Acute Exacerbation and Respiratory InfectionS in COPD (AERIS): A prospective, observational cohort study of the dynamics of airway pathogens and the seasonal aetiology of exacerbations in chronic obstructive pulmonary disease

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Supplementary appendix

METHODS

Inclusion and exclusion criteria

All subjects must satisfy all the following criteria at study entry:

- Subjects who the investigator believes can and will comply with the requirements of the protocol.
- Written informed consent obtained from the subject.
- Male or female subjects between, and including, 40 and 85 years of age, at the time of consent.
- Subjects with confirmed diagnosis of COPD (based on post-bronchodilator spirometry)[1] with forced expiratory volume of air expired in 1 second (FEV_1) of ≤80% of predicted normal and FEV_1/forced expiratory vital capacity <0·7.
- Subjects have moderate, severe, or very severe COPD, according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging.[1]
- Subjects have a current or prior history of ≥10 pack-years of cigarette smoking. Former smokers are defined as those who have stopped smoking for at least 6 months. Number of pack years = (number of cigarettes per day/20) x number of years smoked.
- Subjects present a documented history of ≥1 exacerbation requiring antibiotics and/or oral corticosteroids or hospitalization in the previous 12 months. Subjects with recent COPD exacerbations, in stable condition, and having stopped antibiotics, can be enrolled one month post exacerbation.

The following criteria should be checked at the time of study entry. If any exclusion criterion applies, the subject must not be included in the study:

- Subject has a confirmed diagnosis of asthma (as only cause of obstructive respiratory disorder), cystic fibrosis, pneumonia risk factors or other respiratory disorders (e.g. tuberculosis, lung cancer).
- Subjects having undergone lung surgery.
- Subject has a α-1 antitrypsin deficiency as underlying cause of COPD.
- Subject who experienced a moderate or severe COPD exacerbation not resolved at least 1 month prior to enrolment visit and at least 30 days following the last dose of oral corticosteroids (subjects can be enrolled when their AECOPD or pneumonia has resolved).
- Subject using any antibacterial, antiviral, or respiratory investigational drug or vaccine up to 30 days prior to the enrolment visit.
- Subject has other conditions that the principal investigator judges may interfere with the study findings, such as:
  - Subject at risk of non-compliance or unable to comply with the study procedures.
  - Evidence of alcohol or drug abuse.
- Women who are pregnant or lactating or are planning on becoming pregnant during the study.

Procedures

AECOPD was defined as worsening of at least two major symptoms (dyspnoea, sputum volume, and sputum purulence) or worsening of at least one major symptom and one minor symptom (wheeze, sore throat, cold

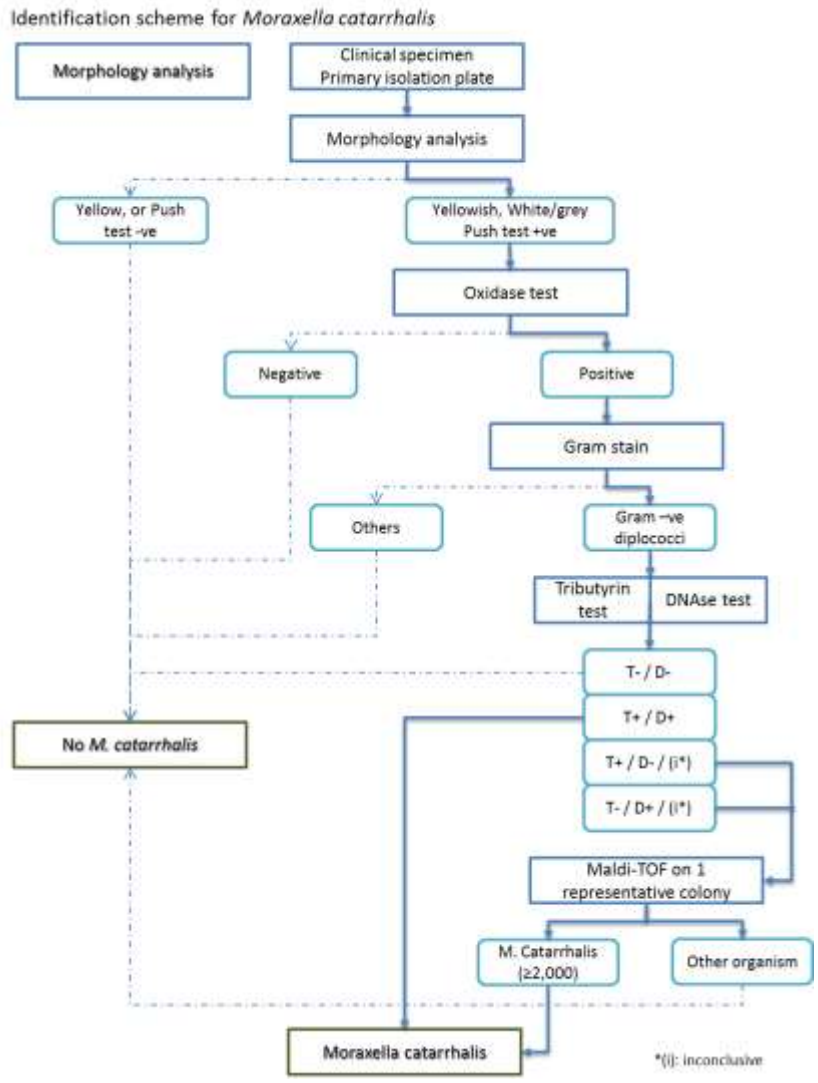
symptoms, cough, and fever without other cause).[2] An exacerbation was considered mild if self-managed by the patient using inhaled therapy, moderate if it required treatment with oral corticosteroids or antibiotics, and severe if the patient required hospitalisation or a home care intervention.[3]

