Evaluation of real-time PCR for the early detection of *Legionella pneumophila* DNA in serum samples

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Legionella pneumonia can be difficult to diagnose. Existing laboratory tests all have shortcomings, especially in the ability to diagnose Legionnaires' disease (LD) at an early stage of the disease in a specimen that is readily obtainable. The aim of this study was to assess the performance of PCR as a rapid diagnostic method and to compare the results of different PCR assays of serum samples from patients with LD. Samples included 151 serum samples from 68 patients with proven LD and 60 serum samples from 36 patients with respiratory tract infections other than *Legionella*. PCR assays were based on the 5S rRNA gene, 16S rRNA gene and the *mip* gene. The samples from patients with infections caused by pathogens other than *Legionella* all tested negative in PCR. Among the patients with proven LD 54.4 % (37/68) tested positive in 5S rRNA PCR, 52.9 % (36/68) in *mip* gene PCR and 30.9 % (21/68) in 16S rRNA PCR in the first available serum sample. The association between threshold cycle value in 5S PCR positive serum samples ($n=49$) and C-reactive protein value was determined, and showed a strong negative correlation (Pearson correlation coefficient $r=-0.63$, $P<0.0001$). In addition to existing tests for the diagnosis of LD, detection of *Legionella* DNA in serum could be a useful tool for early diagnosis of LD caused by any *Legionella* species and serogroup, and has the potential to provide a diagnosis in a time frame that could affect initial infection management.

Received 8 May 2006

Accepted 22 September 2006

INTRODUCTION

Legionnaires' disease (LD) is an acute pneumonia caused by *Legionella* spp., which are Gram-negative bacteria ubiquitous in both man-made and natural aquatic reservoirs. *Legionella* spp. are responsible for 1 to 5 % of cases of community-acquired pneumonia (CAP) (Breiman & Butler, 1998; Van der Eerden *et al.*, 2005). Although currently 48 *Legionella* species have been described, more than 90 % of culture-confirmed clinical cases are caused by *Legionella pneumophila* (Yu *et al.*, 2002). *Legionella* pneumonia can be difficult to diagnose because the signs and symptoms are nonspecific and do not distinguish *Legionella* infection from other common causes of pneumonia (Den Boer & Yzerman, 2004). A definite diagnosis is important because it enables adequate antimicrobial treatment; a delay in starting appropriate therapy has been associated with increased mortality (Heath *et al.*, 1996). Currently, the diagnosis of LD in patients with pneumonia is based on phenotypic (culturing, serological testing, antigen detection in urine) and genotypic methods

(PCR) using respiratory specimens. Isolation of *Legionella* from respiratory secretions is considered the gold standard in case definition, but is not very sensitive (10 to 80 % sensitivity) and a positive culture is not normally available until 3 days post incubation (Murdoch, 2003; Den Boer & Yzerman, 2004). Another major limitation of culturing is that fewer than one half of patients with LD produce sputum (Murdoch, 2003). Serological testing for *Legionella* infection is a valuable epidemiological tool but has little impact on clinical decision making because of the time delay before a result is available (Den Boer & Yzerman, 2004). For the detection of *L. pneumophila* serogroup 1, urinary antigen tests have sensitivities in the range of 56–99 % and specificities approaching 100 %. A major disadvantage with these tests is their inability to reliably detect organisms other than *L. pneumophila* serogroup 1 (Den Boer & Yzerman, 2004; Yzerman *et al.*, 2002).

PCR-based methods can detect *Legionella*-specific DNA in respiratory secretions, with varying degrees of sensitivity and specificity (Murdoch, 2003; Hayden *et al.*, 2001; Cloud *et al.*, 2000; Ballard *et al.*, 2000). Application of PCR to nonrespiratory samples is particularly attractive, because this could circumvent the problem of patients who do not

Abbreviations: CAP, community-acquired pneumonia; CRP, C-reactive protein; Ct, threshold cycle; LD, Legionnaires' disease.

produce sputum samples (Diederer *et al.*, 2006; Murdoch *et al.*, 1996). In a recent report, a *Legionella* specific 5S rRNA PCR on patient serum was positive in 80.5 % of cases (54/67 patients), with a peak positivity at 6–10 days after disease onset (Lindsay *et al.*, 2004). Unlike urinary antigen, the number of PCR positives decreased very slowly, perhaps as a result of the slow release of DNA from *Legionella* surviving in macrophages.

The aim of this study was to assess the performance of PCR as a rapid diagnostic method and to compare the results of different PCR assays in serum samples from patients with LD.

