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***Streptococcus Gallolyticus* Subsp. *Pasteurianus* Infection in a neonatal intensive care unit**

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**Abbreviated title: *S. pastuerianus* cluster in neonatal intensive care unit**

**Running title : virulence of a *S. pasteurianus* cluster**

**Key words : *Streptococcus gallolyticus* subsp. *pasteurianus*, neonatal, transmission, genome sequencing, virulence factors**

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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.

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

11  
12 **Patient 1.** A preterm male neonate was born at 30 weeks postmenstrual age, after premature  
13 rupture of membranes and preterm labor. On day 7, the boy became cardiorespiratory  
14 instable. Vancomycin and amikacin were initiated for suspected late-onset sepsis.  
15 Cerebrospinal fluid showed protidorrachia and leucorrachia but culture remained sterile. Blood  
16 culture grew *S. gallolyticus* subsp. *pasteurianus* susceptible to penicillin. The boy recovered  
17 and was discharged at a postnatal age of 57 days.

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

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

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27 1, the patients' rooms were additionally cleaned and disinfected, extra focus on hand hygiene  
28 compliance and rectal screening on the ward were initiated. Accordingly, patient 3 was  
29 detected.

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

### 35 **Methods**

36 The *S. gallolyticus* subsp. *pasteurianus* strains were identified by means of MALDI-TOF  
37 mass spectrometry (Bruker Daltonics, Bremen, Germany).  
38 They were typed with pulsed-field-gel electrophoresis (PFGE) adapted after Descheemaker et  
39 al<sup>5</sup> at the Belgian national reference laboratory for *Enterococcus* spp. UZA Antwerp.  
40 To confirm the identification, to definitely demonstrate the clonality of the three isolates and  
41 to search for possible virulence factors explaining the invasiveness of the strains in patients 1  
42 and 2, the genome sequences of the three strains were determined by using the Illumina  
43 technology. Libraries were constructed by using the Illumina Nextera XT kit. Sequencing was  
44 performed on a Illumina MiSeq, 300 bases single read run. Sequences were assembled with  
45 Velvet.<sup>6</sup> The complete 16S rRNA gene sequences were extracted from the genome sequence  
46 assembly of the three clinical isolates. The genomic data of the second patient's strain (HC-  
47 2909-2) were deposited at EMBL (genome project ERS578714). Illumina reads alignments  
48 were conducted with BWA.<sup>7</sup> Single nucleotide polymorphism (SNP) calling was done using  
49 GATK.<sup>8</sup> To identify specific features of these strains, we compared their genomes to that of  
50 strain ATCC 43144 (genbank accession number AP012054).<sup>9</sup> Regions specific to the three  
51 strains were extracted by assembly of the BWA unmapped reads. Contigs larger than 300 bp

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

## 57 **Results**

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

62 Genome sequencing showed that the three strains were virtually identical, confirming PFGE  
63 data: the patients' strains differed by maximum 4 SNPs from each other. The sequence of the  
64 16S rRNA genes were 100 % identical over the whole length (1462 bp) to that of the published  
65 ATCC strain *S. gallolyticus* subsp. *pasteurianus* 43144.<sup>9</sup> Whole genome comparisons with  
66 strain ATCC 43144 confirmed the species identification and showed that the patients' strains  
67 are rather distantly related to the ATCC strain with 0.4% of polymorphism. Interestingly,  
68 analysis of the SNP distribution by SyntView  
69 ([http://genopole.pasteur.fr/SynTView/flash/Streptococcus\\_pasteurianus/SynWeb.html](http://genopole.pasteur.fr/SynTView/flash/Streptococcus_pasteurianus/SynWeb.html)) along  
70 the genome alignment shows an uneven distribution of SNP density with alternate pattern of  
71 regions of high and low SNP density, suggesting a high recombination rate involving large  
72 genomic regions as previously described in *Streptococcus agalactiae*.<sup>11</sup>

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

## 76 **Discussion**

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77 We analyzed the specific genes of the three patients' *S. pasteurianus* strains in search for their  
78 virulence potency. These strains had a complete operon for the utilization of L-fucose and L-  
79 fucosyl oligosaccharides. Fucose, a mucin component is both a carbon source and a signal  
80 molecule. Blast search revealed that this locus is missing in sequenced *S. pasteurianus* and *S.*  
81 *gallolyticus* strains but that it is present in *Streptococcus suis* pointing at a recent acquisition  
82 of the locus by lateral gene transfer. This locus encodes a protein highly similar to the  $\alpha$ -L-  
83 fucosidase from *Bifidobacterium*. Likewise, Stahl et al showed enhanced survival in the piglet  
84 gut of *Campylobacter jejuni* strains that use L-fucose as a substrate of growth.<sup>12</sup> The CovRS  
85 two component system regulating the transition from commensalism to invasiveness in group  
86 A and group B streptococci is missing in the ATCC 43144 strain but is present in these *S.*  
87 *gallolyticus* isolates. Furthermore the ATCC 43144 strain expresses a different capsule operon  
88 and a different locus for the synthesis of bacteriocin and competence compared with the  
89 sequenced strains. How the specific gene content of these three strains might contribute to  
90 their virulence remains to be analyzed. Probably more significant is the identification of  
91 antibiotic resistance genes in all three strains: *tet(M)*: tetracycline resistance determinant,  
92 *ermB*: an adenine N-6-methyltransferase conferring resistance to erythromycin, *aad(E)*: a  
93 streptomycin aminoglycoside 6-adenyltransferase, and *aphA3*: a putative spectinomycin  
94 adenytransferase. All these genotypic resistance determinants were associated with minimum  
95 inhibitory concentrations of at least 64  $\mu\text{g/mL}$ , suggesting a phenotypical resistance to these  
96 antibiotics for all three strains.