Sputum samples were collected at study entry, monthly, and at exacerbation. Within two hours of expectoration, sputum plugs were separated from saliva using sterile forceps. Samples were kept at room temperature and sent to the Public Health England laboratory for culture-based microbiology.

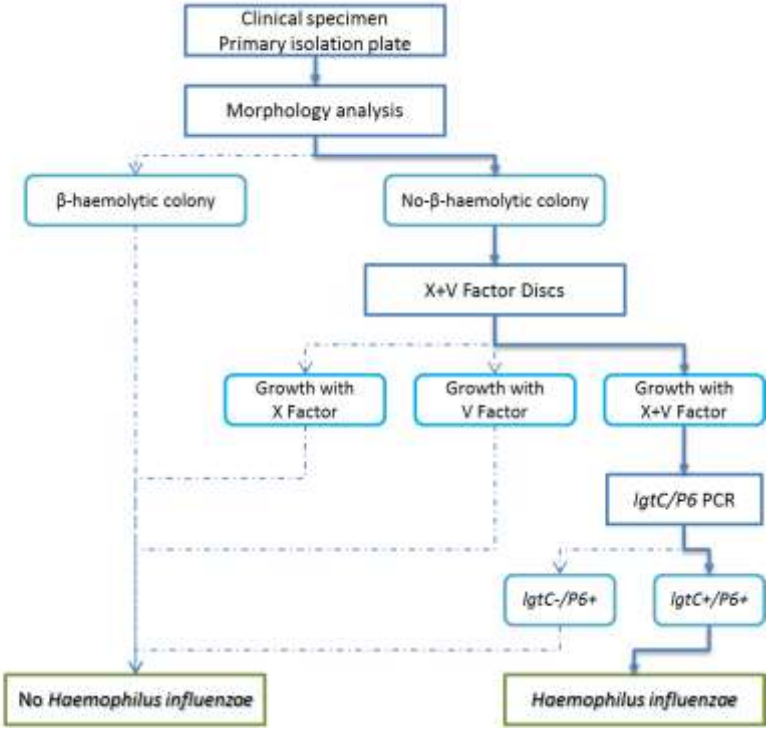
Sputum samples were also processed for the detection of viruses, including HRV, respiratory syncytial virus, influenza virus, parainfluenza virus, human metapneumovirus, adenovirus, human bocavirus, and coronavirus. The qualitative nucleic acid multiplex test used (xTAG® Respiratory Viral Panel Fast v2; Luminex, Austin, TX, USA) is described below along with methods for calculating bacterial or viral load with the other PCR assays used.

