5

Detection of H influenzae, S pneumoniae and M catarrhalis by real time quantitative PCR

5.1 Introduction

Patients with chronic obstructive pulmonary disease have a high prevalence of lower airway bacterial colonisation (LABC) as presented in chapter 1. This is a dynamic process and changes in the number and the type of bacteria have been linked to lung function decline and exacerbations (Wilkinson et al 2003 and 2006). LABC is also directly related to the degree of lower airway inflammation and to the exacerbation frequency (Patel et al 2002, Sethi et al 2006). Active smoking and the severity of the airway obstruction are recognized risk factors for lower airway colonization include (Zalacin et al 1999, Soler et al 1999). Haemophilus influenzae (HI), Streptococcus pneumoniae (SP) and Moraxella catarrhalis (MC) are the most frequently isolated potentially pathogenic mirco-organisms (PPM) found in the lower airways of COPD patients.

The presence of these micro-organisms in the stable state is associated with a higher total symptom count and more sputum purulence at exacerbation compared with patients who are not colonized (Patel et al 2002). The same strains of HI are known to persist for prolonged periods of time in an individual patient. One study reported prolonged periods of up to six months where these HI strains were not detectable by standard culture methods but where HI DNA could be isolated by polymerase chain reaction (PCR) assays (Murphy et al 2004). Exacerbations associated with a PPM are more severe in terms of symptoms, FEV_1 drop and are linked to higher levels of airway inflammation (Chin et al 2005, Wilkinson et al 2006, Papi et al 2006).

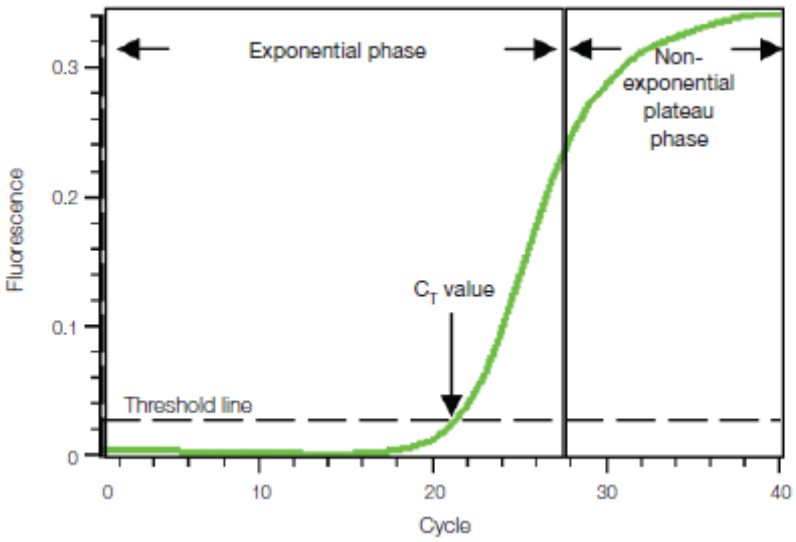
Routine microbiological cultures are currently the gold standard for the detection of bacteria from the sputum samples of COPD patients. Sputum cultures have several pitfalls, their results are influenced by the delay between sample collection and plating of the cultures, the fragility of the organisms, the prior use of antibiotics and the time required to obtain the results. More sensitive, specific and rapid diagnostic methods are necessary for a better understanding of the complex relationship present between bacteria and the COPD lung.

Principles of real time quantitative PCR

Real time polymerase reaction (r-PCR) is a molecular diagnostic technique that has fundamentally changed and improved the way clinical microbiology laboratories diagnose bacterial and viral pathogens from a variety of human samples. In r-PCR, the PCR chemistry is combined with fluorescent probe detection of the amplified product in the same reaction vessel. Furthermore as the nucleic acid amplification and detection steps are performed in the same vessel, the risk of contamination is greatly reduced compared to conventional PCR. Real time PCR has excellent sensitivity, specificity, low contamination risk, and is easy and rapid to perform compared with routine microbiological cultures.

In conventional PCR, the product is the amplified then detected by running the product on an agarose gel. In real time PCR the accumulation of the amplified product is detected and measured as the reaction progresses. This is possible as a DNA binding fluorescently labelled probe is incorporated to the reaction vessel. The thermal cyclers used for r-PCR testing are equipped with fluorescence detection modules and monitor the fluorescence as amplification occurs as shown in **Figure 24**. The measured fluorescence represents the amount of amplified product in each cycle. Real time PCR allows us to determine the starting template copy number with accuracy.

Figure 24: Real time PCR amplification plot



The PCR cycle number is represented on the x axis and the fluorescence which is proportional to the amount of amplified DNA in the tube is represented on the y axis.

During the exponential phase the amount of PCR product doubles in each cycle. As the reaction proceeds, the components are consumed and the reaction slows and enters the plateau phase.

The threshold value or Ct value is the cycle number at which enough amplified product has accumulated to yield a detectable florescent signal. The Ct value of a reaction is determined by the amount of template present at the start of the amplification process. If a large amount of template is present fewer amplification cycles would be necessary to give a signal above background, and the Ct value will be low. In contrast if a small amount of template is present at the beginning of the reaction, more amplification cycles will be necessary to obtain a signal above background and the Ct value will be high. This relationship forms the basis for the quantitative aspect of r-PCR.

In quantitative r-PCR absolute quantification is achieved by comparing the Ct values of the test samples to a standard curve. The standard curve is generated by running serial dilutions of a target with known concentrations and plotting the log of the starting quantity against the Ct value obtained during amplification of each dilution. The equation of linear regression line along with Pearson's correlation coefficient (r) or the coefficient of determination (R^2) is then used to evaluated how optimized the quantitative r-PCR is. A $r>0.990$ or $R^2 >0.98$ is necessary for the r-PCR to be optimized.

