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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 approximately. 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 clinical features and symptoms. Identification of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* from other agents and their differentiation are important to suggest 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-consuming procedures and low sensitivity. Now, the Nucleic Acid Amplification Techniques (NAATs) with Real-Time PCR techniques have many benefits in the detection of respiratory pathogens, such as high sensitivity and specificity and rapidity.We are offering a new real-time PCR based on a 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

Respiratory 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) is 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 the extracted sample are added to 15µL of ready-to-use Chla/Myco amplification premix. The reading signal is at 530nm for *Chlamydophila pneumoniae* and at 560nm for *Mycoplasma pneumoniae*.

QCMD EQA Programme 2010/2011

Chlamydophila pneumoniae and *Mycoplasma pneumoniae* panels 2010 & 2011 were extracted on NucliSENS® easyMAG® extraction of 200µL of sample eluted in 50µL. PK pre-treatment is performed.
QCMD 2010 panel was amplified on LightCycler 480 (Roche), RotorGene 6000 (Corbett) and ABI StepOne (Applied Biosystems).
QCMD 2011 panel was amplified on ABI 7500 Fast (Applied Biosystems) and Dx Real-Time System (Bio-Rad).

Analytical Sensitivity

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

Specificity

The specificity of the kit Chla/Myco pneumo r-gene™ was determined experimentally on samples containing various viruses/bacteria that may be involved in respiratory diseases or present in respiratory samples. NucliSENS® easyMAG® extraction of 400µL of sample eluted in 100µL was performed then amplification was done on Versant kPCR AD (Siemens).

Results

QCMD 2010 *Chlamydophila pneumoniae* & *Mycoplasma pneumoniae* EQA programme

QCMD 2010 *Chlamydophila pneumoniae & Mycoplasma pneumoniae* EQA Programme Results					Chla/Myco pneumo r-gene™ 71-044 Results					
					LightCycler 480 (Roche)		StepOne (Applied Biosystems)		RotorGene 6000 (Corbett)	
Panel Code	Sample Contents/ Matrix	Concentration	Expected Results	Sample Type	CT *Chlamydophila pneumoniae*	CT *Mycoplasma pneumoniae*	CT *Chlamydophila pneumoniae*	CT *Mycoplasma pneumoniae*	CT *Chlamydophila pneumoniae*	CT *Mycoplasma pneumoniae*
CP.MP 10-01	*C.pneumoniae*/BAL[1]	0.49 IFU[3]/100µL	Positive (CP)		35.95	NEG	37.36	NEG	34.24	NEG
CP.MP 10-02	*C.pneumoniae*/STM[2]	4.9 IFU[3]/100µL	Positive (CP)	Core (CP)	29.75	NEG	28.88	NEG	25.86	NEG
CP.MP 10-03	*C.pneumoniae*/STM[2]	0.049 IFU[3]/100µL	Positive (CP)		36.08	NEG	35.99	NEG	32.95	NEG
CP.MP 10-04	CP/MP negative/STM[2]	NEG	Negative	Core	NEG	NEG	NEG	NEG	NEG	NEG
CP.MP 10-05	*M.pneumoniae*/STM[2]	50 CCU[4]/100µL	Positive (MP)		NEG	37.52	NEG	38.89	NEG	37.69
CP.MP 10-06	*C.pneumoniae*/STM[2]	4.9 IFU[3]/100µL	Positive (CP)	Core (CP)	29.51	NEG	28.83	NEG	25.66	NEG
CP.MP 10-07	*C.pneumoniae*/BAL[1]	4.9 IFU[3]/100µL	Positive (CP)	Core (CP)	33.03	NEG	32.99	NEG	29.62	NEG
CP.MP 10-08	*M.pneumoniae*/BAL[1]	50 CCU[4]/100µL	Positive (MP)		NEG	36.52	NEG	36.69	NEG	35.97
CP.MP 10-09	*M.pneumoniae*/STM[2]	500 CCU[4]/100µL	Positive (MP)	Core (MP)	NEG	34.72	NEG	34.08	NEG	33.37
CP.MP 10-10	*M.pneumoniae*/STM[2]	5000 CCU[4]/100µL	Positive (MP)	Core (MP)	NEG	32.04	NEG	31.13	NEG	30.70
CP.MP 10-11	*M.pneumoniae*/BAL[1]	500 CCU[4]/100µL	Positive (MP)	Core (MP)	NEG	34.47	NEG	33.91	NEG	33.42
CP.MP 10-12	*C.pneumoniae*/STM[2]	0.49 IFU[3]/100µL	Positive (CP)		32.83	NEG	31.95	NEG	29.01	NEG

The table shows that on the 3 thermocyclers tested :
All 6 positive *Chlamydophila pneumoniae* of QCMD CP.MP 2010 are detected with Chla/Myco pneumo r-gene™.
All 5 positive *Mycoplasma pneumoniae* of QCMD CP.MP 2010 are detected with Chla/Myco pneumo r-gene™.
The "Core" negative sample is undetected as expected with Chla/Myco pneumo r-gene™.
No cross reaction is observed between *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*.
The results, 100% correlated with expected results, show the high sensitivity and specificity of the Chla/Myco pneumo r-gene™.