Potential bacterial respiratory pathogens, including *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were identified using conventional culture techniques and by PCR. Both techniques were used because culture detects only viable bacteria and has been the gold standard in the research of bacteria in COPD, while PCR has higher sensitivity but background signals could have a diluting effect.[4]

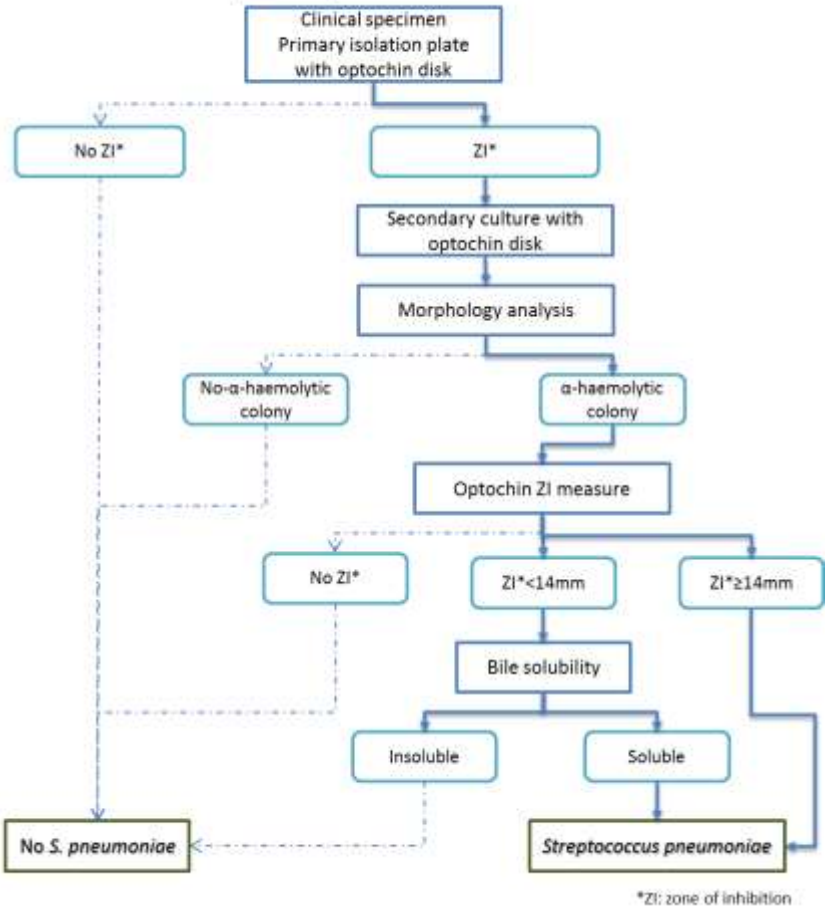
The method for culture of sputum samples was in accordance with Public Health England's UK Standards for Microbiology Investigations.[5] Bacterial isolates phenotypic identification steps were slightly modified as represented in the following figures for *M. catarrhalis*, *H. influenzae* and *S. pneumoniae.*



Identification scheme for *Haemophilus influenzae*



Identification scheme for *Streptococcus pneumoniae*



For PCR, nucleic acids were extracted using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics), as per the manufacturer's instructions. A triplex real-time quantitative PCR assay was used for the detection and quantification of the lipo-oligosaccharide glycosyltransferase encoding gene (*lgtC*) of *Haemophilus influenzae*, the CopB outer membrane protein encoding gene (*copB*) of *M. catarrhalis*, and the autolysin encoding gene (*lytA*) of *S. pneumoniae*. Noteworthy for the latter, the sequences of the primers and probe correspond to the *lytA*-CDC assay.[6]

The presence of *Streptococcus pyogenes*, *S. aureus*, and *P. aeruginosa* was determined using a triplex real-time PCR assay targeting conserved regions of the CDS23 gene, the clumping factor A encoding gene (*clfA*), and the GDP mannose dehydrogenase encoding gene (*algD*), respectively.

The concentration of bacterial DNA in each sample, expressed in copy/mL, was inferred from the calibration curve (made of serial dilutions of a plasmid containing the sequences targeted by the PCR assays) present in each PCR plate and corrected against the dilution factors at each step of the process (DNA extraction and PCR reaction). Positivity thresholds were used for each PCR target. They were set at the limit of detection defined during characterisation of the technical performance of the PCR assays, corresponding to 2000, 15000, 12875, 5000, 3375, and 2750 copies/mL, respectively for *H. influenzae*, *M. catarrhalis*, *S. pneumoniae*, *S. aureus*, *P. aeruginosa*, and *S. pyogenes*. Further details will be presented in a separate paper.

Isolates initially identified as *H. influenzae* by bacteriological methods were later retested by PCR, targeting the glycosyltransferase (*lgtC*) and outer membrane protein P6 (P6) encoding genes[7] to differentiate *H. influenzae* from *H. haemolyticus*. It became clear that for 10·6% of the samples, the isolates identified as *H. influenzae* by conventional microbiological methods were in fact *H. haemolyticus*.[8,9] Also, genetic analysis of the capsule locus indicated that more than 99% of *H. influenzae* isolates were non-typeable (NTHi).

