Development of a new diagnostic tool for the detection of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* in a duplex real-time PCR

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Introduction

Chlamydophila pneumoniae and *Mycoplasma pneumoniae* are two atypical respiratory pathogens. Both bacteria are an important cause of community-acquired pneumonias (CAP), between 10% and 20% of cases approximatively. Symptoms may be mild but the most common Upper and Lower Respiratory Infections (URTI and LRTI) in children and adults include tracheobronchitis, pharyngitis, laryngitis, sinusitis and also more severe illness like atypical pneumonia. *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* can be also implicated in chronic pulmonary diseases such as bronchial asthma. A large number of respiratory agents involved in respiratory tracts infections, including viruses and bacteria share similar clinical features and symptoms. Identification and differentiation of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* from other agents are important to choose or to adapt an appropriate and effective antibiotic therapy. Culture, serological and antigen detection techniques are currently being used for the diagnosis but these methods have many drawbacks such as cost, time to result and low sensitivity. Now, the Nucleic Acid Amplification Techniques (NAATs) with Real-Time PCR techniques have many benefits for the detection of respiratory pathogens like high sensitivity and specificity and are much quicker. We propose a new real-time PCR based diagnostic tool for *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* diagnosis. Our duplex Real-Time PCR kit « Chla/Myco pneumo r-gene™ » allows the simultaneous detection of both bacteria in a single tube reaction.

Materials & Methods

Extraction :
Respiratoty samples are pre-treated with 10µL (for 200µL of sample) of Proteinase K (Novagen) at 20mg/mL and incubated for 15 min at 56°C. NucliSENS® easyMAG® extraction (bioMérieux) are validated for a volume of 400µL of sample eluted in 100µL, or 200µL of sample eluted in 50µL. For both volumes, 50µL of magnetic silica is used.

Amplification :
10µL of extracted sample are added to 15µL of ready-to-use Chla/Myco amplification premix. Signal is read at 530nm for *Chlamydophila pneumoniae* and at 560nm for *Mycoplasma pneumoniae*.

QCMD EQA Programme 2011 *Chlamydophila pneumoniae & Mycoplasma pneumoniae :*

Chlamydophila pneumoniae and *Mycoplasma pneumoniae* panels 2011 are extracted on NucliSENS® easyMAG™ of 200µL of sample eluted in 50µL. PK pre-treatment is performed. QCMD CP.MP 2011 panel is amplified with Chla/Myco pneumo r-gene™ kit (ref.71-044) on ABI 7500 Fast (Applied Biosystems), Dx Real-Time System (Bio-Rad) and LC480 (Roche).

Analytical Sensitivity :

The analytical sensitivity of the Chla/Myco pneumo r-gene™ kit is determined on quantified samples of *Chlamydophila pneumoniae* (at 4.9 IFU/100µL) and *Mycoplasma pneumoniae* (at 5 000 CCU/100µL) from the Panel QCMD CP-MP 2010.
For each bacteria, serial dilutions are performed in a nasopharyngeal sample negative for both bacterial species. Each dilution is extracted 15 times using the NucliSENS® easyMAG® extraction with 200µL of sample eluted in 50µL. PK pre-treatment is performed. Each extract is amplified with Chla/Myco pneumo r-gene™ kit (ref. 71-044) on ABI 7500 Fast.

Specificity :

The specificity of the Chla/Myco pneumo r-gene™ kit is determined experimentally on a panel of various viruses/bacteria representing respiratory pathogens or present in respiratory samples.
NucliSENS® easyMAG® extraction of 400µL of sample eluted in 100µL is performed then amplification is done on Versant kPCR AD (Siemens).