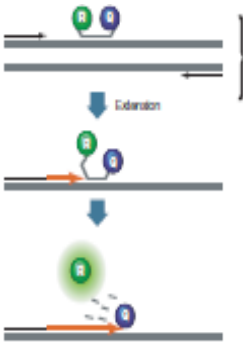
Different types of PCR chemistries can be used to monitor the amplification of the target sequence. The two most common are:

a) DNA-binding dyes (SYBER Green)
b) Fluorescent dye labelled target sequence specific primer or probes

The fluorescence chemistry used in our PCR is a TaqMan probe assay. A sequence of oligonucleotide specific for the organism (= probe) is labelled with a fluorescent reporter dye at it's 5' extremity and a quencher at the 3' extremity. The TaqMan probe uses the 5'exonuclease activity of the thermostable polymerase Taq and during the annealing/extension step of the amplification reaction, the probe hybridizes to the target and the dsDNA-specific 5'→3'exonuclease activity of the Taq cleaves the

reporter dye. This results in emission of fluorescence that is proportional to the amount of amplified product in the sample. The process is illustrated in **Figure 25**.

Figure 25: TaqMan probe assay principles



During annealing the TaqMan probe binds to the target sequence.

During extension, the probe is partially displaced and the reporter dye is cleaved. The free reporter fluoresces.

Reporter Quencher

Certain principles need to be followed when the primers and probes are designed for a r-PCR:

- Primer annealing temperature(Tm) between 55 and 60°C
- Probe with Tm 5-10 °C higher than that of primers
- Probe < 30 nucleotide with GC content of 30-80%
- Primer 75-200 base pairs with GC content of 50-60%
- Avoid secondary structure
- Avoid repeats of Gs and Cs longer than three bases
- Place Gs and Cs on ends of primers
- Check sequence of forward and reverse primers to ensure there is no 3' complementarity (this avoids primer dimer formation)

Once the primers and probes are designed, the sequence specificity can be checked on publicly available databases such as the National Library of Medicine Blast database.

When planning a multiplex assay it is important to use different reporter dyes for the different probes. As all the fluorescent labelled probes will be in the same reaction tube, each individual amplification reaction can thus be distinguished from the others. The Rotor-Gene Corbett used in our lab was able to detect fluorescence on 3 different channels.

The next step in the setting up the multiplex r-PCR assay is to optimize individual assays and determine the Ct for each target. Then the multiplex assay can be validated. Running the singleplex and multiplex assays for the targets in the same run allows validation of the multiplex. The Ct for each individual target should be similar in the singleplex and multiplex assay.

All the information above was summarized from Espy et al 2006, Mackay et al 2002 and 2004 and Real-Time PCR: Applications guide Bio-Rad Laboratories Inc.

5.2 Aim

We established a multiplex quantitative real time (qr)-PCR assay for the detection of HI, SP and MC from sputum samples.
We used this multiplex assay on samples collected for the time course study. First we compared our assay with conventional microbiological cultures. Secondly we assessed the relationship between the detection of micro-organisms by PCR and airway inflammation in patients with COPD sampled in the stable state and at exacerbation. Finally we assessed the relationship between bacterial eradication by PCR and airway inflammation 7 days after exacerbation.

5.3 Methods

The first part of this methods section will describe the steps involved in setting up the multiplex quantitative r-PCR. The sputum samples used were from a group of patients from the time course study.

5.3.1 Establishing the real time PCR assay

The PCR work was a new collaboration between the Academic Unit of Microbiology and the Academic Unit of Respiratory Medicine, University College London, Royal Free Campus. I spent six months working in the Academic Unit of Microbiology under the supervision of Drs Claire Ling and Tim McHugh.
The PCR assay was performed on a Corbett Research Rotorgen analyzer.

5.3.2 Probes and primers

The target sequences for the three organisms were chosen after a literature review on published studies of r-PCR and according to the microbiology laboratory's own experience with nested PCR.

The target sequences chosen were the pneumolysin gene for S pneumoniae, the CopB gene for M catarrhalis and the Hel gene for H influenzae. The probes and primers were designed using the Primerquest and MFold programs. The specificity of the chosen sequences was checked using NCBI BLAST database. The target sequences, probes and primers are presented in **Table 19.**

The primers were designed to have around 20 base pairs, a Tm around 60 °C and a GC content of 40-50%. The probes were designed to have around 30 base pairs, a Tm around 70 °C and a GC content of 50%.

The probes and primers were all manufactured by T.I.B. MOLBIOL (Syntheselabor GmbH, Berlin, Germany).

The Rotor-Gene instrument we used for our assay was able to recognize 3 different dyes on 3 channels simultaneously. As our laboratory had experience working with them we chose the reporter dyes and quenchers presented in **Table 20** for our probes.

Table 19: Primers and probes used for multiplex quantitative r-PCR

Organism	Target gene	Forward primer Reverse Primer	Probe
S. pneumoniae	ply gene	5' ATggTggCgTACggTTTATgA 3' 5' TCTACCTTATCCTCTACCTgAg 3'	5' ROX-ATTTgCCACTAgTgCgTAAgCgACgATT-BHQ2 3'
H. influenzae	hel gene	5' CCATTCgATggTAAAgATTggACTC 3' 5' CAAAACgAgCCgCTTTAgC 3'	5' 6FAM-ACAgCCACAACggTAAAgTgTTCTACgT-Dabcyl 3'
M. cattarhalis	copB gene	5' TgTACCgAAACgACTTTgACCA 3' 5' TAATgCCATTgACCTAAAAATTgACA 3'	5' YAK-TCTAgCATAAAgCgACCTTggTggTAgTgT- Dabcyl 3'

Table 20: Reporter dyes and quenchers used in the assay

Gene	Reporter dye	Quencher
Hel	FAM	DABCYL
Ply	ROX	DABCYL
CopB	YAK	BHQ1

5.3.3 Determination of sensitivity and specificity of the assay

The sensitivity of each set of primers and probes was determined individually then multiplexed together. For the sensitivity determination we used DNA extracted from pure cultures of National Culture Type Collection (NCTC) strains of S pneumoniae (NCTC 49619), H influenzae (NCTC 11931) and M catarrhalis (NCTC 11020).