The xTAG® Respiratory Viral Panel (RVP) Fast v2 (Luminex) is a qualitative nucleic acid multiplex test intended for the simultaneous detection and identification of multiple respiratory virus nucleic acids in respiratory specimens.[10] It detects influenza A, including subtypes of influenza A (H1 and H3), and distinguishes between 2009 H1N1 and other H1N1 (seasonal) strains, influenza B, respiratory syncytial virus, human metapneumovirus, parainfluenza virus 1–4, coronavirus (OC43, 229E, NL63, HKU1), rhinovirus/enterovirus, adenovirus, and bocavirus.

A quantitative real-time PCR (RT-PCR) assay was used for the detection and quantification of a fragment of a conserved region of the 5' noncoding region of rhinovirus[11] in samples displaying a positive signal for rhinovirus/enterovirus by xTAG® RVP Fast v2.

The concentration of rhinovirus RNA in each sample, expressed in copies per mL, was inferred from the calibration curve (made of serial dilutions of an *in vitro* transcript containing the sequence targeted by the RT-PCR assay) present in each RT-PCR plate and corrected against the dilution factors at each step of the process (nucleic acid extraction and RT-PCR reaction).

References

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Table S1. Number of exacerbations recorded during the study (full cohort year 1).

	Acute exacerbation severity			
	Any	**Mild**	**Moderate**	**Severe**
Number of exacerbations	355	31	304	20
Exacerbation rate per patient-year[a], mean (95% CI)	3·0 (2·6–3·5)	0·3 (0·2–0·4)	2·6 (2·2–3·0)	0·2 (0·1–0·4)
Number of exacerbations		Number of subjects (N=127)		
0	19 (15·0%)	99 (78·0%)	27 (21·3%)	113 (89·0%)
1	30 (23·6%)	25 (19·7%)	29 (22·8%)	11 (8·7%)
2	18 (14·2%)	3 (2·4%)	20 (15·7%)	1 (0·8%)
3	18 (14·2%)	0 (0%)	18 (14·2%)	1 (0·8%)
4	9 (7·1%)	0 (0%)	8 (6·3%)	1 (0·8%)
5	15 (11·8%)	0 (0%)	12 (9·4%)	0 (0%)
6	8 (6·3%)	0 (0%)	4 (3·1%)	0 (0%)
7	5 (3·9%)	0 (0%)	8 (6·3%)	0 (0%)
>7	5 (3·9%)	0 (0%)	1 (0·8%)	0 (0%)

[a] Negative binomial model. Total exposure time was 117 years.

Table S2. Bacterial and viral pathogen incidence in exacerbation-state sputum samples (full cohort year 1).

	Number stable-state samples/total number samples	Number exacerbation-state samples/total number samples	Rate per patient-year of exacerbations with samples containing pathogen[a] (95% CI)
Bacteria: culture-positive			
Any	466/952 (48·9%)	188/320 (58·8%)	1·59 (1·30–1·95)
NTHi	287/952 (30·1%)	129/320 (40·3%)	1·10 (0·84–1·43)
M. catarrhalis	50/952 (5·3%)	40/320 (12·5%)	0·34 (0·24–0·48)
S. pneumoniae	177/952 (18·6%)	47/320 (14·7%)	0·39 (0·27–0·58)
S. aureus	40/952 (4·2%)	18/320 (5·6%)	0·16 (0·07–0·36)
P. aeruginosa	49/952 (5·1%)	21/320 (6·6%)	0·18 (0·09–0·35)
Bacteria: PCR-positive			
Any	515/910 (56·6%)	206/307 (67·1%)	1·75 (1·43–2·14)
NTHi	405/910 (44·5%)	165/306 (53·9%)	1·41 (1·12–1·77)
M. catarrhalis	102/910 (11·2%)	60/306 (19·6%)	0·50 (0·37–0·69)
S. pneumoniae	120/910 (13·2%)	26/306 (8·5%)	0·22 (0·14–0·35)
S. aureus	40/910 (4·4%)	12/307 (3·9%)	0·10 (0·03–0·32)
P. aeruginosa	52/910 (5·7%)	22/307 (7·2%)	0·19 (0·09–0·39)
Virus			
Any	124/910 (13·6%)	126/305 (41·3%)	1·07 (0·86–1·32)
Enterovirus	83/910 (9·1%)	80/305 (26·2%)	0·68 (0·51–0·89)
Human rhinovirus	56/908 (6·2%)	70/305 (23·0%)	0·59 (0·45–0·78)
Human coronavirus	22/910 (2·4%)	21/305 (6·9%)	0·18 (0·11–0·29)
Influenza virus	5/910 (0·5%)	15/305 (4·9%)	0·07 (0·03–0·14)
Human metapneumovirus	2/910 (0·2%)	6/305 (2·0%)	0·05 (0·02–0·11)
Parainfluenza virus	5/910 (0·5%)	6/305 (2·0%)	0·05 (0·02–0·11)
Adenovirus	6/910 (0·7%)	3/305 (1·0%)	0·03 (0·01–0·08)
Respiratory syncytial virus	2/910 (0·2%)	3/305 (1·0%)	0·03 (0·01–0·08)
Human bocavirus	2/910 (0·2%)	3/305 (1·0%)	0·02 (0·00–0·12)

