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Original article

Benzalkonium tolerance genes and outcome in *Listeria monocytogenes* meningitis

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ABSTRACT

Objectives: *Listeria monocytogenes* is a food-borne pathogen that can cause meningitis. The listerial genotype ST6 has been linked to increasing rates of unfavourable outcome over time. We investigated listerial genetic variation and the relation with clinical outcome in meningitis.
Methods: We sequenced 96 isolates from adults with listerial meningitis included in two prospective nationwide cohort studies by whole genome sequencing, and evaluated associations between bacterial genetic variation and clinical outcome. We validated these results by screening listerial genotypes of 445 cerebrospinal fluid and blood isolates from patients over a 30-year period from the Dutch national surveillance cohort.
Results: We identified a bacteriophage, phiLMST6 co-occurring with a novel plasmid, pLMST6, in ST6 isolates to be associated with unfavourable outcome in patients (p 2.83e-05). The plasmid carries a benzalkonium chloride tolerance gene, *emrC*, conferring decreased susceptibility to disinfectants used in the food-processing industry. Isolates harbouring *emrC* were growth inhibited at higher levels of benzalkonium chloride (median 60 mg/L versus 15 mg/L; p <0.001), and had higher MICs for amoxicillin and gentamicin compared with isolates without *emrC* (both p <0.001). Transformation of pLMST6 into naive strains led to benzalkonium chloride tolerance and higher MICs for gentamicin.
Conclusions: These results show that a novel plasmid, carrying the efflux transporter *emrC*, is associated with increased incidence of ST6 listerial meningitis in the Netherlands. Suggesting increased disease severity, our findings warrant consideration of disinfectants used in the food-processing industry that select for resistance mechanisms and may, inadvertently, lead to increased risk of poor disease outcome.
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Introduction

Listeriosis is caused by eating food contaminated with the bacterium *Listeria monocytogenes* and poses an important public health problem [1]. In humans it can cause a range of infections including gastroenteritis, bacteraemia, sepsis and meningitis [1]. The decline since the 1990s in incidence of invasive disease caused by *L. monocytogenes* has been attributed to a decrease in contamination through ready-to-eat food following improvements in the food-processing industry [2]. However, food-processing plants have increasingly been implicated as an infection source of

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listeriosis [3]. The official sources of information probably underestimate the true burden of disease attributable to listeria [4]. The Emerging Infections Programmes Network reported the incidence of listerial meningitis to be 0.05 cases per 100 000 population [5]; but the true incidence can be estimated to be higher [6].

We previously reported an increasing rate of unfavourable outcome among adults with listerial meningitis over a 14-year period, from 27% in 1998–2002 [7,8], to 61% in 2006–2012 [9]. The emerging bacterial genotype sequence type (ST) 6 was identified as the main factor leading to this poorer prognosis [9]. Recently, *L. monocytogenes* clonal complex 6, comprising ST6, was associated with invasive disease, in particular meningitis, but data on outcome were not available [10]. *Listeria monocytogenes* ST6 isolates are typed as serotype B lineage II by classic serotyping methods. ST6 isolates are relatively infrequently cultured from contaminated food sources [10].

We investigated variation in the *L. monocytogenes* genome, in particular in the ST6 genotype, and its relation with clinical outcome in meningitis. We used whole bacterial genome sequencing of isolates from two nationwide cohort studies on listerial meningitis, and validated results in a nationwide surveillance study over a 30-year period.

Materials and methods

Nationwide clinical cohort

We identified adults as >16 years of age who had *L. monocytogenes* meningitis, as established by positive cerebrospinal fluid (CSF) culture, and were listed in the database of the Netherlands Reference Laboratory for Bacterial Meningitis between 1 October 1998 and 1 April 2002 [6,7], and between 1 March 2006, and 1 April 2012 [9]. During these two periods, a prospective national cohort study ran in which patients with bacterial meningitis were included and their clinical characteristics were scored [9]. Patients or their legal representatives received written information concerning the study and were asked to give written informed consent for participation. Patients with hospital-acquired bacterial meningitis, neurosurgical procedures, or those within 1 month following neurosurgical procedure or neurotrauma were excluded. Patients with an altered immune status owing to splenectomy, diabetes mellitus, cancer, alcoholism, or the use of immunosuppressive drugs were considered immunocompromised, as were patients infected with human immunodeficiency virus. Neurological examination was performed at discharge, and outcome was scored according to the Glasgow Outcome Scale, with scores varying from 1 (death) to 5 (good recovery) [11]. A favourable outcome was defined as a score of 5, and an unfavourable outcome was defined as a score of 1–4. Adequate treatment was defined as amoxicillin 2 g six times per day or penicillin G 3 million units six times daily. If adequate treatment was not initiated on admission, number of days to adequate treatment was recorded. The studies were approved by the Medical Ethics Committee of the Academic Medical Centre, University of Amsterdam, the Netherlands.