97  
98 In conclusion, nosocomial transmission of *S. gallolyticus* subsp. *pasteurianus* in a NICU  
99 resulted in screening and isolation measures. No further infections/colonizations with this  
100 species arose. This shows the importance of awareness of possible transmission of  
101 microorganisms other than the familiar multi-drug resistant organisms.

102 Genome analysis of the three strains showed a specific pattern of metabolic and regulatory  
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2 103 functions as well as of expressed antigens that might have contributed to the specific  
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4 104 virulence of these strains. In addition, the number of antibiotic resistance genes present in its  
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7 105 genome possibly witness a long history of interaction with humans in a disease or hospital  
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10 106 context which also might be associated with an increased capacity for dissemination.  
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2 109 **Table legend**

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7 111 **Table 1:** Major functions encoded by genes specific to *S. pasteurianus* strains ATCC 43144

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9 112 and the strain from the second patient (HC-2909-2).

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

Functional categories	Strain ATCC 43144	HC-2909-2
Carbon metabolism	<ul style="list-style-type: none"> <li>- Allulose <sup>2</sup>PTS permease and metabolism</li> <li>- keto desoxygluconate permease</li> <li>- Didhydroxyacetone operon</li> <li>- Alcohol dehydrogenase</li> </ul>	<ul style="list-style-type: none"> <li>- Putative sugar ABC transporter system.</li> <li>- Oligo-1,6-glucosidase</li> <li>- Alpha galactosidase and sucrose specific PTS system</li> <li>- PTS system, ascorbate family</li> <li>- L-fucose and L- fucosyl oligosaccharides utilization operon</li> </ul>
DNA metabolism and regulators	<ul style="list-style-type: none"> <li>- CRISPR-<i>cas</i> (type 2-B)</li> <li>- DNA cytosine methyltransferase</li> <li>- DNA binding protein</li> </ul>	<ul style="list-style-type: none"> <li>- CRISPR-<i>cas</i> (type 2-A)</li> <li>- <sup>3</sup>TCS (x2)</li> <li>- CovR CovS, virulence TCS</li> <li>- Transcription regulator coupled with an ABC transporter</li> <li>- Type 2 restriction modification system</li> <li>- DNA-cytosine methyltransferase</li> </ul>
Surface polysaccharides	<ul style="list-style-type: none"> <li>- <sup>4</sup>Capsular polysaccharide</li> <li>- Glycosyl transferase</li> </ul>	<ul style="list-style-type: none"> <li>- <sup>4</sup>Capsular polysaccharide</li> <li>- D-alanyl-D-alanine carboxypeptidase</li> </ul>
Antibiotics	<ul style="list-style-type: none"> <li>- L-antibiotic, Nisin U biosynthesis</li> <li>- L-antibiotic ABC transporter</li> </ul>	<ul style="list-style-type: none"> <li>- rRNA (Adenine-N(6)-) - methyltransferase (EryR)</li> <li>- Aminoglycoside phosphotransferase</li> <li>- Aminoglycoside 6-adenylyltransferase</li> <li>- Adenine phosphoribosyltransferase</li> <li>- Spectinomycin 9-O-adenylyltransferase</li> <li>- <sup>5</sup><i>tet</i>(M), tetracycline resistance gene</li> <li>- L-antibiotic ABC transporter</li> <li>- Bacteriocin locus and competence, including <i>comDE</i> TCS</li> </ul>
Surface proteins	<ul style="list-style-type: none"> <li>- CnaB motif LPXTG protein</li> <li>- sortase C, cell wall ribonuclease</li> </ul>	<ul style="list-style-type: none"> <li>- Aggregation substance LPXTG protein</li> <li>- S-layer homology domain protein</li> <li>- LPXTG surface proteins of unknown function (x2)</li> <li>- Sortase A (paralogous gene)</li> <li>- Collagen adhesion protein</li> </ul>
Ion transport	<ul style="list-style-type: none"> <li>- <i>trk</i> K<sup>+</sup> uptake</li> </ul>	

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