[a] Negative binomial model. Total exposure time was 117 years.

Table S3. Proportion of patients with at least one sputum sample that was positive for a specific bacterial pathogen (by culture or PCR) at stable and exacerbation states (full cohort year 1).

	Culture (95% CI)		PCR (95% CI)	
	Stable (N=119)	**Exacerbation (N=104)**	**Stable (N=118)**	**Exacerbation (N=101)**
Any bacteria	83·2% (75·2–89·4)	76·9% (67·6–84·6)	91·5% (85·0–95·9)	82·2% (73·3–89·1)
NTHi	63·9% (54·6–72·5)	56·7% (46·7–66·4)	82·2% (74·1–88·6)	70·3% (60·4–79·0)
M. catarrhalis	23·5% (16·2–32·2)	28·8% (20·4–38·6)	39·8% (30·9–49·3)	37·6% (28·2–47·8)
S. pneumoniae	47·9% (38·7–57·2)	27·9% (19·5–37·5)	36·4% (27·8–45·8)	20·8% (13·4–30·0)
S. aureus	15·1% (9·2–22·8)	8·7% (4·0–15·8)	13·6% (8·0–21·1)	5·0% (1·6–11·2)
P. aeruginosa	15·1% (9·2–22·8)	11·5% (6·1–19·3)	13·6% (8·0–21·1)	10·9% (5·6–18·7)

N = number of subjects with culture/PCR results available.

Table S4. Proportion of patients (95% CI) with at least one sputum sample that was positive for a specific viral pathogen at stable and exacerbation states (full cohort year 1).

	Stable (N=118)	Exacerbation (N=101)
Any virus[a]	51·7% (42·3–61·0)	68·3% (58·3–77·2)
Enterovirus	39·0% (30·1–48·4)	48·5% (38·4–58·7)
Human rhinovirus	26·3% (18·6–35·2)	46·5% (36·5–56·7)
Human coronavirus HKU1	2·5% (0·5–7·3)	3·0% (0·6–8·4)
Human coronavirus NL63	6·8% (3·0–12·9)	6·9% (2·8–13·8)
Human coronavirus 229E	1·7% (0·2–6·0)	1·0% (0·0–5·4)
Human coronavirus OC43	5·1% (1·9–10·7)	7·9% (3·5–15·0)
Influenza A virus	2·5% (0·5–7·3)	6·9% (2·8–13·8)
Influenza A H1N1 virus	0% (0·0–3·1)	0% (0·0–3·6)
Influenza A H3N2 virus	1·7% (0·2–6·0)	6·9% (2·8–13·8)
Influenza B virus	0% (0·0–3·1)	1·0% (0·0–5·4)
Human metapneumovirus	1·7% (0·2–6·0)	5·9% (2·2–12·5)
Parainfluenza virus 1	0% (0·0–3·1)	0% (0·0–3·6)
Parainfluenza virus 2	0% (0·0–3·1)	0% (0·0–3·6)
Parainfluenza virus 3	0·8% (0·0–4·6)	4·0% (1·1–9·8)
Parainfluenza virus 4	1·7% (0·2–6·0)	2·0% (0·2–7·0)
Adenovirus	5·1% (1·9–10·7)	3·0% (0·6–8·4)
Respiratory syncytial virus	1·7% (0·2–6·0)	3·0% (0·6–8·4)
Human bocavirus	1·7% (0·2–6·0)	2·0% (0·2–7·0)

[a] Virus detection via the xTAG® Respiratory Viral Panel Fast v2 (Luminex) qualitative nucleic acid multiplex test. For samples displaying a positive signal for rhinovirus/enterovirus, a quantitative real-time PCR assay was used to confirm identification.
N = number of subjects with PCR results available.