METHODS

Patients. Samples were collected between June 1999 and May 2005 at the following hospitals in The Netherlands: St Elisabeth Hospital and TweeSteden Hospital, Tilburg; Pasteur Hospital, Oosterhout; TweeSteden Hospital, Waalwijk; Lievensberg Hospital, Bergen op Zoom; Franciscus Hospital, Roosendaal and Amphia Hospital, Breda. The samples were stored at -70°C until they were tested. A proven case of LD was defined as a patient with pneumonia, who showed radiological signs of infiltration, and who showed laboratory evidence of infection with *L. pneumophila*. Laboratory evidence included (i) a single high value of IgM and/or IgG antibodies or seroconversion for *L. pneumophila* serogroup 1 to 7 in paired acute-phase and convalescent-phase sera using a commercial ELISA (Serion ELISA; Institut Virion\Serion) (Boshuizen *et al.*, 2003), plus (ii) one or more of the following: isolation of *L. pneumophila* from a respiratory tract sample, a positive urinary antigen test (Binax NOW), a positive PCR result for *L. pneumophila* on a respiratory tract sample using a 16S rRNA assay (Van der Zee *et al.*, 2002).

In addition, 60 serum samples from 36 patients with respiratory tract infections other than *Legionella* were tested in a similar manner to serve as controls. All control patients tested negative for *Legionella* antigen in urine (Binax NOW) and *L. pneumophila* serology (Serion ELISA). Controls consisted of 5 patients with CAP and positive blood cultures with *Streptococcus pneumoniae* (5 samples) and 31 patients with respiratory tract infections and a fourfold rise in (complement-fixing) antibody titre against influenza A virus, adenovirus, *Chlamydia psittaci* or *Mycoplasma pneumoniae* (55 samples).

Bacterial strains. Validation of PCR assays was carried out using a panel of human-pathogenic and environmental *Legionella* spp. The following bacterial strains were used: Philadelphia-1 (SG1) ATCC33152, Togus-1 (SG2) ATCC33154, Bloomington-2 (SG3) ATCC33155, L.A.-1 (SG4) ATCC33156, Dallas-1E (SG5) ATCC33216, Chicago-2 (SG6) ATCC33215, Chicago-8 (SG7) ATCC33823, Concord-3 (SG8) ATCC35096, IN-23 (SG9) ATCC35289, Leiden-1 (SG10) ATCC43283, 797/PA/H (SG11) ATCC43130, 570-CO-H (SG12) ATCC43290, 82A3105 (SG13) ATCC43736, 1169-MN-H (SG14) ATCC43703, *Legionella anisa* ATCC35292, *Legionella birminghamensis* ATCC43702, *Legionella bozemanii-1* ATCC33217, *Legionella bozemanii-2* ATCC35545, *Legionella brunensis*, *Legionella cherii* ATCC35252, *Legionella dumoffii* ATCC35850, *Legionella erythra* ATCC35303, *Legionella feeleyi-1* ATCC35072, *Legionella feeleyi-2* ATCC35849, *Legionella gormanii* ATCC33297, *Legionella hackeliae-1* ATCC35250, *Legionella israelensis* ATCC43119, *Legionella jamestowniensis* ATCC35298, *Legionella jordanis* ATCC33623, *Legionella longbeachae-1* ATCC33462, *Legionella longbeachae-2* ATCC33484, *Legionella maceachernii* ATCC35300, *Legionella micdadei* ATCC33218, *Legionella moravica* ATCC43877, *Legionella oakridgensis* ATCC33761, *Legionella parisiensis*

ATCC35299, *Legionella rubrilucens* ATCC35304, *Legionella sainthe-lensi* ATCC35248, *Legionella santicrusis* ATCC35301, *Legionella spiri-tensis* ATCC35249, *Legionella steigerwaltii* ATCC35302, *Legionella tucsonensis* ATCC49180, *Legionella wadsworthii* ATCC33877. In addition, cultures of non-*Legionella* bacteria were analysed; *Streptococcus pneumoniae* ATCC49619, *Bordetella pertussis* Tohama strain, *Bordetella parapertussis* B24, *Mycoplasma pneumoniae* ATCC15293, *Chlamydia pneumoniae* ATCCVR1355, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* ATCC27853 and *Lactobacillus casei*. In addition, clinical specimens were tested from hospitalized patients with community-acquired respiratory tract infections (Schneeberger *et al.*, 2004) and laboratory evidence for infection with *Streptococcus pneumoniae* (15), *Haemophilus influenzae* (10), *Moraxella catarrhalis* (4), *Streptococcus pyogenes* (1), *Mycoplasma pneumoniae* (10) and *Chlamydia pneumoniae* (5).