The specificity was also tested for each organism individually then all three multiplexed together. The specificity of the assay was tested against the organisms shown in **Table 21.** These bacteria were extracted from pure cultures strains isolated from routine sputum specimens in the microbiology department.

Table 21: List of bacteria used to assess the specificity of our assay

Bacterial species	
Moraxella catarrhalis	Proteus mirabilis
Haemophilus influenzae	Neisseria meningitides
MRSA	Bordetella pertussis
Echerishia coli	Corynebacterium diphteriae
Coagulase negative Staphyloccocus (S & R)	Gr A Streptococcus
Pseudomonas aeruginosa	Oxford Staphylococcus
Candida albicans	Legionella pneumophiliae
Klebsiella pneumoniae	Haemophilus parainfluenzae
Neisseria gonorrhoea	Acinetobacter

To achieve the best sensitivity and specificity of the assay we conducted experiments where we changed:

- the concentrations of magnesium chloride: $MgCl_2$ 5 and 6 mM
- the cycling temperatures: 60 and 62 °C
- the cycle duration:
    - Short cycle: 95°C for 3 min
        - 95°C for 10 seconds } for 40
        - 60°C for 5 seconds } cycles
    - Long cycle: 95°C for 3 min
        - 95°C for 10 seconds } for 40
        - 60°C for 45 seconds } cycles
- probe concentrations: 0.2 and 0.25 µM
- primer concentrations: 0.3 and 0.5 µM
- Taq polymerase concentration: 1 and 1.25 U/µl

When using NTCT bacterial strain cultures, the sensitivity of our multiplex assay was:

- M catarrhalis 10^4 organisms
- S pneumoniae 10^4 organisms
- H influenzae 10^1 organisms

The specificity of the multiplex assay was 100% against the organisms in **Table 21** with cycles cropped at 30 Ct for S pneumoniae.

5.3.4 Mastermix and cycling

The experiments above allowed us to optimize the in-house PCR mastermix which we then used for all experiments and samples.

The mastermix used for our assay contained:

- 2.5 µL PCR reaction buffer (Promega, Southampton, UK)
- 1.5 mM of the four deoxyribuonucleotide triphosphate (dNTPs) (Promega, Southhampton, UK)
- 6mM $MgCl_2$

- 0.5µM of each of the primers
- 0.25 µM of each of the probes
- 1.25 U/ µL of Taq DNA Polymerase (Promega)
- 1.5 µL of sterile radiated PCR grade water

We used the 72 well rotor with 0.1 ml ependorf tubess. We added 20µL of our in-house PCR mastermix and 5 µL of sample DNA to each tube.

The optimum PCR cycling was with initial denaturation at 95 °C for 3 minutes followed by 40 cycles held at 95 °C for 10 seconds and 60 °C for 45 seconds.

5.3.5 Sputum sample preparation for PCR assay and DNA extraction

PCR sputum sample preparation

The sputum was diluted 10 fold in phosphate buffered saline (PBS) and manually agitated with 1-2mm in diameter siliconised glass beads (BDH Chemicals Ltd, Poole, Dorset, UK). Then 0.5 mililiters of the supernatant was frozen at -70 °C till DNA extraction.

Bacterial DNA extraction

The DNA extraction was performed using an in-house protocol containing Chelex 10%. In brief, heat-killed sputum samples were washed twice with sterile PBS, centrifuged at 13000 rpm and the pellets re-suspended by vortexing for 15 seconds with 250 µl of sterile distilled water and 250 µl of Chelex 10%. The samples was then incubated at 56°C for 30 min followed by 95°C for 5 min. Finally the samples were centrifuged at 13000 rpm for 10 minutes and the supernatant taken for qr-PCR. Positive and negative extraction controls were also performed with each batch.

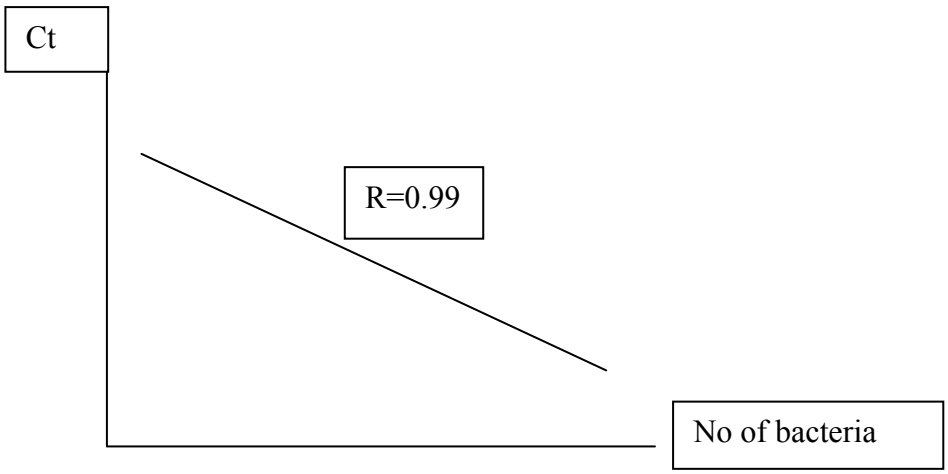
5.3.6 Detection of bacteria with the real time multiplex PCR assay

Our multiplex r-PCR was developed to give quantitative results for the 3 bacteria from sputum samples. Therefore we established standard curves for each organism using r-PCR negative sputa spiked with known amounts of each of the NCTC strains.

In brief we collected a pooled sample of culture negative sputum. We extracted bacterial DNA with the in house Chelex protocol. We confirmed the sputum was also negative for the concerned organisms by r-PCR. In separate tubes we added 500µl of r-PCR negative sputum with a whole loop(= about 10^8 CFU/ml) of pure culture from each of the NTCT strains. **Figure 26** is a diagram of the process used to establish the standard curve.

