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Multi-locus sequence typing scheme for *Laribacter hongkongensis*, a novel bacterium associated with freshwater fish-borne gastroenteritis and traveller's diarrhoea

Key Messages

1. A multilocus sequence typing (MLST) system was developed for *Laribacter hongkongensis*, an emerging pathogen associated with fish-borne gastroenteritis and traveller's diarrhoea.
2. A website for *L hongkongensis* MLST at http://mlstdb.hku.hk:14206/MLST_index.html enables comparison of strains from different localities.
3. The same species of freshwater fish in a particular market is usually obtained from the same fish farm. The presence of multiple sequence types in *L hongkongensis* strains isolated from the same species of freshwater fish from the same market implies that multiple clones of *L hongkongensis* probably exist in the same fish farm in mainland China or Hong Kong.
4. The clustering of fish and human isolates into different groups observed previously using pulsed-field gel electrophoresis and the present MLST studies suggest that some clones of *L hongkongensis* are more virulent than others.

Introduction

Laribacter hongkongensis was first discovered in Hong Kong in 2001 from the blood and empyema pus of a 54-year-old man with alcoholic cirrhosis and bacteraemic empyema thoracis.¹ It is a facultative anaerobic, motile, non-sporulating, urease-positive, Gram-negative, S-shaped bacillus. Genotypically, *L hongkongensis* belongs to the *Neisseriaceae* family of the β -subclass of Proteobacteria. It was subsequently discovered in the stool of six patients with community-acquired gastroenteritis. In a multi-centre prospective study using cefoperazone MacConkey agar as the selective medium, *L hongkongensis* was associated with community-acquired gastroenteritis.² Freshwater fish were confirmed to be a reservoir.²

L hongkongensis has been found in the intestines of healthy freshwater fish but not other studied animals that are commonly used for cooking in Hong Kong.³ The bacterium was isolated from the guts of 24% out of 360 freshwater fish that were studied.^{3,4} *L hongkongensis* has also been isolated from drinking water reservoirs in Hong Kong.⁵ The presence of a heterogeneous population of *L hongkongensis* identified using pulsed-field gel electrophoresis (PFGE) of freshwater fish isolates³ and the association of *L hongkongensis* gastroenteritis with fish consumption² suggests that freshwater fish are a likely major reservoir of the bacterium and the source of infections.

A highly reproducible and discriminative typing system is essential for better understanding of the epidemiology of *L hongkongensis*. We have used PFGE for typing *L hongkongensis*.^{2,3,5} Due to experimental variations, PFGE patterns are difficult to compare among different laboratories. As multi-locus sequence typing (MLST) is highly reproducible and discriminative for bacteria, we developed such a typing system for *L hongkongensis*.

Methods

This study was conducted from December 2005 to December 2007. In order to have better understanding of the epidemiology of *L hongkongensis*, we developed an MLST system for *L hongkongensis*, using 146 human and fish isolates.⁶ Seven housekeeping genes were selected for cross comparison: transcription termination factor Rho, aconitate hydratase, cell division protein, anthranilate synthase component I, ketol-acid reductoisomerase, thiamin biosynthesis protein ThiC, and enolase. The nucleotide sequences of seven housekeeping genes in all the *L hongkongensis* strains were aligned and compared.⁶

Regarding the 146 *L hongkongensis* isolates, 39 were from humans and 107 from fish. The fish isolates were recovered from the gut of freshwater fish sampled from retail food markets in Hong Kong,^{2,3} including 50 isolated from grass carp, 42 from bighead carp, 12 from mud carp and three from large-mouth bass.

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Extracted DNA of the 146 isolates of *L hongkongensis* was used as the template for amplification of seven housekeeping genes.⁶ The nucleotide sequences of the seven gene loci in all the *L hongkongensis* strains were aligned and compared with those of strain HLHK1 using Clustal W multiple alignment implemented in BioEdit (version 7.0.5.2). The ratio of non-synonymous to synonymous base substitutions (d_n/d_s) was calculated with START2 (Sequence Type Analysis and Recombinational Tests Version 2) [<http://pubmlst.org/software/analysis/>]. A dendrogram was constructed using the unweighted pair group method with the arithmetic mean using START2. Grouping of STs into lineages was performed with eBURST. The BURST algorithm first identifies mutually exclusive groups of related genotypes in the population (typically an MLST database), and attempts to identify the founding genotype (ST) of each group. The algorithm then predicts the descent from the predicted founding genotype to the other genotypes in the group. The linkage disequilibrium between alleles at the seven gene loci was measured using the standardised index of association (I^s_A) with LIAN 3.5 (<http://www.mlst.net>).

Results

Variations at the seven MLST loci

Among the 3068 bp of the seven loci, 332 polymorphic sites were observed in the 146 isolates of *L hongkongensis*.⁶ Allelic profiles were assigned to the 146 isolates. The alleles defined for the MLST system were based on sequence lengths of between 362 bp (ilvC) and 504 bp (acnB).⁶ No strong positive selective pressure was present at all seven loci.

Relatedness of *L hongkongensis* isolates

A total of 97 different STs were assigned to the 146 *L hongkongensis* isolates.⁶ Their discriminatory power was 0.9861. eBURST grouped the isolates into 12 lineages, with 14 STs in group 1, 12 in group 2, seven in group 3, three in groups 4 to 6, and two in groups 7 to 12, whereas 43 STs did not belong to any of the 12 groups.⁶ A website for *L hongkongensis* MLST at http://mlstdb.hku.hk:14206/MLST_index.html was set up.

No relationships were observed among the *L hongkongensis* isolates with respect to their year of isolation, location of the respective hospitals, age and gender of the patients, the presence of plasmids in the patient strains, the species of fish, and the locations of markets where the fish were purchased.

Discussion

The clustering of fish and human isolates into different groups observed in both the previous PFGE and the present MLST studies suggested that some clones of *L hongkongensis* were more virulent than others. Differential gene expression experiments using strains of human- and fish-derived isolates may reveal *L hongkongensis* virulence factors.

A heterogeneous population of *L hongkongensis* existed in the same ecosystem. *L hongkongensis* isolated from neither the same species of fish nor the same fish market was clustered together. Over 80% of freshwater fish consumed in Hong Kong are imported from fish farms in mainland China, whereas the remaining 20% are locally reared in fish farms in rural areas of Hong Kong. The same species of freshwater fish in a particular market was usually obtained from the same fish farm, but multiple STs were present in *L hongkongensis* strains isolated from the same species of freshwater fish purchased from the same market. This implies that multiple clones of *L hongkongensis* probably exist in the same fish farm in mainland China or Hong Kong.

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