Intra&inter-assay reproducibility :

The reproducibility is performed on different concentrations of ATCC culture (ATCC VR-1355 and ATCC29342) or quantified samples from the Panel QCMD CP-MP 2011 of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*.
For the intra-assay reproducibility , both bacteria are diluted at 10x, 5x and 2x the LOD in a nasopharyngeal negative sample. Each dilution is extracted 10 times on NucliSENS® easyMAG® with 200µL of sample eluted in 50µL. PK pre-treatment is performed. Each extract is amplified in a same run with Chla/Myco pneumo r-gene™ kit (ref.71-044) on LC480.
For the inter-assay reproducibility, both bacteria are diluted at 1000x, 100x and 10x the LOD in a nasopharyngeal sample negative. Each dilution is extracted 10 times on 10 independant runs of extraction using extraction NucliSENS® easyMAG® with 200µL of sample eluted in 50µL. PK pre-treatment is performed. Each extract is amplified in 10 independant runs with Chla/Myco pneumo r-gene™ kit (ref.71-044) on Dx Real Time System.

Results

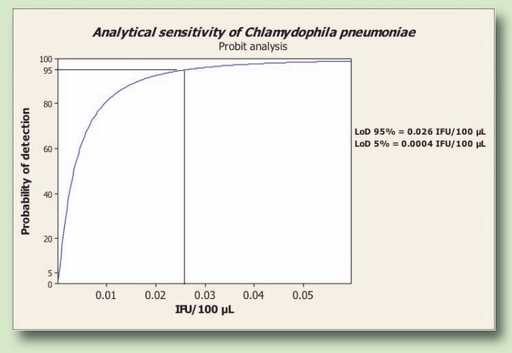
QCDM 2011 *Chlamydophila pneumoniae* & *Mycoplasma pneumoniae* EQA programme

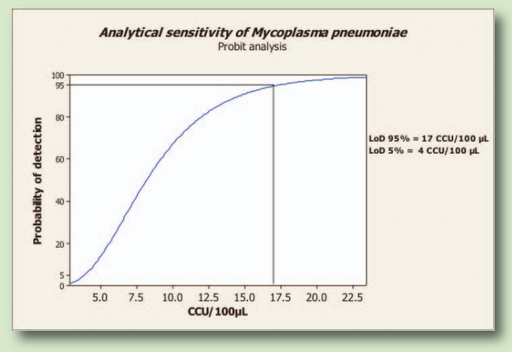
QCMD Expected Results						Chla/Myco pneumo r-gene™ Results					
						ABI 7500 Fast (Applied Biosystems)		Dx Real Time System (Bio-Rad)		LC480 (Roche)	
Panel Code	Sample Content	Sample Matrix	Sample Conc.	Expected Result	Sample Type	CT *Chlamydophila pneumoniae*	CT *Mycoplasma pneumoniae*	CT *Chlamydophila pneumoniae*	CT *Mycoplasma pneumoniae*	CT *Chlamydophila pneumoniae*	CT *Mycoplasma pneumoniae*
CPMP11-01	*C. pneumoniae*	STM	4.9 IFU/100µL	Positive (CP)	Core (CP)	**30.67**	neg	**30.03**	neg	**30.6**	neg
CPMP11-02	*M. pneumoniae*	STM	5 CCU/100µL	Positive (MP)		neg	**40.94**	neg	**neg**	neg	**> 40**
CPMP11-03	*C. pneumoniae*	BAL	4.9 IFU/100µL	Positive (CP)	Core (CP)	**30.49**	neg	**29.71**	neg	**30.43**	neg
CPMP11-04	*C. pneumoniae*	STM	0.049 IFU/100µL	Positive (CP)		**36.77**	neg	**36.63**	neg	**37.01**	neg
CPMP11-05	*M. pneumoniae*	BAL	500 CCU/100µL	Positive (MP)	Core (MP)	neg	**35.15**	neg	**34.17**	neg	**34.78**
CPMP11-06	*M. pneumoniae*	STM	250 CCU/100µL	Positive (MP)	Core (MP)	neg	**36.64**	neg	**35.31**	neg	**36.28**
CPMP11-07	*M. pneumoniae*	STM	50 CCU/100µL	Positive (MP)		neg	**37.68**	neg	**36.97**	neg	**38.43**
CPMP11-08	*CP/MP Negative*	STM	-	Negative	Core	neg	neg	neg	neg	neg	neg
CPMP11-09	*M. pneumoniae*	STM	500 CCU/100µL	Positive (MP)	Core (MP)	neg	**35.62**	neg	**34.42**	neg	**35.29**
CPMP11-10	*CP/MP Negative*	BAL	-	Negative	Core	neg	neg	neg	neg	neg	neg
CPMP11-11	*C. pneumoniae*	STM	0.49 IFU/100µL	Positive (CP)		**33.93**	neg	**34.10**	neg	**33.57**	neg