For each organism the standard curve was established in triplicate.
The RotorGene software allowed us to determine the R value and threshold for each standard curve as illustrated in **Figure 27**.

Figure 27: Standard curve given by Rotor gene



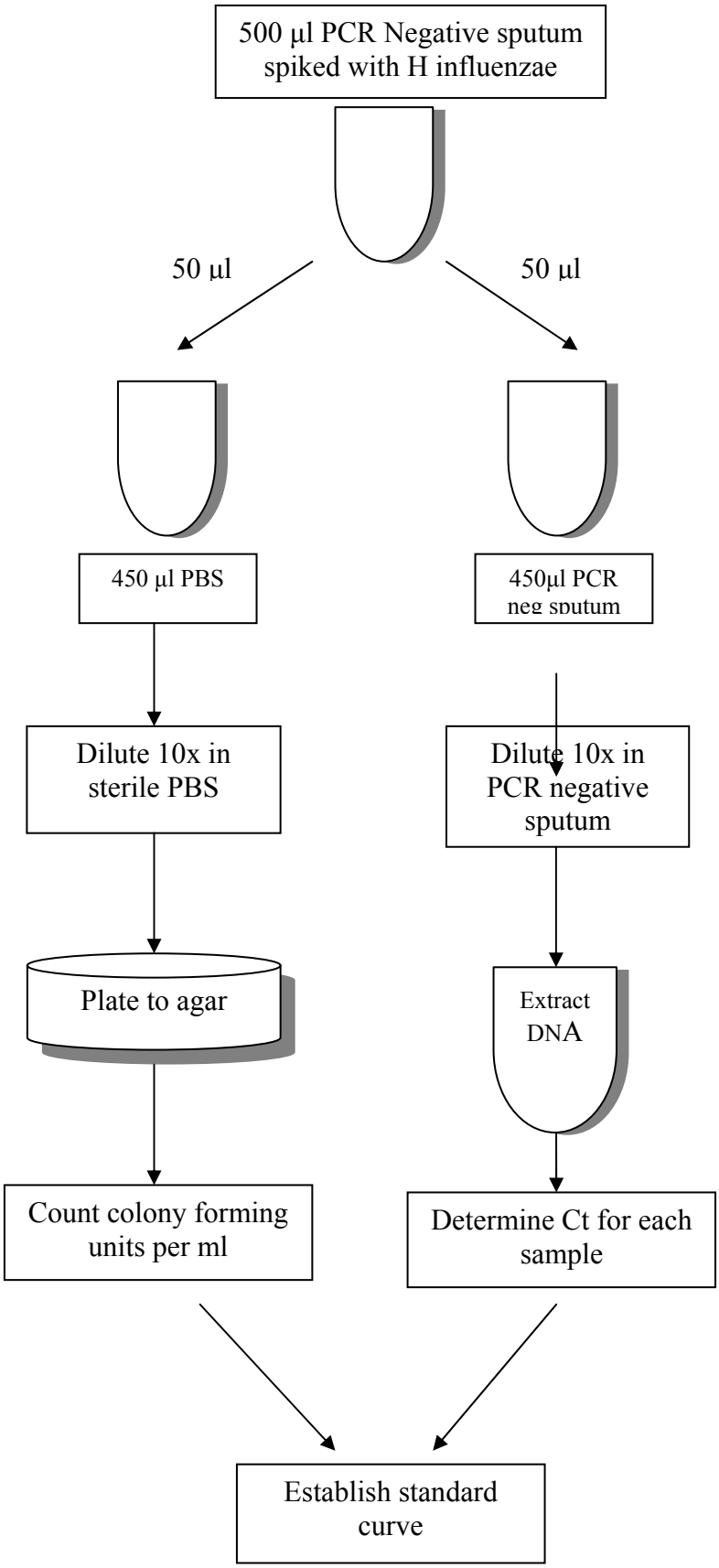
The R value was 0.99 for all three and the threshold values (Ct) were:

H influenzae 0.01

S pneumoniae 0.02

M catarrhalis 0.03

Figure 26: Establishing the standard curve

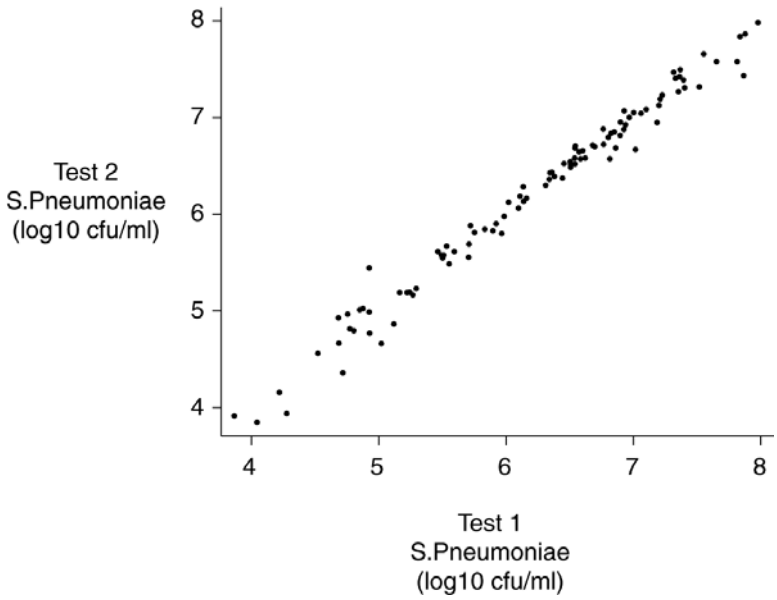


Using these standard curves the sensitivity of the multiplex quantitative r-PCR assay was:

H influenzae	$2.6x10^3$	or	$10^{3.41}$	CFU/ml
S pneumonia	$7.2x10^5$	or	$10^{5.85}$	CFU/ml
M catarrhalis	$7.2x10^4$	or	$10^{4.85}$	CFU/ml

Each sputum sample was run in duplicate in the same r-PCR assay and each sample was run on two separate tests. The inter-assay correlation for the 2 tests was $r^2=0.99$; $p<0.001$ and is represented in F**igure 28**.