Table S5. Seasonality of exacerbation visits overall and according to detection or no detection of bacterial or viral aetiology.

	Exacerbations		Exacerbation with aetiology		Exacerbation without aetiology	
Month/season	**n**	**% (95% CI)[a]**	**n**	**% (95% CI)[a]**	**n**	**% (95% CI)[a]**
July	17	16·8% (10·8–25·2)	12	11·8% (6·7–19·9)	5	5·1% (2·3–11·1)
August	21	21·6% (14·5–30·9)	15	15·7% (10·0–23·7)	6	6·5% (2·7–14·6)
September	18	17·0% (11·0–25·5)	14	13·7% (8·2–21·8)	4	3·8% (1·5–9·4)
October	31	28·4% (20·8–37·4)	26	24·9% (17·4–34·3)	2	2·0% (0·5–7·6)
November	33	28·8% (22·2–36·5)	29	26·4% (19·8–34·3)	4	3·7% (1·4–9·3)
December	40	36·8% (29·4–45·0)	34	32·1% (24·8–40·3)	5	4·4% (1·8–10·2)
January	43	36·0% (28·8–43·9)	35	30·0% (23·1–37·9)	8	7·5% (4·0–13·8)
February	19	19·2% (12·8–27·7)	16	16·6% (10·7–24·9)	2	2·0% (0·3–10·4)
March	28	25·1% (19·1–32·4)	23	20·9% (14·8–28·5)	4	3·6% (1·5–8·7)
April	23	24·4% (17·2–33·4)	15	15·8% (10·2–23·7)	8	8·8% (4·5–16·5)
May	29	26·3% (19·2–34·9)	25	23·3% (16·7–31·5)	2	1·9% (0·5–7·2)
June	18	17·4% (11·3–25·8)	15	14·8% (9·6–22·3)	3	2·9% (0·9–8·9)
Monthly effect p value[b]	p=0·0033		p=0·00141		p=0·43529	
High season[c]	194	29·3% (25·5–33·3)	163	25·3% (21·8–29·2)	25	3·9% (2·5–6·1)
Low season[c]	126	20·6% (17·0–24·7)	96	15·9% (12·6–19·9)	28	4·6% (3·0–7·0)
High vs. low season p value[b]	p=0·00033		p=0·00009		p=0·51317	

[a]Percentages and confidence intervals estimated from generalised estimating equation (GEE) regression model with logit link and assuming an exchangeable correlation matrix. Only exacerbation, enrolment, and stable visits were taken into account in the total visit number; not recovered visits (in which the subject was recorded by the physician as not recovered from a previous exacerbation) were excluded.
[b]Tests of the effect of month or season were obtained via likelihood ratio tests.
[c]High season, October to March; low season, April to September.

Table S6. Seasonality of the presence or new occurrence of bacteria (NTHi or *M. catarrhalis* [Mcat]) or viruses.