Notes: BAL: Human Bronchoalveolar Lavage / STM: Sample Transport Medium / IFU: Inclusion Forming Units / CCU: Colour-Changing Units / CT: Crossing-Threshold (cycles)

The results obtained with Chla/Myco pneumo r-gene™ kit show a correlation of 100% on 7500Fast and LC480 and 91.67% on Dx Real-Time System with expected results. All 4 positive *Chlamydophila pneumoniae* and all 3 positive "Core" *Mycoplasma pneumoniae* samples from QCMD CP.MP 2011 are detected with Chla/Myco pneumo r-gene™ kit. On the 2 low *Mycoplasma pneumoniae* samples, one is detected by the 3 themocyclers and the lowest (5CCU/100µL) is detected on 7500Fast and LC480. No cross-reaction is observed between *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*.
The results show the good sensitivity and specificity of the Chla/Myco pneumo r-gene™ kit.

Analytical Sensitivities of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*





Notes: IFU: Inclusion Forming Units / CCU: Colour-Changing Units / LOD: Limit Of Detection

Specificity

None of following viruses or bacteria is amplified with Chla/Myco pneumo r-gene™, which attests of the good specificity of the assay.

Viruses	Quantification (copies/mL) or Crossing Threshold (cycles)	*Chlamydophila pneumoniae*	*Mycoplasma pneumoniae*
Adenovirus 12	2E+05	-	-
Adenovirus 3	7E+04	-	-
Adenovirus 11	6E+04	-	-
Adenovirus 5	4E+04	-	-
Adenovirus 8	3E+04	-	-
Adenovirus 4	6E+05	-	-
Adenovirus 40	6E+05	-	-
Cytomegalovirus	4E+04	-	-
Epstein Barr Virus	1E+06	-	-
BK Virus	3E+06	-	-
Herpes Simplex Virus 1	2E+05	-	-
Herpes Simplex Virus 2	3E+05	-	-
Varicella Zoster Virus	2E+05	-	-
Human Herpes Virus 6	5E+03	-	-
Human Herpes Virus 7	30.50 cycles	-	-
Human Herpes Virus 8	5E+04	-	-
Influenza A/PR/8/34	23.70 cycles	-	-
Influenza B/Ann Arbor	22.31 cycles	-	-
Respiratory Syncytial Virus A	24.14 cycles	-	-
Respiratory Syncytial Virus B	23.75 cycles	-	-
Human Metapneumovirus type A	26.24 cycles	-	-
Human Metapneumovirus type B	24.23 cycles	-	-
Human Bocavirus 1	26.42 cycles	-	-
Parainfluenza Type 1	21.09 cycles	-	-
Parainfluenza Type 2	25.34 cycles	-	-
Parainfluenza Type 3	22.86 cycles	-	-
Parainfluenza Type 4	24.54 cycles	-	-
NL63	30.67 cycles	-	-
Rhinovirus 14	23.13 cycles	-	-
Rhinovirus 87	26.07 cycles	-	-
Rhinovirus 1B	32.96 cycles	-	-
Echovirus 25	25.18 cycles	-	-
Coxsackie B2	26.74 cycles	-	-
Coxsackie A9	27.06 cycles	-	-
Echovirus 9	29.42 cycles	-	-
Poliovirus S3	32.61 cycles	-	-
Echovirus 30	28.57 cycles	-	-
Parechovirus 1	29.05 cycles	-	-
Parechovirus 2	27.49 cycles	-	-