Figure 28: Inter-assay correlation on two separate occasions for SP with r-PCR assay



5.3.7 Detection of bacteria with conventional cultures

The sputum was homogenized by adding an equal weight of sputolysin (Sputasol; Unipath, Hampshire, UK) and several glass beads (1-1.5mm in diameter) and incubating for 30 minutes at 37°C, time during which they were intermittently vortexed for 5-10 seconds. Tenfold serial dilutions of the homogenized sample were

made in brain heart infusion broth and 100 μL aliquots were plated out onto the surface of a range of different media including blood agar, chocolate agar, MacConkey agar and cysteine lactose electrolyte-deficient agar. These plates were then incubated for 18 to 36 hours in an atmosphere of air +5% carbon dioxide. After incubation, bacterial colonies were counted and sub-cultured for identification by standard methods (Barrow et al 1993). The number of colony forming units per millilitre of sputum was calculated from the number of colonies obtained and the dilution of the sputum. The bacteriology data is expressed as the total bacterial count in log base 10.

5.3.8 Patient recruitment and sampling

Forty-nine patients were recruited from the London COPD Cohort. COPD was defined as a post-bronchodilator forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC) ratio below 70% and a β_2-agonist reversibility on predicted FEV_1 of less than 15% or 200ml. Patients with a history of other significant respiratory diseases were excluded, as were those unable to complete daily diary cards. Patients were recruited when stable, at least six weeks away from their last exacerbation. At recruitment, patients' daily respiratory symptoms, smoking history, COPD exacerbation history and drug history were recorded. Height and weight were measured, in addition to baseline lung function using a rolling seal spirometer (Sensor Medic Corp, Yorba Linda, US). Reversibility to β_2-agonists was measured after inhaling 400μg salbutamol from a metered dose inhaler via spacer. Partial pressures of arterial oxygen and carbon dioxide were measured on arterialised ear lobe blood gases (Model 278 Blood Gas Analyser, Ciba-Corning, Medfield, US). Patients also completed the St. George's Respiratory Questionnaire (SGRQ), which is a disease-specific measure of health-status (Jones et al 1992).

All the samples from the present analysis were used for the time course of airway and systemic inflammation analysis presented in Chapter 3. However, the bacteriology data was not addressed in that chapter and all the current analyses are original.

Patient follow-up and exacerbation

All patients were asked to keep daily diary cards on which they recorded their morning post-medication peak expiratory flow rate (PEFR) with a mini-Wright peak-flow meter (Clement-Clarke International Ltd, Harlow, UK) and any increase in their daily respiratory symptoms. Patients were routinely followed up every three months in the research clinic, their diary cards were reviewed and spirometry was recorded. They were asked to contact the study team when they experienced any increase in their daily respiratory symptoms. They were generally seen within 48 hours by a study-physician, their symptoms were reviewed and the exacerbation was confirmed according to our symptomatic definition described below. Patients were sampled before starting any additional treatment for the exacerbation.

Patient sampling

Patients were seen and sampled in the stable state, at exacerbation prior to treatment, and seven days, 14 days and 35 days later. At each visit, the patients' symptoms were documented, lung function was recorded, and sputum and serum were collected. Spontaneous sputum was used, or when no spontaneous sputum was available, patients were induced according to our previously described methodology (Bhowmik 1998). We have previously reported on the equivalence of induced and spontaneous samples for the assessment of airway inflammatory markers (Bhowmik 1998). All exacerbations were treated with bronchodilators, antibiotics and or oral steroids depending on the clinical severity of the episode, as judged by the attending physician.

Sputum processing

Sputum samples were processed within two hours of collection. Sputum was separated from the contaminating saliva using sterile forceps and one third of the sputum sample was used for quantitative bacterial analysis, one third was used for the bacteriology PCR and the remainder was used for inflammatory cytokines analysis. Only sputum samples containing <25 squamous epithelial cells per low powered field and > 25 leukocytes per high powered field were used for the present analysis.

Airway inflammatory markers

The sputum interleukin (IL)-6 and IL-8 levels were measured using an enzyme-linked immunosorbent assay kit(R&D Systems; Abingdon, UK). The sputum sample was prepared by diluting it tenfold in PBS, homogenizing and filtering through a 50µm nylon gauze to remove mucus and debris. Afterwards it was centrifuged at 2000rpm for 10min and the supernatant was stored at -70 °C till analysis. The limit of detection for sputum IL-6 was 0.7pg/ml and for sputum IL-8 it was 10 pg/ml.

5.3.9 Statistical analysis

The Kolmogorov-Smirnov test of normality was applied and normally distributed data are presented as mean (SD) values and skewed data as median (inter quartile range, IQR) values. Continuous variables with normal distributions were compared by t test, and non-normal distributions were compared with the Mann-Whitney U or Wilcoxon signed-ranks test. Chi-square test was used to assess the results by standard cultures and PCR. The data analysis was performed using statistical software SPSS version 10 (SPSS; Chicago, Illinois, USA).

5.3.10 Ethics

All patients gave written informed consent and the project was approved by the East London and City Health Authority Ethics committee.

5.4 Results

5.4.1 Patient characteristics

Samples from 49 patients who participated in the time course study were available for the present analysis. The patients had moderate to severe COPD and their baseline characteristics are presented in **Table 22**. The mean (SD) FEV_1 was 1.06 (0.41) litres or 44 (18) % predicted. One hundred and seventy five sputum samples were analysed by quantitative r-PCR, 23 were baseline samples, 39 were exacerbation samples, 43 were samples taken after 7 days, 36 were samples taken after 14 days and 34 were samples taken 35 days after exacerbation. One hundred and fifty eight of those samples had a matched conventional culture result available.