PCR assays. For the detection of *Legionella* in serum samples three assays were used, targeted at specific regions within the 5S rRNA gene, the 16S rRNA gene and the *mip* gene (Table 1). The 5S PCR was based on the primers described by Lindsay *et al.* (2004) and detected in real-time using a TaqMan probe Leg5S. Real-time PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). DNA was amplified according to the following parameters: after 2 min incubation at 50°C and 10 min denaturation at 95°C , amplification consisted of 50 cycles of 15 s denaturation at 95°C followed by 1 min annealing and extension at 60°C .

DNA extraction. A sample of 200 μl was processed with a MagNA Pure LC (Wilson *et al.*, 2004), using the total nucleic acid kit (Roche Diagnostics) with an elution volume of 50 μl . A 5 μl aliquot of the eluate was used as template in PCR.

Controls. Serum samples from two healthy volunteers were included as negative controls and were processed after every four clinical samples. As an internal control Phocid herpesvirus (PhoHV) was added to the samples to monitor processing as well as inhibition of PCR (Niesters, 2001). Detection of PhoHV was included in the *mip* gene PCR. In each run a no-template control (mix control) was included. Sensitivity controls for the PCR consisted of 10-fold dilutions of *L. pneumophila* DNA ranging from 1000 to 10 fg (approximately 230 to 2.3 genome equivalents). Primers and probes were synthesized by Applied Biosystems.

Interpretation of results. Results obtained in real-time PCR were expressed as threshold cycle (Ct) values, corresponding to the cycle at which PCR entered the exponential phase. If no increase in fluorescent signal was observed after 50 cycles, the sample was assumed to be negative.

Statistical analysis. To determine the degree of linear relationship between inflammation [C-reactive protein (CRP) value] and Ct value, a Pearson correlation coefficient was calculated (<http://statpages.org/>).

RESULTS AND DISCUSSION

Although more than 90 % of culture-confirmed clinical cases of LD are caused by *L. pneumophila*, some non-*pneumophila* *Legionella* species are also known to cause disease in humans (Muder & Yu, 2002). PCR based diagnosis of *Legionella* is a very sensitive method, but can be difficult with regard to discrimination of *L. pneumophila* from other *Legionella* species. Theoretically, 5S rRNA gene based PCR can detect all *Legionella*, but the primers have been reported to detect other microorganisms (Maiwald *et al.*, 1995). The *mip* gene based PCR can detect all *L.*

Table 1. Primers and probes for PCR assays

Primer/probe	Sequence	Product size
5S rRNA PCR*	5S1 (forward 5'-ACTATAGCGATTTGGAACCA-3') 5S2 (reverse 5'-GCGATGACCTACTTTCGCAT-3')	104 bp
<i>mip</i> PCR	Leg5S (5'-FAM-CCGCGCCAATGATAGTGTGAGGC-TAMRA-3') Mip-F1 (forward 5'-GCCAAGTGGTTTGAATACAAA-3') Mip-R1 (reverse 5'-CTCGACAGTGACTGTATCCGATTT-3')	80 bp
16S rRNA PCR†	LPN- <i>mip</i> (5'-FAM-TAATCAATGCTGGAAATGGTGTAAACCCG-TAMRA-3') Leg-F1 (forward 5'-TACCTACCCCTTGACATACAGTG-3') Leg-R1 (reverse 5'-CTTCCTCCGGTTTGTAC-3')	150 bp
PhoHV PCR‡	LPN-1 (5'-FAM-AGTCCCCACCATCACAT-MGB-3') LSPP-1 (5'-VIC-CCGTAACGAGCGCAA-MGB-3') PhHV-F1 (forward 5'-GGCGAATCACAGATTGAATC-3') PhHV-R1 (reverse 5'-GCGGTTCCAAACGTACCAA-3')	80 bp
	PhHV-1 (5'-VIC-TTTTTTATGTGTCCGCCACCATCTGGATC-TAMRA-3')	

FAM, 6-carboxyfluorescein; MGB, minor groove binding protein (non-fluorescent); TAMRA, 6-carboxytetramethylrhodamine.

*Primers according to Lindsay *et al.* (2004).

†Primers according to Van der Zee *et al.* (2002).

