

DETECTION AND
QUANTIFICATION OF
LEGIONELLAE IN HOSPITAL
WATER SAMPLES BY
QUANTITATIVE REAL-TIME PCR

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Background

- Risk assessment of nosocomial *Legionella* infection, requires surveillance and rapid monitoring of suspected hospital water supplies.
- The *Legionella pneumophila* strain is responsible for ~ 90 % of the pathogenic infections.
- Conventional culture methods for detection of *Legionella* spp. and *Legionella pneumophila* in water samples present several disadvantages including low sensitivity, and long incubation periods (10 to 13 days of analysis for positive samples).
- Several assays based on the polymerase chain reaction (PCR) have been evaluated. The advent of real time PCR technology has made possible reductions in analysis time.

Materials and Methods (1)

- One hundred environmental water samples from various sites of the CHU Nancy were investigated by *Legionella pneumophila* real-time PCR assay, of which 80 were also tested by *Legionella* spp. real-time PCR assay.
- In parallel conventional culture method (NF T90 431) for detection and count of “viable” *Legionella* cells was performed.

Materials and Methods (2)

PROTOCOL

- Sampling : water samples of 1 liter.
- Water sample filtration (concentration).
- DNA extraction.
- Purification on columns.
- Quantitative detection of *L. pneumophila* and *Legionella* spp. performed on each DNA extracted samples using the iCycler™ iQ thermal cycler (Bio-Rad) and Bio-Rad reagents, following producer instructions.

iQ-Check *Legionella* product range

- iQ-Check Screen *Legionella* spp.
- iQ-Check Screen *L. pneumophila*
- iQ-Check Quanti *L. pneumophila*
- iQ-Check Quanti *Legionella* spp.

Reagents composition

- Amplification mix :

- Primers
- Taq polymerase
- dNTP
- UDG
- Internal control

- Fluorescent probes :

- Molecular beacons, labelled with FAM
- Molecular beacons for the internal control, labelled with Texas red

Positive control or 4 quantification standards :
Extracted DNA from *L. pneumophila* 1 ATCC 33152 strain

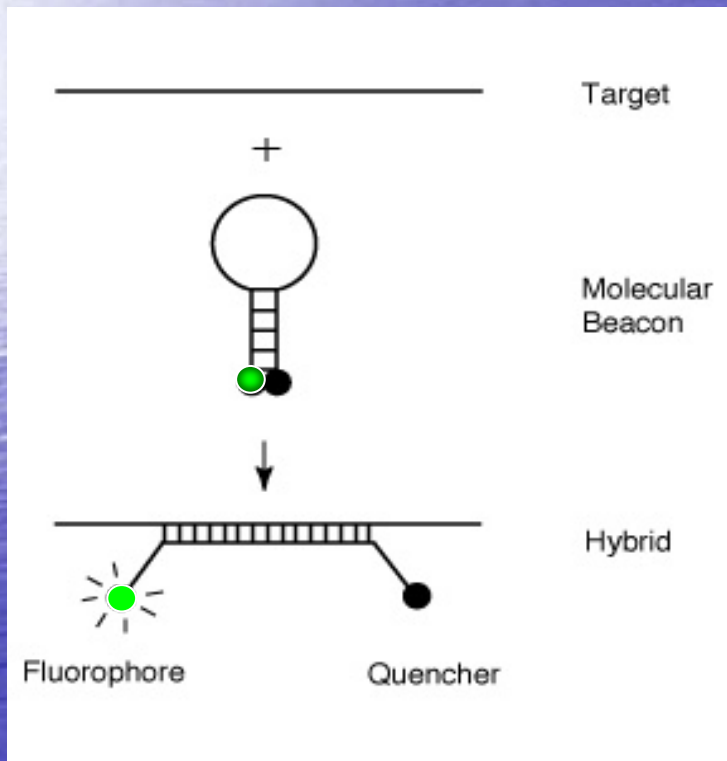
Negative control : TE

The target sequences

Legionella species
rRNA 5S gene

Legionella pneumophila
mip gene

Molecular beacon : specific fluorescent probe

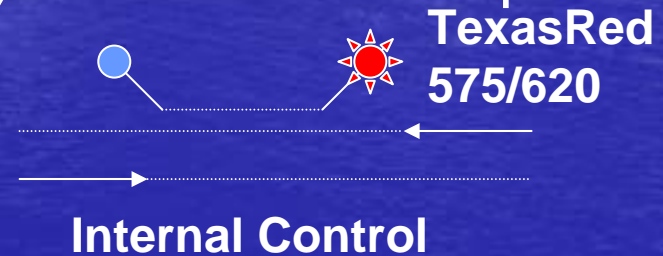


- The molecular beacon is a short oligonucleotide that forms a hairpin structure with a loop and a stem.
- During the PCR, the molecular beacon hybridizes to the amplicons.
- The fluorophore emits fluorescence only when hybridized to the amplicons.

