Molecular detection of *Legionella*: moving on from *mip*

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INTRODUCTION

Bacteria of the genus *Legionella* are ubiquitous in soil and water environments where they persist and multiply in free living protozoa. Despite the pathogen being adapted for an environmental niche, humans may become infected with *Legionella* through the inhalation of contaminated aerosols. The ability of *Legionella* to replicate in environmental protozoa has equipped the bacteria with the capacity to replicate in human alveolar macrophages (Newton et al., 2010). Intracellular replication requires a specialized type IV secretion system termed the effecitve in organelle trafficking/intracellular multiplication (Dot/Icm) system. Although ancestrally related to DNA conjugation systems, the Dot/Icm system transports multiple effecter proteins into the host cell to establish a vacuole that evades lysosome fusion and interacts instead with membranes and vesicles of the secretory pathway (Shin and Roy, 2008; Franco et al., 2009; Isberg et al., 2009; Nora et al., 2009; Newton et al., 2010). The formation of the specialized *Legionella* containing vacuole (LCV) is critical for pathogen replication and spread to new host cells.

Although several *Legionella* species have been associated with human infection, *Legionella pneumophila* is the most common cause of Legionnaire’s disease (Fields et al., 2002). In particular, serogroup 1 isolates of *L. pneumophila* represent the majority of clinical strains and many diagnostic tests are specific for the detection and diagnosis of this serogroup (Yu et al., 2002; Tronel and Hartemann, 2009). The difficulty of culturing of *Legionella* isolates from clinical and environmental samples has led to the development of rapid molecular tests for the detection of *Legionella* DNA (Rantakokko-Jalava and Jalava, 2001; Reischl et al., 2002; Templeton et al., 2003; Wilson et al., 2003; Bencini et al., 2007; Wilson et al., 2007). The current gold standard in molecular diagnosis is based on detection of the *mip* gene specific for *L. pneumophila* and 16S rRNA for identification of the *Legionella* genus (Ratcliff et al., 1998; Templeton et al., 2003). The *mip* gene was one of the first genes associated with the ability of *L. pneumophila* to replicate in euakaryotic cells and encodes a surface located peptidylprolyl cis/trans isomerase (PPlase) (Cianciotto et al., 1990; Cianciotto and Fields, 1992; Fischer et al., 1992; Wintermeyer et al., 1995). The 24 kDa *mip* product shares amino acid sequence similarity and is a structural mimic of the mainly euakaryotic family of FK-506 binding proteins, a class of immunophilins (Fischer et al., 1992; Hacker and Fischer, 1993; Riboldi-Tunnicliffe et al., 2001).

The limitation of using *mip* and 16S rRNA for molecular detection or the 23S–5S rRNA gene spacer region is that without nucleotide sequencing or other post-PCR analysis, these targets cannot distinguish serogroup 1 *L. pneumophila* from other serogroups and/or cannot detect non-*pneumophila* species of *Legionella* (Maurin et al., 2010; Yang et al., 2010). Since other serogroups of *L. pneumophila* and other species, such as *L. longbeachae* cause a significant burden of disease in many parts of the world (Yu et al., 2002; Gobin et al., 2009), their diagnosis and detection should be incorporated into any new molecular test. Non-serogroup 1 *L. pneumophila* and other species are currently likely to be significantly underrepresented given the bias of available tests such as the urine antigen test to the detection of *L. pneumophila* serogroup 1 (Benin et al., 2002). Therefore, there is significant scope to expand and improve current testing for *Legionella*.

**CAN LEGIONELLA GENOMICS INFORM MOLECULAR DETECTION METHODS?**

The recent *L. pneumophila* serogroup 1 genome sequences are an invaluable resource for molecular epidemiology and analysis of *L. pneumophila* genetic diversity. The six available *L. pneumophila*
genome sequences are all serogroup 1 human clinical isolates with worldwide distribution, and include endemic and epidemic strains (McDade et al., 1977; Jepras et al., 1985; Aurell et al., 2003; Nguyen et al., 2006; D’Auria et al., 2010; Schroeder et al., 2010). The core *L. pneumophila* genome contains many of the factors associated with the ability of the bacteria to replicate in eukaryotic cells but there is also great variability between strains (Cazale et al., 2008). Comparative analysis of the *L. pneumophila* genomes has revealed a diverse species where 7–11% of the genes in each *L. pneumophila* isolate are strain specific (Gomez-Valero et al., 2009). The genome exhibits high plasticity which presumably reflects the ability of the pathogen to acquire new genetic factors that enhance environmental survival and bacterial replication in eukaryotic cells. Some of the diversity occurs among genes encoding Dot/Icm effectors, including those within the same family (Cazale et al., 2008). Nevertheless, many elements of the *L. pneumophila* genome are highly conserved and these less variable factors may constitute useful targets for molecular detection and typing. In contrast to *L. pneumophila*, the *L. longbeachae* genome appears more highly conserved with few differences between strains and serotypes (Cazale et al., 2010; Kozak et al., 2010).