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

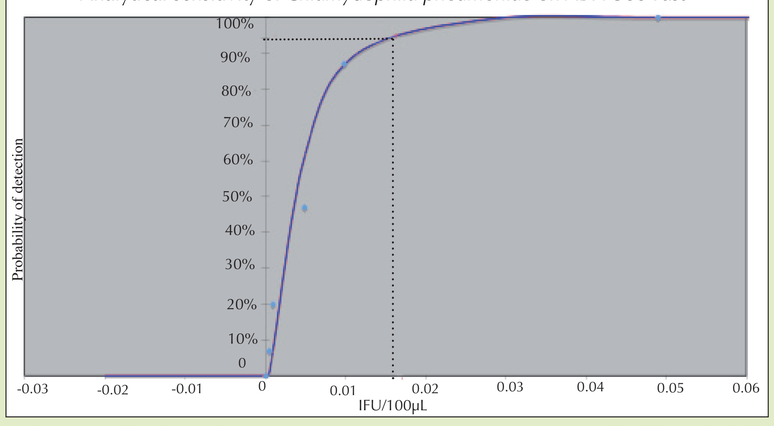
QCMD 2011 *Chlamydophila pneumoniae & Mycoplasma pneumoniae* EQA Programme Results					Chla/Myco pneumo r-gene™ 71-044 Results			
					ABI 7500Fast (Applied Biosystems)		DX Real Time System (Bio-Rad)	
Panel Code	Sample Contents/ Matrix	Concentration	Expected Results	Sample Type	CT *Chlamydophila pneumoniae*	CT *Mycoplasma pneumoniae*	CT *Chlamydophila pneumoniae*	CT *Mycoplasma pneumoniae*
CP.MP 11-01	*C.pneumoniae*/STM[2]	4.9 IFU[3]/100µL	Positive (CP)	Core (CP)	30.67	NEG	30.04	NEG
CP.MP 11-02	*M.pneumoniae*/STM[2]	5 CCU[4]/100µL	Positive (MP)		NEG	40.94	NEG	NEG
CP.MP 11-03	*C.pneumoniae*/BAL[1]	4.9 IFU[3]/100µL	Positive (CP)	Core (CP)	30.49	NEG	29.71	NEG
CP.MP 11-04	*C.pneumoniae*/STM[2]	0.049 IFU[3]/100µL	Positive (CP)		36.77	NEG	36.64	NEG
CP.MP 11-05	*M.pneumoniae*/BAL[1]	500 CCU[4]/100µL	Positive (MP)	Core (MP)	NEG	35.15	NEG	34.18
CP.MP 11-06	*M.pneumoniae*/STM[2]	250 CCU[4]/100µL	Positive (MP)	Core (MP)	NEG	36.64	NEG	35.32
CP.MP 11-07	*M.pneumoniae*/STM[2]	50 CCU[4]/100µL	Positive (MP)		NEG	37.68	NEG	36.97
CP.MP 11-08	CP/MP Negative/STM[2]	NEG	Negative	Core	NEG	NEG	NEG	NEG
CP.MP 11-09	*M.pneumoniae*/STM[2]	500 CCU[4]/100µL	Positive (MP)	Core (MP)	NEG	35.62	NEG	34.42
CP.MP 11-10	CP/MP Negative/BAL[1]	NEG	Negative	Core	NEG	NEG	NEG	NEG
CP.MP 11-11	*C.pneumoniae*/ STM[2]	0.49 IFU[3]/100µL	Positive (CP)		33.93	NEG	34.11	NEG

The table shows that on both thermocyclers tested :
The 4 positive *Chlamydophila pneumoniae* of QCMD CP.MP 2011 are detected with Chla/Myco pneumo r-gene™.
The 3 positive "Core" *Mycoplasma pneumoniae* of QCMD CP.MP 2011 are detected with Chla/Myco pneumo r-gene™.
On the 2 lows *Mycoplasma pneumoniae* samples, one is detected by both themocyclers and the lowest (5CCU/100µL) is detected on ABI 7500Fast.
The 2 "Core" negative samples are undetected as expected with Chla/Myco pneumo r-gene™.
No cross reaction is observed between *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*.
The results, 100% and 91.67% correlated (respectively on ABI 7500Fast & Dx Real Time System) with expected results, show the high sensitivity and specificity of the Chla/Myco pneumo r-gene™.