‡Primers according to Niesters (2001).

pneumophila but also may detect some non-*pneumophila* *Legionella* species. The 16S rRNA gene based PCR may provide the most clear discrimination between *L. pneumophila* and other *Legionella* species (Van der Zee *et al.*, 2002) although its theoretical specificity is no longer 100 % due to the ongoing expansion of sequence data available in recent years. In this study the three PCR assays were revalidated. The analytical specificities and sensitivities of PCR assays were investigated as described in Methods. With the 5S rRNA gene based PCR only *Legionella* species were detected. With the *mip* gene based PCR all serogroups of *L. pneumophila* were detected and *L. israelensis*. With 16S rRNA based PCR only *L. pneumophila* and *Legionella* species were detected; a 100 % specificity of discrimination was observed. No PCR positives were found among non-*Legionella* bacteria tested. In clinical materials from patients with CAP of known aetiology no *Legionella* spp. DNA was detected in any of the three assays. The sensitivity of the 5S rRNA based PCR assay was highest, with a lower detection limit of 10 fg chromosomal DNA. In the *mip* gene based assay, the lower detection limit was 100 fg, and 1000 fg was found as lower detection limit for the 16S rRNA based PCR.

In order to study the usefulness of serum for PCR-based diagnosis, we included 68 patients with proven LD, with 151 serum samples taken in total. The cases included were 68 adults (48 males and 20 females, male-female ratio 2.4) between 32 and 78 years old (mean age 54.0 years). The laboratory results of these patients were as follows: [positive/number tested (%)] serology 68/68 (100 %), isolation 15/29 (52 %), PCR 26/26 (100 %) and urinary antigen test 55/60 (92 %). Since no records were made of the first day of illness, we arbitrarily designated the 'acute-phase' serum sampling as the starting point of the disease (day 0). Among the patients with proven LD 54.4 % (37/68) tested positive in 5S

rRNA PCR, 52.9 % (36/68) in *mip* gene PCR and 30.9 % (21/68) in 16S rRNA PCR in the first available serum sample. Among LD cases, 20/68 (29.4 %) tested positive in all three PCRs (Table 2), with mean Ct values of 29.3, 31.5 and 32.5, respectively, for 5S rRNA, *mip* and 16S rRNA gene based PCRs. Another 15 (22.1 %) patients were PCR positive in 5S rRNA and *mip* gene based PCR (mean Ct values of 35.2 and 37.8) but negative in 16S rRNA based PCR, 1 patient (1.5 %) was positive in both 5S rRNA and 16S rRNA based PCR (Ct values of 35.4 and 45.0, respectively), 1 patient was positive only in 5S rRNA based PCR (Ct value 36.9) and 1 patient was positive only in *mip* based PCR (Ct value 38.5). The detection rate for all (non-inhibited) samples was 34.9 % (49/149), 35.6 % (48/149) and 16.8 % (25/149) for 5S rRNA, *mip* gene and 16S rRNA based PCR, respectively. In two samples (obtained from two patients) inhibition of PCR occurred.

Failure to diagnose and treat LD early has been found to contribute to increased morbidity and mortality (Heath *et al.*, 1996). Existing laboratory tests lack sensitivity in detecting all cases of LD or provide only a retrospective diagnosis. The theoretical advantages of PCR for the detection of *L. pneumophila* DNA in serum are evident: serum samples are readily obtainable and can be processed within a working day. Our findings are largely in concordance with results previously reported (Diederer *et al.*, 2006). For an illness of low prevalence such as LD the specificity of a test is an important parameter, and our results suggest that the positive predictive value obtained using PCR on serum is high. In contrast to the high sensitivity found by Lindsay *et al.* (2004), the sensitivity of PCR found in our study was relatively low. Sensitivity of PCR is likely to increase when testing samples that are obtained early in the course of illness, when testing of more