One of the most striking features of the *L. pneumophila* genome is the number and type of genes predicted to encode products that share similarity with eukaryotic proteins (Cazale et al., 2004; Gomez-Valero et al., 2009; Lomma et al., 2009; Schroeder et al., 2010). For example, *L. pneumophila* produces two enzymes that belong to the mammalian CD39 family of ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) (Sansom et al., 2007; Galka et al., 2008). NTPDases are associated almost exclusively with eukaryotes and so the *L. pneumophila* proteins, LpgI905, and Lpg0971 are likely to contribute to the way the bacteria interact with eukaryotic cells by mimicking eukaryotic NTPDases. Indeed, we showed recently that the bacterial protein is a conserved structural mimic of mammalian NTPDases (Vivian et al., 2010) and that LpgI905 enhances *L. pneumophila* intracellular replication (Sansom et al., 2007; Sansom et al., 2008). Virulence genes of *L. pneumophila* such as the eukaryotic type effectors or even genes of the Dot/Icm type IV secretion system have not yet been used widely as targets for molecular detection. However, since much of the detailed genetic information on *L. pneumophila* has only been obtained in recent years, the field of molecular diagnostics and detection is perhaps yet to capitalize on the usefulness of this information to inform molecular testing. Given the limited scope of the current PCR based tests, we believe that a knowledge of *Legionella* genomics could be used to improve rapid molecular detection of *Legionella* in environmental and clinical samples.

**DEVELOPMENT OF NEW GENERATION MOLECULAR TESTS: AN EXAMPLE IN PROGRESS**

Although the identification of *Legionella* species and serogroup is important for clinical and environmental management as well as epidemiological analysis, few rapid molecular tests can differentiate isolates of *L. pneumophila* serogroup 1 from other serogroups as well as *L. pneumophila* from other *Legionella* species (Tronel and Hartemann, 2009). Prior to determination of the *Legionella* genome sequences, we identified genes that were specific to *L. pneumophila* by experimental genomic subtractive hybridization of *L. pneumophila* with *L. micdadei* (Newton et al., 2006; Sansom et al., 2007). Two targets emerged as potentially useful for discriminating *Legionella* species and serogroups based on the fact that one, lpg0774 (lpp0839) (Cazale et al., 2008), was associated with the serogroup 1 LPS biosynthesis region and another, lpg1905 (lpp1880) encoding one of the *L. pneumophila* ecto-NTPDases (Sansom et al., 2007; Sansom et al., 2008), was specifically associated with *L. pneumophila*. We then assessed the suitability of these genes for the detection of *L. pneumophila* in clinical and environmental samples by designing a multiplex PCR to include lpg0774, lpg1905, and 16S rRNA. This multiplex PCR allowed the simultaneous identification of the genus *Legionella*, *L. pneumophila*, and serogroup 1 isolates of *L. pneumophila* when tested against a culture collection that comprised 36 strains of *L. pneumophila* of various serogroups and 20 non-*pneumophila* species (Figure 1 and not shown). The PCR was also effective when tested on a limited number of environmental and clinical samples (Figure 2). To our knowledge this is the first attempt to distinguish *L. pneumophila* from other *Legionella* species that does not rely on knowledge of the mip or 16S rRNA nucleotide sequence and that can identify serogroup 1 *L. pneumophila* without post-PCR analysis.