Table 22: Baseline characteristics of the 49 COPD patients (21 female, 14 active smokers)

	MEAN	SD
Age (years)	68.7	8.0
FEV_1 (l)	1.06	0.41
FEV_1 (% predicted)	44	18
FVC (l)	2.38	0.84
BMI (kgm^{-2})	26.2	6.80
PaO_2 (kPa)	8.72	0.96
$PaCO_2$ (kPa)	5.68	0.76
Smoking (pack years)	49	29
SGRQ (total score)	60.3	16.6

5.4.2 Detection of bacteria by qr-PCR compared to conventional cultures

The results from the 158 samples with conventional bacteriological cultures and multiplex qr-PCR assay are presented in **Table 23**.

Table 23: Qualitative results of the conventional cultures and the qr-PCR

Sample		Culture positive			PCR positive		
Type	Total	Spneu	Hinf	Mcat	Spneu	Hinf	Mcat
All	158	7	37	15	65	46	27
Baseline	21	0	7	1	10	8	1
Exacerbation	37	3	10	6	13	11	10
Day 7	40	1	5	1	14	6	3
Day 14	32	1	8	6	18	10	8
Day 35	28	2	7	1	10	11	5

By conventional culture 62/158 samples grew an identifiable pathogen. A PPM was present in 59/158 samples, 2 of the other samples grew Pseudomonas aeruginosa and 1 sample grew Haemophilus parainfluenzae. No specific growth was found in 96/158 samples. However, in all these samples with no specific growth the total bacterial load was above 10^5 CFU/ml.

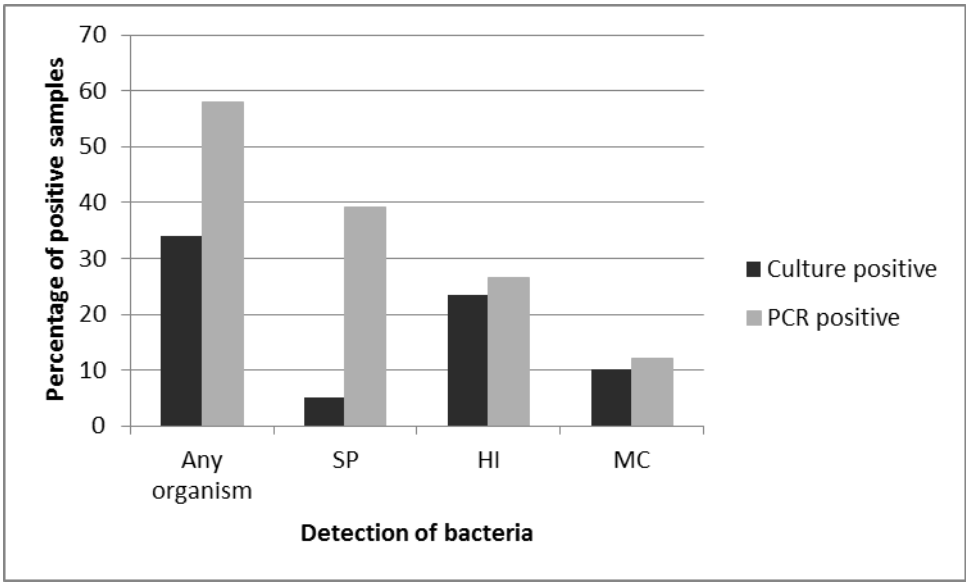
In 8 samples we detected 2 PPM's. No samples detected 3 pathogens by culture. By qr-PCR we detected 2 PPM's in 21 samples and all 3 PPM's in 5 samples.

All the samples that grew SP by conventional cultures were also positive by qr-PCR. For MC, 14/15 samples that were culture positive were also qr-PCR positive. For HI, 37 samples were culture positive, a matched qr-PCR was available for 32/37 samples. In these samples, 27/32 samples were culture and qr-PCR positive for HI.

When all the samples were considered together, the detection rate of any organism was significantly higher by qr-PCR compared to conventional culture, 58% by qr-PCR versus 34% by culture, $p<0.001$ (Chi-square test).

The detection of S pneumoniae was significantly higher by qr-PCR compared to conventional culture, 39.2 % by qr-PCR versus 5.0% by culture, $p<0.001$. There was no statistically significant difference in the detection rates of the other 2 organisms between qr-PCR and cultures. Detection of HI was 24.8% by qr-PCR versus 22.1% by culture and detection of MC was 11.7% by qr-PCR versus 9.7% by culture, $p>0.05$ for both. These results are presented in **Figure 29.**