	Visits with NTHi present in culture		Visits with Mcat present in culture		Visits with any virus present		Visits with HRV present		Visits with any virus other than HRV present		Visits with NTHi new occurrence in culture	
Month/Season	n	% (95% CI)[a]	n	% (95% CI)[a]	n	% (95% CI)[a]	n	% (95% CI)[a]	n	% (95% CI)[a]	n	% (95% CI)[a]
July	32	31·3% (22·9–41·0)	5	4·6% (1·8–11·6)	22	20·7% (13·4–30·6)	16	14·4% (8·4–23·6)	6	6·0% (2·7–12·5)	10	11·0% (6·2–18·7)
August	29	28·2% (20·2–37·9)	6	6·6% (3·1–13·4)	13	14·7% (8·9–23·3)	6	7·6% (3·7–14·8)	7	7·6% (3·4–16·1)	8	9·3% (4·8–17·4)
September	32	29·8% (21·7–39·4)	1	0·8% (0·1–7·7)	15	15·1% (9·4–23·3)	9	9·4% (5·2–16·4)	6	5·9% (2·7–12·6)	7	7·5% (3·6–15·0)
October	44	37·0% (28·5–46·3)	6	5·5% (2·4–12·1)	22	22·2% (15·3–31·0)	15	15·0% (9·5–22·9)	7	7·0% (3·5–13·5)	15	14·3% (8·9–22·1)
November	41	35·8% (26·9–45·8)	13	11·3% (6·8–18·1)	21	21·4% (14·6–30·1)	13	14·5% (9·3–22·0)	8	7·5% (3·8–14·2)	11	10·5% (5·9–17·9)
December	34	32·0% (23·9–41·4)	8	7·4% (3·8–14·1)	32	31·9% (22·9–42·4)	15	15·0% (9·0–24·1)	17	16·6% (10·4–25·4)	8	8·3% (4·2–15·5)
January	47	33·9% (25·1–44·0)	12	9·9% (5·4–17·3)	31	27·0% (19·8–35·6)	12	11·0% (6·0–19·4)	18	16·3% (10·4–24·8)	16	14·3% (9·2–21·6)
February	33	34·2% (25·8–43·6)	8	7·7% (3·7–15·1)	24	25·3% (17·3–35·4)	7	7·4% (3·7–14·2)	16	16·9% (10·6–25·8)	7	7·9% (3·9–15·4)
March	42	40·7% (32·1–50·0)	12	9·7% (5·5–16·5)	22	20·1% (13·9–28·2)	7	6·0% (2·8–12·5)	16	15·3% (9·8–22·9)	15	14·7% (9·2–22·6)
April	28	31·6% (23·2–41·4)	3	2·7% (0·7–9·8)	19	20·2% (12·8–30·4)	8	8·9% (4·6–16·6)	11	11·4% (5·9–21·0)	8	9·1% (4·6–17·0)
May	27	25·5% (18·4–34·2)	11	9·0% (4·8–16·3)	21	19·9% (13·5–28·4)	12	11·9% (6·9–19·6)	10	9·1% (5·1–15·7)	6	6·2% (2·9–12·8)
June	27	26·1% (18·8–35·1)	5	5·2% (2·4–11·2)	8	8·8% (4·8–15·7)	6	7·3% (3·8–13·4)	2	2·0% (0·5–7·8)	7	8·1% (4·0–15·9)
Monthly effect p value[b]		p=0·2748		p=0·00276		p=0·00107		p=0·0814		p=0·0001		p=0·42186
High season[c]	241	35·6% (29·5–42·1)	59	8·7% (6·5–11·6)	152	24·5% (20·5–29·0)	69	11·5% (8·5–15·3)	82	13·2% (10·7–16·2)	72	11·8% (9·6–14·5)
Low season[c]	175	28·6% (22·9–35·0)	31	4·9% (3·0–7·9)	98	16·6% (12·8–21·3)	57	10·1% (7·0–14·3)	42	7·0% (4·9–9·9)	46	8·5% (6·5–11·0)
High vs. low season p value[b]		p=0·0098		p=0·00484		p=0·00098		p=0·42315		p=0·00193		p=0·04309

[a]Percentages and confidence intervals estimated from generalised estimating equation (GEE) regression model with logit link and assuming an exchangeable correlation matrix. Only exacerbation, enrolment, and stable visits were taken into account in the total visit number; not recovered visits (in which the subject was recorded by the physician as not recovered from a previous exacerbation) were excluded.
[b]Tests of the effect of month or season were obtained via likelihood ratio tests.
[c]High season, October to March; low season, April to September.

Figure S1. Seasonal distribution of AECOPD cases with sputum samples: total number and number of cases positive for NTHi or *M. catarrhalis* (detected by culture or PCR), HRV, or any viral species, and cases negative for bacteria and viruses (full cohort year 1; month of follow-up considered regardless of year).