Table 2. Data for patients with LD

Patient no.	Serum no.	Age (years)/gender	UAG	PCR	Serology	Culture	Interval	5S* (Ct)	mip* (Ct)	16S (Ct)			
1	1	64/male	+	+	+	+	0	+	(24.4)	+	(24.5)		
	2						22	+	(35.1)	+	(41.5)	—	
	3						80	—	—	—	—		
	4						89	—	—	—	—		
2	5	46/male	+	+	+	ND	0	+	(31.2)	+	(33.7)	+	(33.2)
	6						13	—	—	—	—		
	7						21	—	—	—	—		
3	8	57/male	+	+	+	ND	0	+	(37.0)	+	(40.8)	—	
	9						10	—	—	—	—		
4	10	59/female	+	+	+	+	0	—	—	—	—		
	11						10	—	—	—	—		
5	12	72/female	+	+	+	+	0	+	(35.7)	+	(36.9)	—	
	13						19	—	—	—	—		
	14						51	—	—	—	—		
6	15	54/female	+	+	+	ND	0	+	(30.5)	+	(32.9)	+	(34.7)
7	16	41/male	+	+	+	ND	18	+	(36.2)	+	(41.8)	—	
8	17	41/male	+	ND	+	ND	0	+	(34.8)	+	(38.2)	+	(43.7)
	18						20	—	—	—	—		
9	19	64/male	+	ND	+	ND	0	+	(35.4)	—	—	+	(45.0)
	20						16	—	—	—	—		
10	21	55/male	+	+	+	+	0	—	—	—	—		
	22						16	—	—	—	—		
11	23	44/male	+	ND	+	ND	0	—	—	—	—		
12	24	44/male	+	+	+	+	0	+	(38.0)	+	(41.8)	—	
	25						57	—	—	—	—		
	26						A0	—	—	—	—		
13	27	48/female	+	ND	+	-	28	—	—	—	—		
	28						42	—	—	—	—		
	29						0	—	—	—	—		
14	30	69/female	+	ND	+	ND	0	—	—	—	—		
	31						8	—	—	—	—		
	32						0	+	(26.4)	+	(29.4)	+	(26.9)
16	33	74/male	+	+	+	-	17	—	—	—	—		
	34						0	+	(33.6)	+	(36.3)	+	(39.2)
17	35	50/male	+	ND	+	ND	11	—	—	—	—		
	36						0	+	(30.2)	+	(32.7)	+	(32.5)
18	37	56/female	+	ND	+	ND	21	—	—	—	—		
	38						0	+	(27.5)	+	(29.9)	+	(28.0)
19	39	53/male	+	+	+	ND	23	—	—	—	—		
	40						0	+	(27.4)	+	(29.6)	+	(28.4)
20	41	32/female	ND	+	+	ND	60	—	—	—	—		
	42						0	+	(35.0)	+	(42.9)	—	
21	43	49/male	ND	+	+	ND	19	—	—	—	—		
	44						0	+	(32.8)	+	(35.0)	+	(41.8)
22	45	58/male	+	ND	+	ND	17	—	—	—	—		
	46						0	—	—	—	—		
23	47	57/female	-	+	+	ND	13	—	—	—	—		
	48						0	—	—	—	—		
24	49	62/male	-	+	+	ND	12	—	—	—	—		
	50						0	—	—	—	—		
25	51	43/male	-	+	+	ND	14	—	—	—	—		
	52						0	+	(32.0)	+	(34.5)	+	(36.5)
26	53	61/male	+	ND	+	ND	5	—	—	—	—		
	54						0	—	—	—	—		
27	54	43/male	ND	+	+	ND	0	—	—	—	—		

Table 2. cont.

Patient no.	Serum no.	Age (years)/gender	UAG	PCR	Serology	Culture	Interval	5S* (Ct)	mip* (Ct)	16S (Ct)
	55						15	—	—	—
	56						28	—	—	—
28	57	40/male	+	ND	+	ND	0	+ (31.0)	+ (33.1)	+ (32.7)
	58						16	—	—	—
29	59	42/male	+	ND	+	ND	0	—	—	—
30	60	55/female	+	ND	+	ND	0	+ (29.1)	+ (31.3)	+ (29.8)
	61						13	—	—	—
	62						19	—	—	—
31	63	57/male	+	ND	+	-	0	+ (32.2)	+ (34.2)	+ (35.2)
32	64	51/male	ND	+	+	ND	0	—	—	—
	65						40	—	—	—
33	66	64/male	ND	+	+	ND	0	—	—	—
	67						15	—	—	—
34	68	65/male	ND	+	+	ND	0	+ (30.6)	+ (33.1)	+ (31.9)
	69						33	—	—	—
35	70	45/male	+	ND	+	+	0	+ (38.4)	+ (38.1)	—
	71						11	—	—	—
36	72	69/female	-	+	+	+	0	—	—	—
	73						6	—	—	—
37	74	73/female	+	ND	+	+	0	—	—	—
	75						7	—	—	—
38	76	78/male	+	ND	+	-	0	+ (32.8)	+ (34.6)	—
	77						15	—	—	—
39	78	45/male	+	ND	+	-	0	—	+ (38.5)	—
	79						15	INH	INH	INH
	80						23	—	—	—
40	81	43/female	ND	+	+	ND	0	+ (36.9)	—	—
41	82	57/female	-	+	+	-	0	+ (32.4)	+ (33.1)	—
	83						7	+ (37.3)	—	—
42	84	53/male	+	ND	+	ND	0	—	—	—
43	85	58/male	+	ND	+		0	—	—	—
	86						14	—	—	—
44	87	55/male	+	ND	+	ND	0	—	—	—
	88						58	—	—	—
45	89	34/male	+	ND	+	+	0	+ (27.9)	+ (29.9)	+ (28.9)
	90						5	+ (35.7)	+ (39.4)	—
	91						45	—	—	—
46	92	68/male	+	ND	+	-	0	—	—	—
	93						13	—	—	—
	94						60	—	—	—
	95						75	—	—	—
47	96	33/male	+	ND	+	-	0	—	—	—
48	97	54/male	+	ND	+	ND	0	—	—	—
	98						26	—	—	—
49	99	56/male	+	ND	+	+	0	+ (29.3)	+ (29.4)	—
	100						24	—	—	—
50	101	57/female	+	ND	+	-	0	+ (38.6)	+ (37.7)	—
	102						31	—	—	—
51	103	56/female	+	ND	+	ND	0	+ (32.8)	+ (35.0)	+ (38.8)
	104						28	—	—	—
52	105	47/male	+	ND	+	+	0	+ (32.3)	+ (34.4)	+ (40.1)
	106						29	—	+ (38.1)	—
53	107	54/male	+	ND	+	-	0	—	—	—
	108						13	—	—	—