Note :[1] BAL: Human Bronchoalveolar Lavage / [2] STM: Sample Transport Medium / [3]IFU: Inclusion Forming Units / [4]CCU: Colour-Changing Units

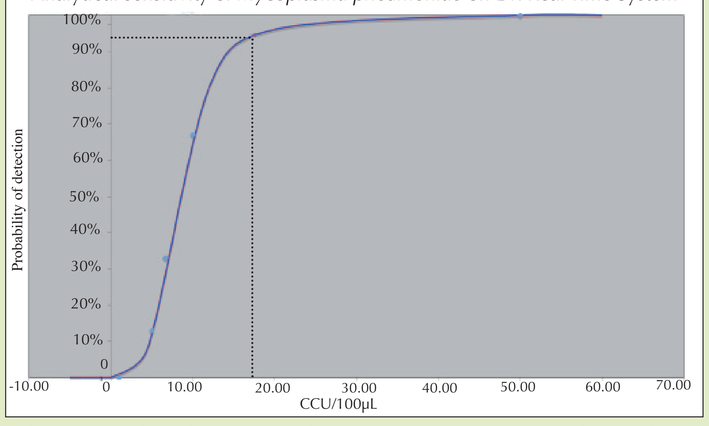
Analytical Sensitivities on *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*

Analytical sensitivity of *Chlamydophila pneumoniae* on ABI 7500 Fast



These graphs show that on ABI 7500Fast, 0.016 IFU/100µL of ***Chlamydophila pneumoniae*** are detected in 95% and 0.0001 IFU/100µL are detected in 5%. Analytical sensitivity on Dx Real Time System is the same.
The limit of detection at 95% of ***Chlamydophila pneumoniae*** is **0.16 IFU/mL** with Chla/Myco pneumo r-gene™ kit.

Analytical sensitivity of *Mycoplasma pneumoniae* on Dx Real Time System



These graphs show that on Dx Real Time System, 18 CCU/100µL of ***Mycoplasma pneumoniae*** are detected in 95% and 4 CCU/100µL are detected in 5%. Analytical sensitivity on ABI 7500Fast is the same.
The limit of detection at 95% of ***Mycoplasma pneumoniae*** is **180 CCU/mL** with Chla/Myco pneumo r-gene™ kit.

Specificity

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

Viruses

HSV1, HSV2, VZV, EBV, CMV, HHV6, HHV7, HHV8, *Influenza A*, *Influenza B*, RSV A, RSV B, hMPV A, hMPV B, Adenovirus3 (AdV), AdV4, AdV5, AdV8, AdV11, AdV12, AdV40 , Bocavirus1, Parainfluenza1, Parainfluenza2, Parainfluenza3, Parainfluenza4, Coronavirus NL63, Rhinovirus 1B, Rhinovirus 14, Rhinovirus 87, Coxakievirus A9, Coxakievirus B2, Echovirus 9, Echovirus 25, Echovirus 30, Poliovirus S3, Parechovirus 1, Parechovirus 2, Parvovirus B19, JC Virus & BK Virus.

Bacteria

Acinetobacter baumanii, Bordetella bronchiseptica, Bordetella holmesii, Bordetella parapertussis, Bordetella pertussis, Branhamella catarrhalis, Candida albicans, Candida glabrata, Candida non albicans (tropicalis), Candida non albicans (utilis), Citrobacter freundi, Citrobacter koseri, Enterobacter cloacae, Enterobacter kobei, Escherichia coli, Haemophilus influenzae, Haemophilus parainfluenzae, Klebsiella pneumoniae, Klebsiella oxytoca, Legionella micadadei, Legionella pneumophila, Morganella morganii, Proteus mirabilis, Pseudomonas aeruginosa, Raoultella ornithinolytica, Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis, Stenotrophomonas maltophilia, Streptococcus agalactiae, Streptococcus constellatus, Mycobaterium avium, Mycobaterium chelonae, Mycobaterium fortuitum, Mycobaterium gordonae, Mycobaterium intracellulare, Mycobaterium kansasii, Mycobaterium lentiflavum, Mycobaterium tuberculosis, Mycobaterium xenopi & Nocardia asteroides.

Conclusion

Results presented in this study show the sensitivity, robustness and reliability of **Chla/Myco pneumo r-gene™ kit ref. 71-044**. 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 belong to the Respiratory MWS r-gene™ brand range which represents an innovative solution in response to the challenges in respiratory infections. These PCR assays can assist clinical laboratories in identifying 12 respiratory pathogens or a family of respiratory pathogens in hospitalized patients and aid in patient management.