using BRESEQ.

Results

All three strains belonged to the same PFGE type A, indicating clonality knowing that PFGE is the most discriminative technique for this species. Moreover, clustering of cases with this uncommon invasive pathogen on the same hospital ward in this short three-week period is a strong indication of nosocomial transmission.

Genome sequencing showed that the three strains were virtually identical, confirming PFGE data: the patients' strains differed by maximum 4 SNPs from each other. The sequence of the 16S rRNA genes were 100 % identical over the whole length (1462 bp) to that of the published ATCC strain *S. gallolyticus* subsp. *pasteurianus* 43144.[9] Whole genome comparisons with strain ATCC 43144 confirmed the species identification and showed that the patients' strains are rather distantly related to the ATCC strain with 0.4% of polymorphism. Interestingly, analysis of the SNP distribution by SyntView (http://genopole.pasteur.fr/SynTView/flash/Streptococcus_pasteurianus/_SynWeb.html) along the genome alignment shows an uneven distribution of SNP density with alternate pattern of regions of high and low SNP density, suggesting a high recombination rate involving large genomic regions as previously described in *Streptococcus agalactiae*.[11]

Both the ATCC 43144 and the patients' strains possessed specific genes. However, the three patient strains analyzed here showed unique features possibly associated with their fitness and virulence (Table 1).

Discussion

We analyzed the specific genes of the three patients' *S. pasteurianus* strains in search for their virulence potency. These strains had a complete operon for the utilization of L-fucose and L-fucosyl oligosaccharides. Fucose, a mucin component is both a carbon source and a signal molecule. Blast search revealed that this locus is missing in sequenced *S. pasteurianus* and *S. gallolyticus* strains but that it is present in *Streptococcus suis* pointing at a recent acquisition of the locus by lateral gene transfer. This locus encodes a protein highly similar to the α–L-fucosidase from Bifidobacterium. Likewise, Stahl et al showed enhanced survival in the piglet gut of *Campylobacter jejuni* strains that use L-fucose as a substrate of growth.[12] The CovRS two component system regulating the transition from commensalism to invasiveness in group A and group B streptococci is missing in the ATCC 43144 strain but is present in these *S. gallolyticus* isolates. Furthermore the ATCC 43144 strain expresses a different capsule operon and a different locus for the synthesis of bacteriocin and competence compared with the sequenced strains. How the specific gene content of these three strains might contribute to their virulence remains to be analyzed. Probably more significant is the identification of antibiotic resistance genes in all three strains: *tet*(M): tetracycline resistance determinant, *ermB*: an adenine N-6-methyltransferase conferring resistance to erythromycin, *aad*(E): a streptomycin aminoglycoside 6-adenyltransferase, and *aph*A3: a putative spectinomycin adenyltransferase. All these genotypic resistance determinants were associated with minimum inhibitory concentrations of at least 64 µg/mL, suggesting a phenotypical resistance to these antibiotics for all three strains.

In conclusion, nosocomial transmission of *S. gallolyticus* subsp. *pasteurianus* in a NICU resulted in screening and isolation measures. No further infections/colonizations with this species arose. This shows the importance of awareness of possible transmission of microorganisms other than the familiar multi-drug resistant organisms.

Genome analysis of the three strains showed a specific pattern of metabolic and regulatory functions as well as of expressed antigens that might have contributed to the specific virulence of these strains. In addition, the number of antibiotic resistance genes present in its genome possibly witness a long history of interaction with humans in a disease or hospital context which also might be associated with an increased capacity for dissemination.

Table legend

Table 1: Major functions encoded by genes specific to *S. pasteurianus* strains ATCC 43144 and the strain from the second patient (HC-2909-2).

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Table 1: Major functions encoded by genes[1] specific to *S. pasteurianus* strains ATCC 43144 and the strain from the second patient (HC-2909-2)

Functional categories	Strain ATCC 43144	HC-2909-2
Carbon metabolism	- Allulose [2]PTS permease and metabolism - keto desoxygluconate permease - Didhydroxyacetone operon - Alcohol dehydrogenase	- Putative sugar ABC transporter system. - Oligo-1,6-glucosidase - Alpha galactosidase and sucrose specific PTS system - PTS system, ascorbate family - L-fucose and L- fucosyl oligosaccharides utilization operon
DNA metabolism and regulators	- CRISPR-*cas* (type 2-B) - DNA cytosine methyltransferase - DNA binding protein	- CRISPR-*cas* (type 2-A) - [3]TCS (x2) - CovR CovS, virulence TCS - Transcription regulator coupled with an ABC transporter - Type 2 restriction modification system - DNA-cytosine methyltransferase
Surface polysaccharides	- [4]Capsular polysaccharide - Glycosyl transferase	- [4]Capsular polysaccharide - D-alanyl-D-alanine carboxypeptidase
Antibiotics	- L-antibiotic, Nisin U biosynthesis - L-antibiotic ABC transporter	- rRNA (Adenine-N(6)-) – methyltransferase (EryR) - Aminoglycoside phosphotransferase - Aminoglycoside 6-adenylyltansferase - Adenine phosphoribosyltransferase - Spectinomycin 9-O-adenylyltransferase - [5]*tet*(M), tetracycline resistance gene - L-antibiotic ABC transporter - Bacteriocin locus and competence, including *comDE* TCS
Surface proteins	- CnaB motif LPXTG protein - sortase C, cell wall ribonuclease	- Aggregation substance LPXTG protein - S-layer homology domain protein - LPXTG surface proteins of unknown function (x2) - Sortase A (paralogous gene) - Collagen adhesion protein
Ion transport	- trk K+ uptake	

[1]Phages, remnant phages and mobile genomic elements are not reported; [2]PTS: phosphotransferase sugar transport system; [3]TCS: two component regulatory system; [4]corresponds to two different capsular operons; [5]present but inactivated by a frameshift mutation in the ATCC 43144 strain.