The internal control

➤ Multiplex reactions :

- a synthetic DNA is added in each reaction tube called Internal Control.
- Target DNA and internal control are amplified in the same time, but are detected by 2 different fluorophores.



➤ Why ? :

- Allows to detect any inhibitory process during PCR.
- Assures the reliability of negative results.

Standards for quantification

- 4 Quantification standards

- Extracted *L. pneumophila* ATCC 33152 DNA

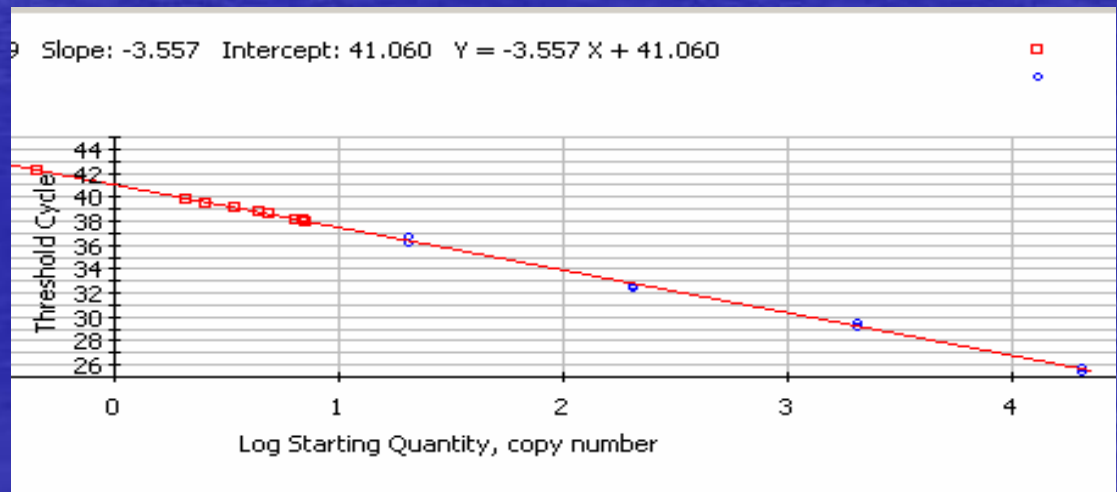
- Target levels :