Figure 29: Comparison of samples positive by culture and qr-PCR



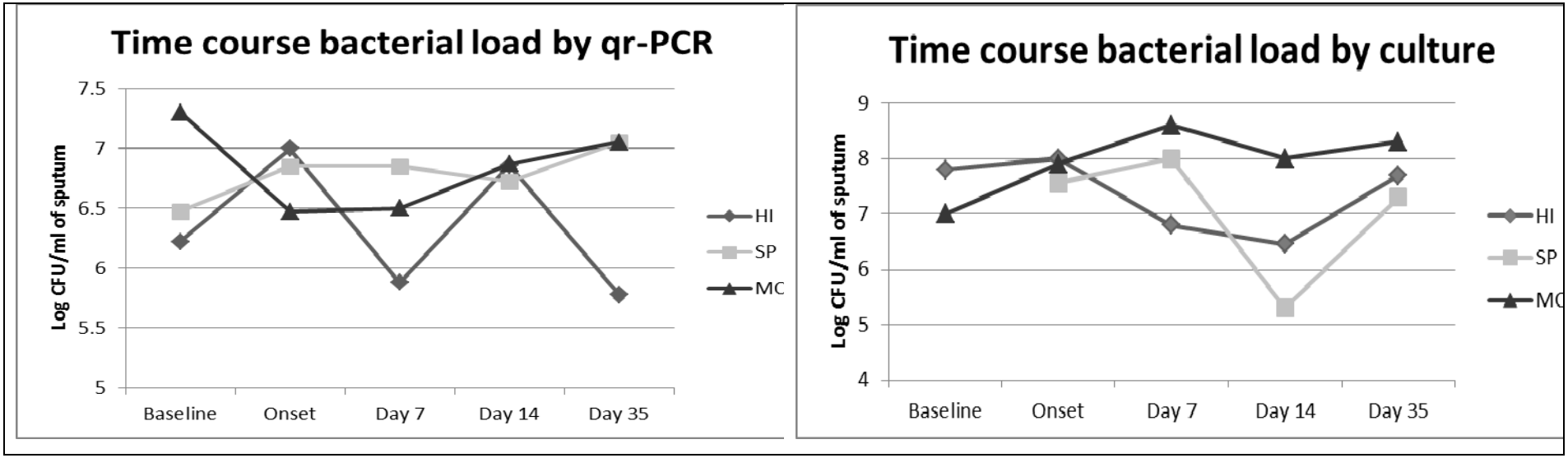
5.4.3 Time course of the bacteria

The changes in bacterial load between baseline, exacerbation and the recovery at day 7, 14 and 35 as measured by conventional cultures and qr-PCR are shown in **Table 24** and **Figure 30**. There were no statistically significant changes in bacterial load between baseline and exacerbation by either of the bacterial detection methods.

	Baseline		Onset		Day 7		Day 14		Day 35	
Bacteria	Culture	qr-PCR	Culture	qr-PCR	Culture	qr-PCR	Culture	qr-PCR	Culture	qr-PCR
HI	7.8	6.22	8	7	6.8	5.88	6.45	6.86	7.7	5.78
SP	0	6.47	7.55	6.85	8	6.85	5.3	6.72	7.3	7.05
MC	7	7.3	7.9	6.47	8.6	6.5	8	6.87	8.3	7.05

Table 24: Bacterial load by culture and qr-PCR in log CFU/ml of sputum

Figure 30: Bacterial load time course



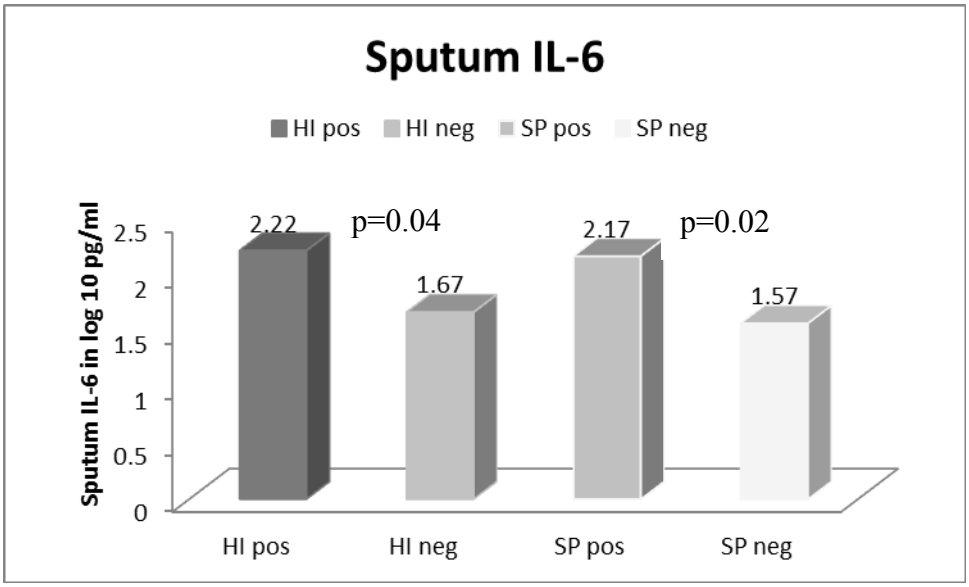
There were a sufficient number of samples to compare the mean bacterial loads by culture and qr-PCR for HI at all sampling time points, for SP at exacerbation and at day 35 and for MC at exacerbation and day 14 (paired sample t test).

The mean bacterial load for HI detected by culture was significantly higher at baseline and day 35 (p=0.001 for both). For MC, the mean bacterial load was higher by culture only at day 14 (p=0.04).

5.4.4 Airway inflammation and bacterial detection by qr-PCR

The sputum levels of IL-6 and IL-8 were determined in all our patients. Patients with H influenzae or S pneumoniae detected by qr-PCR in the stable state had a significantly higher level of sputum IL-6 than those without, p=0.04 and p=0.02 respectively as shown in **Figure 31**. No similar relationship was observed between PPM detected by routine culture and sputum IL-6.

Figure 31: Sputum IL-6 levels in patients with and without HI or SP detected by qr-PCR



At exacerbation, the detection of any of the 3 organisms by qr-PCR was associated with a higher level of sputum IL-8, $p<0.05$ for all 3, as presented in **Table 25**. No similar relationship was observed with standard cultures.

Table 25: Relationship between sputum IL-8 and PPM detected by qr-PCR

	Sputum IL-8 if PCR+ log 10 pg/ml	**Sputum IL-8 if PCR- log10 pg/ml**	**p=**
HI	3.6	3.43	0.03
SP	3.59	3.42	0.01
MC	3.62	3.44	0.03

The presence of M catarrhalis at exacerbation was associated with higher levels of sputum IL-6 at 2.2 versus 1.74 log10 pg/ml, p=0.01. No statistically significant relationships were present at any of the other time points.

5.4.5 Bacterial eradication and airway inflammation

Thirty four patients had sputum qr-PCR performed at exacerbation prior to treatment and 7 days after treatment. Among these patients, 19/34 had a PPM detected by qr-PCR at exacerbation, 12/34 Patients had a PPM detected by qr-PCR at exacerbation and at day 7. The 7 patients who had a PPM at exacerbation but had eradicated it by day 7, showed a trend towards lower sputum IL-8 levels at the second visit compared to those who had not eradicated the organism. Mean (SD) sputum IL-8 was 3.40(0.20) versus 3.55(0.09) log10 pg/ml, p=0.06.