**WHAT IS THE SCOPE FOR FUTURE MOLECULAR DETECTION OF LEGIONELLA?**

The recent advances in *Legionella* genomics offer the possibility to rethink the targets currently used for molecular detection and diagnostics. Analysis of the nucleotide sequence of the LPS biosynthesis region and another, lpg0774 (Gene Bank: AY680227) the upstream primer started at base 46: 5\'-TGCTTAAACACACTATCCCAGAA3' and downstream primer started at base 202: 5\'-TGCTAACAACCACTATCCCAAA-3'. The 16S rRNA primers included in the triplex PCR to identify the genus *Legionella* have been described previously (Myamoto et al., 1997). Triplex PCR was performed using 20 ng of template DNA in a 25 μL PCR reaction mix containing 1× Green GoTaq® Flexi Buffer (Promega), 2 mM MgCl2, 200 μM dNTP, 0.5 μM of each primer and 1 U GoTaq® DNA polymerase. The optimized triplex PCR condition was performed in MyCycler™ (BIORAD) at initial denaturation of 95°C for 4 min followed by 35 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 5 min. The amplified products were then analyzed by DNA gel electrophoresis.

![Figure 1](representative gel showing simultaneous detection of lpg1905, lpg0774, and 16S rRNA by multiplex PCR. Std. 100 base pair marker (Promega); Lane 1, no DNA control; Lane 2, *L. pneumophila* serogroup 1 strain 02/41; Lane 3, *L. pneumophila* serogroup 1 strain B6; Lane 4, *L. pneumophila* serogroup 1 strain CS1; Lane 5, *L. pneumophila* serogroup 1 strain 02/41; Lane 6, *L. pneumophila* serogroup 2–14 strain C11(1); Lane 7, *L. pneumophila* serogroup 2–14 strain C4(1); Lane 8, *L. gormanii* strain C9; Lane 9, *L. anisa* strain L041; Lane 10, *L. gormanii* 03/69; Lane 11, Longbeachae A4CS; Lane 12, Longbeachae ATCC30462; Lane 13, *L. pneumophila* strain Lp041; Lane 14, *L. pneumophila* strain Lp042.) The upstream primer of lpg1905 (Gene Bank: AY680227) the upstream primer started at base 46: 5\'-TGCTTAAACACACTATCCCAGAA3' and downstream primer started at base 202: 5\'-TGCTAACAACCACTATCCCAAA-3'. The 16S rRNA primers included in the triplex PCR to identify the genus *Legionella* have been described previously (Myamoto et al., 1997). Triplex PCR was performed using 20 ng of template DNA in a 25 μL PCR reaction mix containing 1× Green GoTaq® Flexi Buffer (Promega), 2 mM MgCl2, 200 μM dNTP, 0.5 μM of each primer and 1 U GoTaq® DNA polymerase. The optimized triplex PCR condition was performed in MyCycler™ (BIORAD) at initial denaturation of 95°C for 4 min followed by 35 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 5 min. The amplified products were then analyzed by DNA gel electrophoresis.
regions for various serogroups could be adapted to the type of multiplexing described above and used to detect less common serogroups that have been associated with infection such as serogroup 3 (Chien et al., 2010). Additionally, with new knowledge of the genomes of other Legionella pathogens, such as L. longbeachae and L. micdadei, simple tests could be developed to identify multiple Legionella species using species-specific genetic targets. Targets drawn from comparative genomics will need to be validated against a large and diverse Legionella culture collection to ensure that they are as sensitive and accurate as possible. For example, although our own work suggested that lpg0774 was exclusively associated with serogroup 1 strains of L. pneumophila, a recent genome screen by microarray suggested that lpg0774 was present in 1 of 66 non-serogroup 1 strains of L. pneumophila (Cazalet et al., 2008). In addition lpg0774 was present in the majority but not all serogroup 1 L. pneumophila (128 of 150 isolates) (Cazalet et al., 2008). The multi-genome analysis performed by Cazalet et al. (2008) did suggest however that other LPS biosynthesis genes, lpg0766 (lpp0831), lpg0772 (lpp0837/wzm), and lpg0773 (lpp0873/wzt) may be useful markers of serogroup 1 strains of L. pneumophila as they were present in all serogroup 1 strains examined (150 isolates) and no non-serogroup 1 strains (66 isolates). Thus multiplex PCR using targets identified from comparative genomics, possibly combined with a recently described PCR based typing scheme that discriminates between monoclonal antibody subgroups of serogroup 1 strains (Thurmer et al., 2009), could significantly enhance our ability to detect and identify species and subgroups of Legionella rapidly and accurately. With an ever increasing knowledge of genomics and gene variation in Legionella, it is timely to update detection procedures to provide more precise and discriminatory testing for Legionella in clinical and environmental samples.

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