- Quantification from ~ 20 up to 20 000 GU/PCR

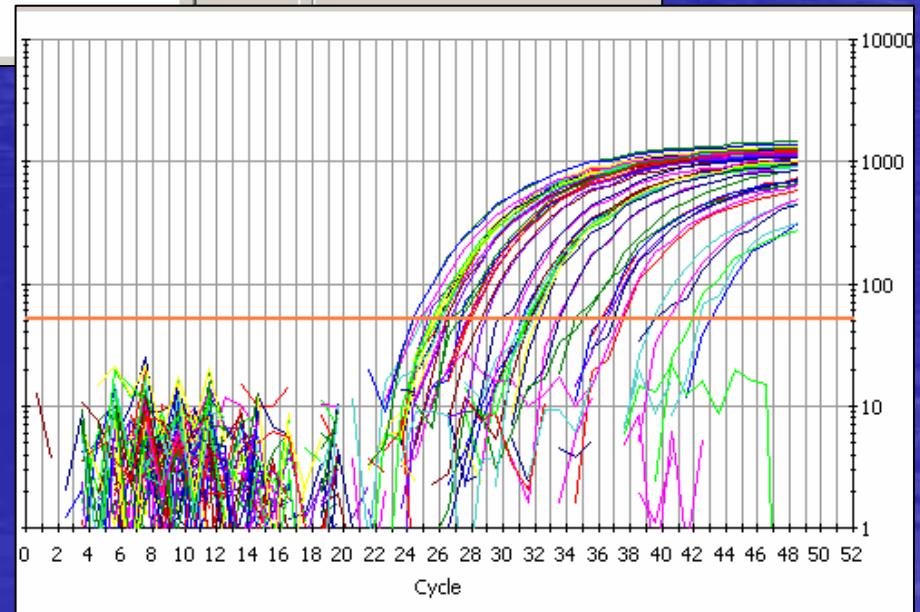
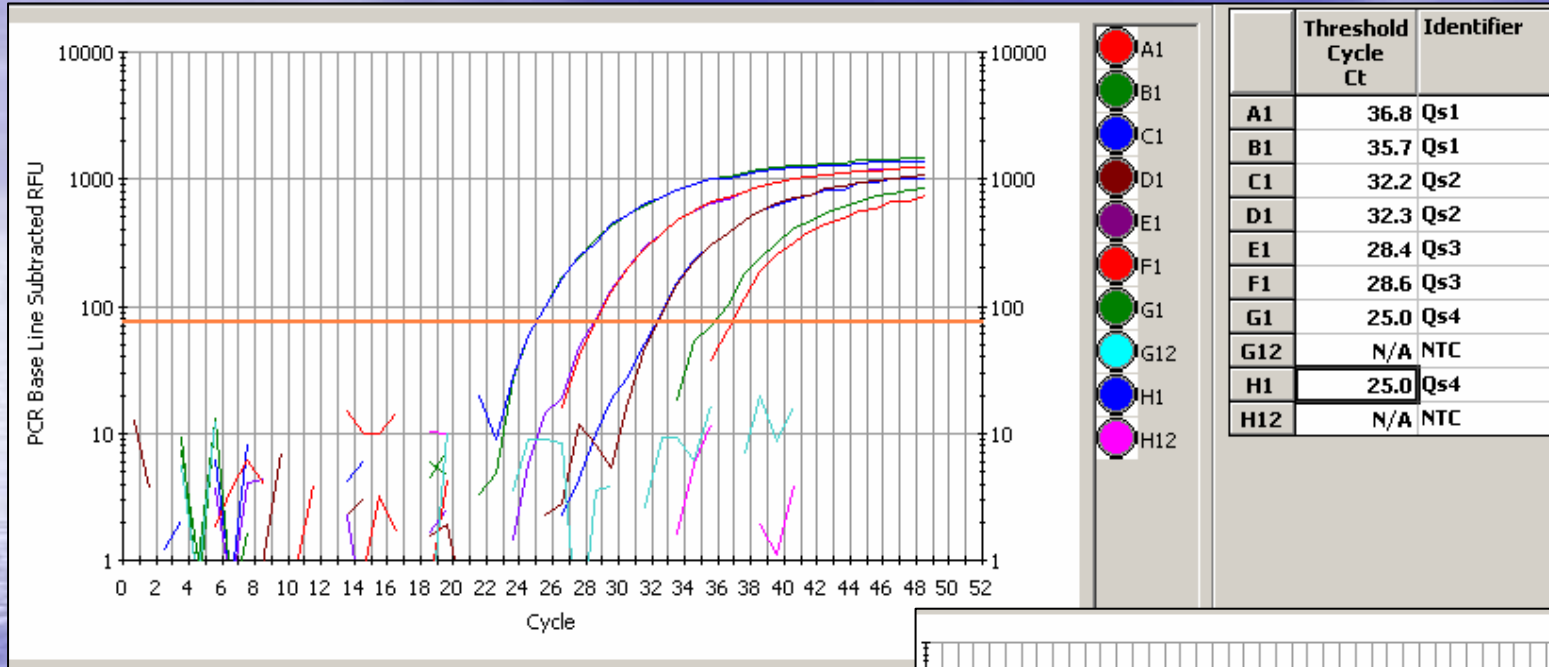
- Quantification from ~ 540 GU/L to 540 000 GU/L

- Ready-to-use

- Calibrated



iQ-Check *L. pneumophila* : examples



Results (1)

20 water samples **positive** for *Legionella pneumophila*
(LP) by culture

(9 LP serogroup 1, 11 LP serogroup 2-14)

PCR results :

iQ-Check™ Quanti *Legionella pneumophila*

20 samples were positive by real time PCR assay

(detection limit (Ld) : 133 genomic units/L)

19 results superior to the quantification limit (Lq)

(Lq : 373 GU/L)

- PCR results :

median	4200 GU/L
mean	4400 GU/L
range	500 to 11000 GU/L

Results (2)

20 water samples **positive** for *Legionella pneumophila* (LP) by culture

(9 LP serogroup 1, 11 LP serogroup 2-14)

PCR results :

iQ-Check™ Quanti *Legionella* spp.