Bacterial whole genome sequencing

DNA from *L. monocytogenes* strains was extracted based on manufacturer's protocol (Promega, Madison, WI, USA). Sequencing was performed using multiplexed libraries on the Illumina HiSeq platform to produce paired end reads of 100 nucleotides in length (Illumina, San Diego, CA, USA). Sequences of the bacterial samples were assembled *de novo* using SPAdes (version 3.6.0) with default parameters [12]. The median number of contigs was 11 (range 5–25), mean GC content 38%, average genome length 2 970 545 bp (range 2 859 080–3 105 945), and mean coverage 239-fold. Sequence types were determined from the whole genome sequences. PacBio sequencing was performed according to manufacturer protocols (Pacific Biosciences, Menlo Park, CA, USA).

Data availability

Fastq sequences of bacterial isolates (accession numbers in see Supplementary material, Table S1) and nucleotide sequences of the bacteriophage (accession number Hx2000053476), plasmid pLMST6 (accession number Hx2000053471) and *emrC* gene (accession number Hx2000053480) have been deposited in the European Nucleotide Archive (ENA).

Pan-genome generation and phylogenetic tree

Genome sequences were annotated with PROKKA, version 1.11 [13]. We used Roary (version 3.5.0) with default parameters to extract clusters of orthologous genes, referred to as gene groups, and create a core gene alignment at a sequence identity threshold of 95% [14]. This process identified a pan-genome of 6360 gene groups and a core genome (shared by 100% of strains) of 2177. The cumulative plot of the pan-genome suggested a closed pan-genome (see Supplementary material, Fig. S1).

A maximum likelihood phylogeny of single-nucleotide polymorphisms (SNPs) in the core genome of all sequenced isolates was produced with RAxML (version 7.8.6) assuming a general time reversible model of nucleotide substitution with a γ-distributed rate heterogeneity [15]. To generate the phylogenetic tree of the ST6 clade, isolates were mapped against an ST6 reference (accession number: NC_021829). For this analysis, the same parameter settings were used as for the phylogeny of all sequenced isolates.

Isolates from each of the four monophyletic groups were mapped to a reference in the same clade with SMALT (version 0.7.4, using default parameters), creating a pseudogenome alignment and phylogenetic tree using RAxML. This pseudogenome alignment and tree were used to infer areas of increased SNP density with Gubbins (version 1.7.4, default parameters) [16]. The algorithm converged to a stable phylogeny after four iterations.

Bacterial genetic association study on clinical outcome

The sequences were mapped against an ST6 reference (accession number: NC_021829). The SNPs were called with bcftools (version 0.1.19) [17]. A matrix containing presence and absence of gene groups was generated from the Roary output [14]. As bacterial populations are generally clonal, standard methods for genome-wide analyses fail [18], GEMMA (version 0.94.1, default parameters) was used to perform a linear mixed model analysis for each SNP or gene group versus unfavourable outcome or mortality phenotype and determine p-values of association [19]. SNPs or gene groups present in <5% or >95% of isolates were excluded. The core SNP alignment was used as the design matrix of random effects to correct for population stratification through the generation of a centred relatedness matrix in GEMMA. No covariates were included. We considered a p-value (α of 0.05) corrected for multiple testing (166 839 tests) to be statistically significant ($p < 2.99 \times 10^{-7}$). Bacteriophage sequences from isolates mapped to the NC_021829 reference isolate were extracted *in silico* by using the conserved gene boundaries of the *comK* gene as primers (see Supplementary material, Table S2). The percentage of sequence identity similarity was determined with BLAST [20]. A Fisher's exact test was performed to assess the association of presence or absence of sequence elements with unfavourable outcome or mortality.