Table 2. cont.

Patient no.	Serum no.	Age (years)/gender	UAG	PCR	Serology	Culture	Interval	5S* (Ct)	mip* (Ct)	16S (Ct)			
54	109	47/female	+	ND	+	+	0	—	—	—			
55	110	53/male	+	ND	+	-	0	—	—	—			
56	111	57/female	+	ND	+	ND	0	+	(33.1)	+	(35.0)	+	(41.1)
	112						21	—	—	—			
57	113	59/female	+	ND	+	+	0	+	(36.3)	+	(40.1)	—	—
	114						28	—	—	—	—	—	—
	115						38	—	—	—	—	—	—
58	116	62/male	+	ND	+	ND	0	+	(34.7)	+	(35.3)	+	(38.5)
	117						29	—	—	—	—	—	—
59	118	50/male	+	ND	+	ND	0	+	(35.1)	+	(35.5)	—	—
	119						12	—	—	—	—	—	—
	120						14	—	—	—	—	—	—
60	121	46/male	+	ND	+	-	0	+	(35.6)	+	(40.7)	—	—
	122						34	—	—	—	—	—	—
61	123	47/male	+	ND	+	-	0	—	—	—	—	—	—
62	124	63/male	+	ND	+	ND	0	—	—	—	—	—	—
	125						22	—	—	—	—	—	—
63	126	43/male	+	ND	+	ND	0	—	—	—	—	—	—
64	127	60/male	ND	+	+	ND	0	+	(33.4)	+	(37.4)	—	—
	128						22	—	—	—	—	—	—
65	129	64/male	+	ND	+	+	0	+	(34.8)	+	(35.6)	—	—
66	130	63/male	+	ND	+	ND	0	—	—	—	—	—	—
	131						21	—	—	—	—	—	—
67	132	59/female	+	+	+	+	0	+	(34.0)	+	(35.2)	+	(35.9)
	133						1	+	(34.2)	+	(34.9)	+	(34.0)
	134						2	+	(35.2)	+	(36.2)	—	—
	135						3	+	(35.8)	+	(37.0)	—	—
	136						4	+	(36.8)	+	(36.5)	+	(37.1)
	137						5	+	(37.5)	+	(37.4)	—	—
	138						6	+	(37.6)	+	(39.7)	+	(37.2)
	139						7	+	(38.6)	+	(40.7)	—	—
	140						9	+	(39.4)	+	(40.7)	—	—
	141						10	—	—	—	—	—	—
	142						11	—	—	+	—	—	—
	143						13	—	—	+	—	—	—
	144						14	—	—	—	—	—	—
68	145	53/male	+	+	+	-	0	—	—	—	—	—	—
	146						1	—	—	—	—	—	—
	147						2	INH	INH	INH	INH	INH	INH
	148						3	—	—	—	—	—	—
	149						4	—	—	—	—	—	—
	150						6	—	—	—	—	—	—
	151						7	—	—	—	—	—	—

UAG, Urinary antigen test; interval, number of days since first serum sample; INH, inhibition of PCR; ND, not determined.

*Among the patients with proven LD 54.4 % (37/68) tested positive in 5S rRNA PCR, 52.9 % (36/68) in *mip* gene PCR and 30.9 % (21/68) in 16S rRNA PCR in the first available serum sample. The detection rate for all (non-inhibited) samples was 34.9 % (49/149), 35.6 % (48/149) and 16.8 % (25/149) for 5S rRNA, *mip* gene and 16S rRNA based PCR, respectively. In two samples (obtained from two patients) inhibition of PCR occurred.