Among these 20 LP culture-positive samples, data for *Legionella* spp. real time PCR assay were available for **15** water samples

- PCR results :

median	15000 GU/L
mean	20000 GU/L
range	900 to 70000 GU/L

Results (3) : PCR results for the 20 positive culture samples

Sample number	CFU/L (Ip1)	CFU/L (Lp2-14)	Quanti <i>L. pneumophila</i> GU/L	Quanti <i>Legionella</i> spp. GU/L
8	250		3067	/
11		300	4481	/
12		550	8108	/
13		350	5707	/
19	350		4161	/
21	50	700	11068	67475
22		1550	3974	39472
29	300		3440	27470
30		500	688	15655
36		800	4534	9281
41		2700	4747	14402
42		1600	5707	21683
44	350		496	1285
48	450		8934	29870
51		300	4774	27203
59	600		3014	12642
72		400	957	3494
73	2300		3360	7574
78	400		1542	12642
94	550		315	896

Results (4)

80 water samples contained fewer viable LP cells than the detection limit (250 CFU/L) of the culture method

PCR results : iQ-Check™ Quanti *Legionella pneumophila*

20 were negative for LP real time PCR assay (with no inhibition of amplification), 19 were under the Ld, 9 between the Ld and Lq, 32 (40%) displayed results superior to the Lq

- PCR results :
 - median 2200 GU/L
 - mean 3100 GU/L
 - range 500 to 10000 GU/L

Results (5)

80 water samples contained fewer viable LP cells than the detection limit (250 CFU/L) of the culture method

PCR results :

iQ-Check™ Quanti *Legionella* spp.

Data were available for 66 samples : two water samples had results superior to the upper quantification limit (UQL : 403783 GU/L) and required adapted dilution of DNA samples for precise quantification ; for the remaining 64 samples, 6 were negative (with no inhibition of amplification), 17 were under the Ld, 3 between the Ld and the Lq, and 38 (59%) displayed results superior to the Lq :

- PCR results :
 - median 4300 GU/L
 - mean 20000 GU/L
 - range 400 to 370000 GU/L

Results (6)

Among the previous 80 samples, 20 water samples displayed results (between the Lq and the UQL) for both LP and *Legionella* spp. real time PCR assays

PCR results :

iQ-Check™ Quanti *Legionella pneumophila*

median 1900 GU/L ; mean 2700 GU/L ;
range 500 to 11000 GU/L

iQ-Check™ Quanti *Legionella* spp.

median 9000 GU/L ; mean 30000 GU/L ;
range 900 to 370000 GU/L

Discussion (1) : culture method

- After any **treatment of the water system**, bacteria are **under stress** and do not grow on selective media.
- In case of mixed bacterial populations, **pre-treatment** of water samples to select *Legionella* species results in **under-evaluation** of CFU counts.
- Our data support previous findings indicating that culture frequently underestimates the presence of *L. pneumophila* in water samples.
- The method does not permit
 - the isolation of viable non-culturable bacteria.
 - the isolation of *Legionella* from samples with high microbiota.
 - the isolation of *Legionella* associated with amoebae.

Discussion (2) : real time PCR

- Real time PCR assays give results **in less than 4 hours after filtration.**
- Sensitivity of real time PCR assays : detection of viable non-culturable *Legionella*, and *Legionella* associated with amoebae.
- According to AFNOR (**Projet** XP T90 471 in progress) standard in preparation : criteria for specificity, detection limit, quantification limit, yield of the extraction are defined.
- PCR assays may therefore be useful for routine **monitoring for *Legionella* contamination**, and for rapid screening of large numbers of water samples during **outbreak investigations**, while the results of culture are still awaited.

Discussion (3)

PCR and culture give different views on the level of *Legionella* load in water

Culture \Rightarrow Growing bacteria

PCR \Rightarrow Total *Legionella* DNA in the sample

Interpretation guidelines for PCR results are awaited