5.5 Discussion

In this pilot study we established a multiplex quantitative real time PCR (qr-PCR) for the detection of HI, SP and MC from sputum samples. We then used the assay to test 158 sputum samples obtained from 49 patients from the London COPD Cohort.

Our main findings were that when all samples were analysed together, the detection of any of the PPM was significantly higher by qr-PCR compared to standard cultures. When the qr-PCR and cultures results for each individual PPM were compared, only SP was detected at significantly higher rates by qr-PCR.

We also found that the detection by qr-PCR of HI or SP in the stable state was associated with higher levels of sputum IL-6. At exacerbation, the detection of any PPM by qr-PCR was associated with higher levels of sputum IL-8. No similar relationship was present with routine cultures.

As we had samples taken at baseline, exacerbation onset, after 7, 14 and 35 days; we were able to establish a time course of the bacterial loads both by standard cultures and qr-PCR. No significant changes in the bacterial load were detected between baseline and exacerbation by either detection method. Our results suggest that the gold standard for bacterial detection in COPD could be qr-PCR.

We used a multiplex qr-PCR assay to simultaneously detect the three PPM. Multiplex assays for the detection of these PPM have previously described for middle ear effusions (Hendoin et al 1997) and lower respiratory tract samples (Kais et al 2006).

In the first study 25 middle ear effusions were examined with a multiplex qr-PCR assay for the detection of SP, HI and MC (Hendolin et al 1997). By conventional cultures 32% of the samples were positive. By multiplex qr-PCR 84% of the samples were positive. In this study none of the culture positive samples were PCR negative. This was equivalent to a 225% relative increase in the detection of a pathogen by qr-PCR.

In the second study 203 consecutive respiratory samples were subjected to detection of SP, HI and MC by a multiplex qr-PCR assay (Kais et al 2006). Significant pathogen findings were defined as the presence of 10^5 or more organisms per CFU/ml. The cultures were positive in 22.2% of the samples and qr-PCR was positive in 33.5% of the samples ($p<0.05$). This corresponds to a 51% relative increase and 11.3% absolute increase in the detection of a pathogen. Our results showed a 70% relative increase and a 24% absolute increase, in keeping with above results.

SP has been successfully isolated by real-time PCR from middle ear fluid, nasopharyngeal aspirate, serum, BAL fluid and respiratory tract samples (=sputum, nasopharyngeal swab and nasopharyngeal aspirate) (Salo et al 1995, Greiner et al 2001, Saukkoriipi et al 2002 and Straelin et al 2006 a & b). Three of these studies also used the pneumoysin gene as a target and the other 2 studies (Straelinet al 2006 a&b) used the lyt A gene as a target. In all of the studies the detection of SP by PCR was higher than by conventional culture, in keeping with our results. The differences were the most pronounced for samples taken after starting antibiotics (Straelin et al 2006 b). However, as the detection rate of SP is significantly higher by qr-PCR, our results should be confirmed and strengthened by detecting SP by a nested PCR.

Only one study has used real time PCR to assess respiratory pathogens obtained exclusively from COPD patients (Curran et al 2006). In this study sputum samples from 30 COPD patients were assessed by real time PCR (not quantitative or multiplex) for the presence of 10 respiratory pathogens. The detection of Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus species and Moraxella catarrhalis was higher by PCR than by culture. However, in this study no clinical details were provided regarding the patients themselves or the severity of the COPD.

Our study is unique as the patients are well characterized and we have matched airway and systemic inflammatory markers available for all the patients. This enabled us to

demonstrate significant relationships between the detection of a PPM by qr-PCR and airway inflammation. It was not possible to detect such relationships with standard cultures.

In matched samples, the mean bacterial load by culture was significantly higher than that detected by qr-PCR for HI and MC at certain time points. These results are the opposite of what one would expect. This could be due to the presence of inhibitors in the sputum. Dithiothreitol and mucolytic agents present in sputum are known to exert an inhibitory effect on PCR amplification (Wilson I 1997). We did not use any Dithiothreitol whilst processing our sputum samples but we did not ask our patients if any of them were using mucolytic agents.

The lower bacterial loads by qr-PCR could also be due to the presence of strains of HI and MC with mutations in our target sequence. Those samples would not be recognized by the qr-PCR assay. The colony morphology of MC can be difficult to distinguish from that of commensal Neisseria species which are also part of the commensal respiratory tract flora (Sethi et al 2000). It is also possible that the loads by culture for HI were overestimated.

Sample preparation and DNA extraction methods are paramount when performing quantitative PCR. When commercially available DNA extraction kits are compared, variable amounts of DNA have been extracted from the same sample (Wilson D et al 2004). We used an in-house Chelex extraction method. Our findings would be strengthened by extracting the DNA from the same sample via several extraction methods and then performing the qr-PCR assay to compare them.

We were able to detect multiple PPM's by qr-PCR more often than by culture. We also had samples with a total bacterial load > 10^5 CFU/ml by culture where no specific growth could be identified. A PPM could be detected in quite a number of those samples by qr-PCR. However, a positive PCR result does not allow us to differentiate dead bacteria from those that are alive.

This project was a pilot study and the results were encouraging. The work has now been taken forward in the department by other research fellows. Their work has

demonstrated that the pneumolysin gene was detecting Streptococcus pseudopneumoniae as well as SP. They have therefore changed the target sequence of SP to the lytA gene. They have also included an internal amplification control into the assay to detect any inhibitors present in the sample.

5.6 Conclusion

In summary we developed a multiplex qr-PCR assay for the detection of HI, SP and MC. We have demonstrated that the detection of any of the PPM is significantly higher by qr-PCR. The assay needs to be further refined with regards to load determination and pathogen specificity. We feel that PCR could become the gold standard for the detection of bacteria from sputum samples in COPD patients in the near future.