Bacteria	Quantification (copies/mL) or Crossing Threshold (cycles)	*Chlamydophila pneumoniae*	*Mycoplasma pneumoniae*
Bordetella pertussis	28.88 cycles	-	-
Bordetella parapertussis	27.63 cycles	-	-
Légionella pneumophila	1E+06	-	-
Bordetella bronchiseptica	2E+06	-	-
Escherichia coli	4E+06	-	-
Staphylococcus epidermidis	1E+06	-	-
Klebsiella pneumoniae	1E+06	-	-
Haemophilus influenzae	3E+06	-	-
Serratia marcescens	6E+06	-	-
Staphylococcus aureus	3E+06	-	-
Proteus mirabilis	3E+07	-	-
Klebsiella oxytoca	3E+06	-	-
Pseudomonas aeruginosa	1E+05	-	-
Stenotrophomonas maltophilia	7E+06	-	-
Bordetella pertussis	3E+05	-	-
Légionella pneumophila	4E+05	-	-
Pseudomonas aeruginosa	8E+05	-	-
Klebsiella pneumoniae	6E+05	-	-
Staphylococcus aureus	5E+05	-	-
Klebsiella oxytoca	2E+06	-	-
Enterobacter kobei	3E+06	-	-
Morganella morganii	9E+06	-	-
Branhamella catarrhalis	2E+05	-	-
Citrobacter freundi	2E+06	-	-
Citrobacter koseri	7E+06	-	-
Streptococcus constellatus	2E+06	-	-
Citrobacter freundi	7E+05	-	-
Raoultella ornithinolytica	3E+05	-	-
Serratia marcescens	3E+06	-	-
Haemophilus parainfluenzae	3E+06	-	-
Haemophilus influenzae	3E+06	-	-
Enterobacter cloacae	6E+05	-	-
Stenotrophomonas maltophilia	7E+05	-	-
Morganella morganii	1E+06	-	-
Acinetobacter baumanii	9E+06	-	-
Pseudomonas aeruginosa	2E+05	-	-
Branhamella catarrhalis	1E+05	-	-
Streptococcus agalactiae	7E+05	-	-

Intra&inter-assay reproducibility

Intra-assay reproducibility

			Mean of Ct (cycles)	Standard deviation	Coefficient of variation
Chlamydophila pneumoniae	10x LoD	2.6 E-1 IFU/100µL	33.98	0.28	0.83 %
	5x LoD	13.0 E-2 IFU/100µL	35.23	0.35	1.00%
	2x LoD	5.2 E-2 IFU/100µL	37.23	0.68	1.77%
Mycoplasma pneumoniae	10x LoD	170 CCU/100µL	34.79	0.53	1.51%
	5x LoD	85 CCU/100µL	36.26	0.92	2.53%
	2x LoD	34 CCU/100µL	38.48	1.40	3.63%

Inter-assay reproducibility

			Mean of Ct (cycles)	Standard deviation	Coefficient of variation
Chlamydophila pneumoniae	1000x LoD	26 IFU/100µL	27.55	0.46	1.66%
	100x LoD	2.6 IFU/100µL	31.24	0.35	1.11%
	10x LoD	2.6 E-1 IFU/100µL	34.15	0.37	1.07%
Mycoplasma pneumoniae	1000x LoD	17000 CCU/100µL	29.54	0.45	1.52%
	100x LoD	1700 CCU/100µL	33.49	1.06	3.16%
	10x LoD	170 CCU/100µL	36.37	0.86	2.37%

The results demonstrate the good intra-assay reproducibility and the good inter-assay reproducibility of Chla/Myco pneumo r-gene™ kit in nasopharyngeal specimens.

Notes: IFU: Inclusion Forming Units / CCU: Colour Changing Units / LOD: Limit Of Detection

Conclusion

Results presented in this study show the sensitivity, robustness and reliability of 71-044 Chla/Myco pneumo r-gene™ kit. The high quality associated with its compatibility with the major extraction and real time PCR platforms allows an immediate integration of Chla/Myco pneumo r-gene™ in most routine diagnostic laboratories. This tool belongs to the respiratory MWS r-gene™ brand new range of product which represents an innovative solution in response to the challenges in respiratory infections diagnosis. These PCR assays can assist clinical laboratories in identifying 12 respiratory pathogens or families of respiratory pathogens in hospitalized patients and aid in patient therapy management.