than one serum sample from each patient is performed and in patients with severe pneumonia, e.g. those with a pneumonia severity index (PSI) of 4 and 5. The urinary antigen test is less reliable in milder cases of LD, and it would

be plausible that the same holds true for the detection of *Legionella* DNA in serum (Yzerman *et al.*, 2002). Because of the retrospective nature of our observations we were not able to investigate the relation between test sensitivity and

the severity of the disease. However, the presence of *L. pneumophila* DNA in serum could correlate with an acute phase response as reflected in high CRP values (Diederer *et al.*, 2006). The Ct value is inversely proportional to the log of the amount of target DNA initially present. In the current study, the association between Ct value in 5S PCR positive serum samples ($n=49$) and CRP value was determined, and showed a strong negative correlation (Pearson correlation coefficient $r=-0.63$, $P<0.0001$). Wever *et al.* (2006) studied the kinetics of *L. pneumophila* DNA in serum samples of patients with LD in relation to CRP using a *mip* gene based PCR. Fifteen hospitalized patients with LD were studied on the day of admission up to 10 days thereafter. Serum samples obtained on the day of admission were *mip* gene PCR positive in 7/11 (64%) patients and negative in 4/11 (36%) patients. Interestingly, the lowest Ct value (26.6) was found in a patient who died on day 5 following admission. On the day of admission, the mean CRP level in the 7 patients with a *mip* PCR positive serum sample was 499 ± 144 mg l⁻¹ (median $\pm$ SD) and 244 ± 97 mg in the 4 patients with a negative PCR result.

Currently, a positive culture, a positive urinary antigen test or a fourfold or greater rise in antibody titre against *L. pneumophila* is definitive of a confirmed case, and PCR-positive samples are only classified as presumptive by the European Working Group on Legionella Infections (EWGLI). There is an apparent need for prospective studies to determine the exact sensitivity and specificity of PCR in patient samples. In our experience, PCR as a diagnostic in patients with severe CAP is a sensitive and specific tool in those who produce sputum. The high specificity is exemplified by a recent investigation conducted by our laboratory (Diederer *et al.*, 2005). In this case-control study, nose and throat samples from patients presenting with acute respiratory tract infections and matched controls were analysed for the presence of *Legionella* spp. by real-time 16S rRNA based PCR. *Legionella* DNA was not detected in any of the cases or controls. Although our definition of a proven case was different from that defined by EWGLI and included a positive PCR, the majority (97%, 65/67) of our patients would also be considered a proven case according to EWGLI criteria (Table 2). We performed a retrospective, laboratory-based study, using serum samples from patients with proven LD and patients with respiratory tract infections other than *Legionella*. Ideally, a prospective study should be conducted to evaluate the value of *Legionella*-specific PCR using consecutive serum samples in patients with (severe) pneumonia. However, our findings suggest that detection of *L. pneumophila* DNA in serum could be a valuable tool in addition to existing diagnostic tests for the rapid diagnosis of LD in the acute phase of disease, and has the potential to detect infections caused by any *Legionella* species and serogroup. The strong association between Ct value and CRP value suggest that application of PCR for the detection of *Legionella* should especially be considered in patients with severe pneumonia of unknown origin. In our view, the best initial testing strategy that will detect all *Legionella*

species and provide results within a time frame that will affect clinical management consists of a urinary antigen test combined with *Legionella* PCR on sputum and serum.

REFERENCES

- Ballard, A. L., Fry, N. K., Chan, L., Surman, S. B., Lee, J. V., Harrison, T. G. & Towner, K. J. (2000). Detection of *Legionella pneumophila* using a real-time PCR hybridization assay. *J Clin Microbiol* **38**, 4215–4218.
- Boshuizen, H. C., Den Boer, J. W., de Melker, H., Schellekens, J. F., Peeters, M. F., van Vliet, J. A. & Conyn-van Spaendonck, M. A. (2003). Reference values for the SERION classic ELISA for detecting *Legionella pneumophila* antibodies. *Eur J Clin Microbiol Infect Dis* **22**, 706–708.
- Breiman, F. R. & Butler, J. C. (1998). Legionnaires' disease: clinical, epidemiological, and public health perspectives. *Semin Respir Infect* **13**, 84–89.
- Cloud, J. L., Carroll, K. C., Pixton, P., Erali, M. & Hillyard, D. R. (2000). Detection of *Legionella* species in respiratory specimens using PCR with sequencing confirmation. *J Clin Microbiol* **38**, 1709–1712.
- Den Boer, J. W. & Yzerman, E. P. F. (2004). Diagnosis of *Legionella* infection in Legionnaires' disease. *Eur J Clin Microbiol Infect Dis* **23**, 871–878.
- Diederer, B. M. W., de Jong, C. M. A., Aarts, I., Peeters, M. F., van Gageldonk-Lafeber, A. B., Wilbrink, B. & Van der Zee, A. (2005). No evidence of *Legionella* infection in general practice patients presenting with acute respiratory infections in The Netherlands. *Clin Microbiol Infect* **11**, 410–412.
- Diederer, B. M. W., de Jong, C. M. A., Kluytmans, J. A. J. W., Van der Zee, A. & Peeters, M. F. (2006). Detection and quantification of *Legionella pneumophila* DNA in serum: case reports and review of the literature. *J Med Microbiol* **55**, 639–642.
- Hayden, R. T., Uhl, J. R., Qian, X., Hopkins, M. K., Aubry, M. C., Limper, A. H., Lloyd, R. V. & Cockerill, F. R. (2001). Direct detection of *Legionella* species from bronchoalveolar lavage and open lung biopsy specimens: comparison of LightCycler PCR, *in situ* hybridization, direct fluorescence antigen detection and culture. *J Clin Microbiol* **39**, 2618–2626.
- Heath, C. H., Grove, D. I. & Looke, D. F. (1996). Delay in appropriate therapy of *Legionella*-pneumonia associated with increased mortality. *Eur J Clin Microbiol Infect Dis* **15**, 286–290.
- Lindsay, D. S., Abraham, W. H., Findlay, W., Christie, P., Johnston, F. & Edwards, G. F. (2004). Laboratory diagnosis of Legionnaires' disease due to *Legionella pneumophila* serogroup 1: comparison of phenotypic and genotypic methods. *J Med Microbiol* **53**, 183–187.
- Maiwald, M., Schill, M., Stockinger, C., Helbig, J. H., Luck, P. C., Witzleb, W. & Sonntag, H. G. (1995). Detection of *Legionella* DNA in human and guinea pig urine samples by the polymouse chain reaction. *Eur J Clin Microbiol Infect Dis* **14**, 25–33.
- Muder, R. R. & Yu, V. L. (2002). Infection due to *Legionella* species other than *L. pneumophila*. *Clin Infect Dis* **35**, 990–998.
- Murdoch, D. R. (2003). Diagnosis of *Legionella* infection. *Clin Infect Dis* **36**, 64–69.
- Murdoch, D. R., Walford, E. J., Jennings, L. C., Light, G. J., Schousboe, M. I., Cheresky, A. Y., Chambers, S. T. & Town, G. I. (1996). Use of the polymerase chain-reaction to detect *Legionella* DNA in urine and serum samples from patients with pneumonia. *Clin Infect Dis* **23**, 475–480.
- Niesters, H. G. (2001). Quantitation of viral load using real-time amplification techniques. *Methods* **25**, 419–429.

- Schneeberger, P. M., Dorigo-Zetsma, J. W., Van der Zee, A., van Bon, M. & van Opstal, J. L. (2004). Diagnosis of atypical pathogens in patients hospitalized with community-acquired respiratory infection. *Scand J Infect Dis* **36**, 269–273.
- Van der Eerden, M. M., Vlassembler, F., de Graaff, C. S., Groot, T., Jansen, H. M. & Boersma, W. G. (2005). Value of intensive diagnostic microbiological investigation in low- and high-risk patients with community-acquired pneumonia. *Eur J Clin Microbiol Infect Dis* **24**, 241–249.
- Van der Zee, A., Verbakel, H., de Jong, C., Pot, R., Bergmans, A., Peeters, M., Schneeberger, P. & Schellekens, J. (2002). Novel PCR-probe assay for detection of and discrimination between *Legionella pneumophila* and other *Legionella* species in clinical samples. *J Clin Microbiol* **40**, 1124–1125.
- Wever, P. C., van de Veerdonk, F., Schellekens, J. J. A., Huijsmans, C. J. J., de Jager, C. P. C. & Hermans, M. H. A. (2006). Presence of *Legionella pneumophila* DNA in serum samples during Legionnaires' disease in relation to C-reactive protein levels. *Ned Tijdschr Med Microbiol* **14** supplement, S87.
- Wilson, D., Yen-Lieberman, B., Reischl, U., Warshawsky, I. & Procop, G. W. (2004). Comparison of five methods for extraction of *Legionella pneumophila* from respiratory specimens. *J Clin Microbiol* **42**, 5913–5916.
- Yu, V. L., Plouffe, J. F., Pastoris, M. C., Stout, J. E., Schousboe, M., Widmer, A., Summersgill, J., File, T., Heath, C. M. & other authors (2002). Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative study. *J Infect Dis* **186**, 127–128.
- Yzerman, E. P., Den Boer, J. W., Lettinga, K. D., Schellekens, J., Dankert, J. & Peeters, M. (2002). Sensitivity of three urinary antigen tests associated with clinical severity in a large outbreak of Legionnaires' disease in the Netherlands. *J Clin Microbiol* **40**, 3232–3236.