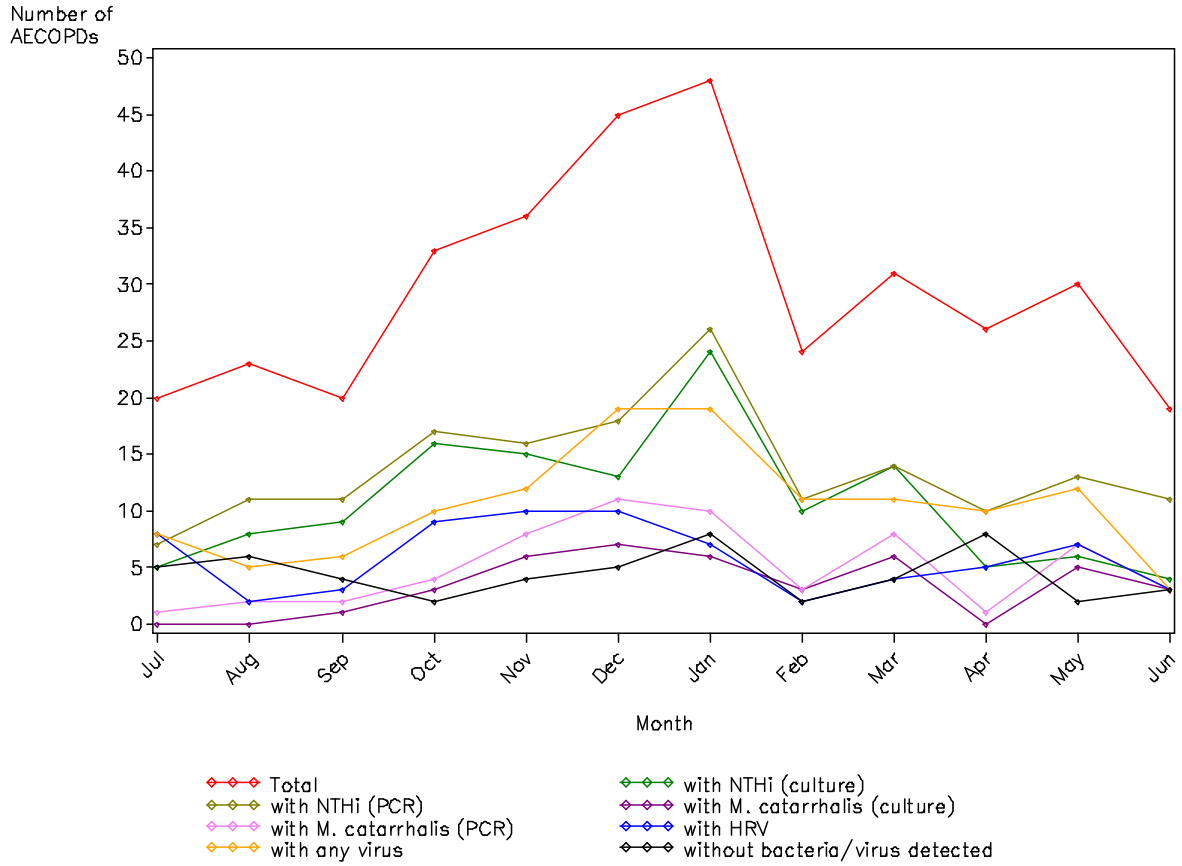
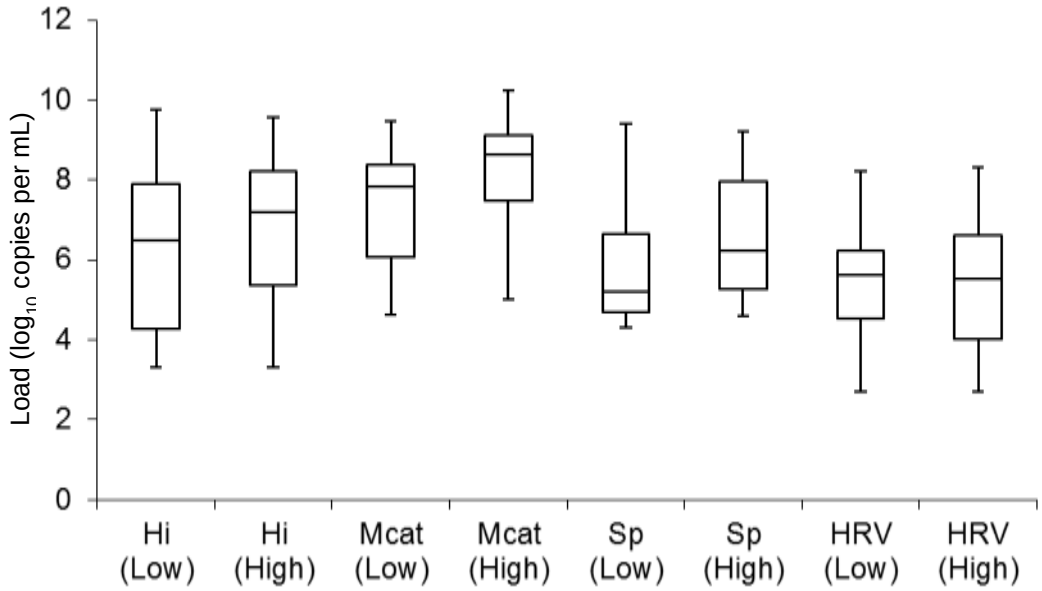
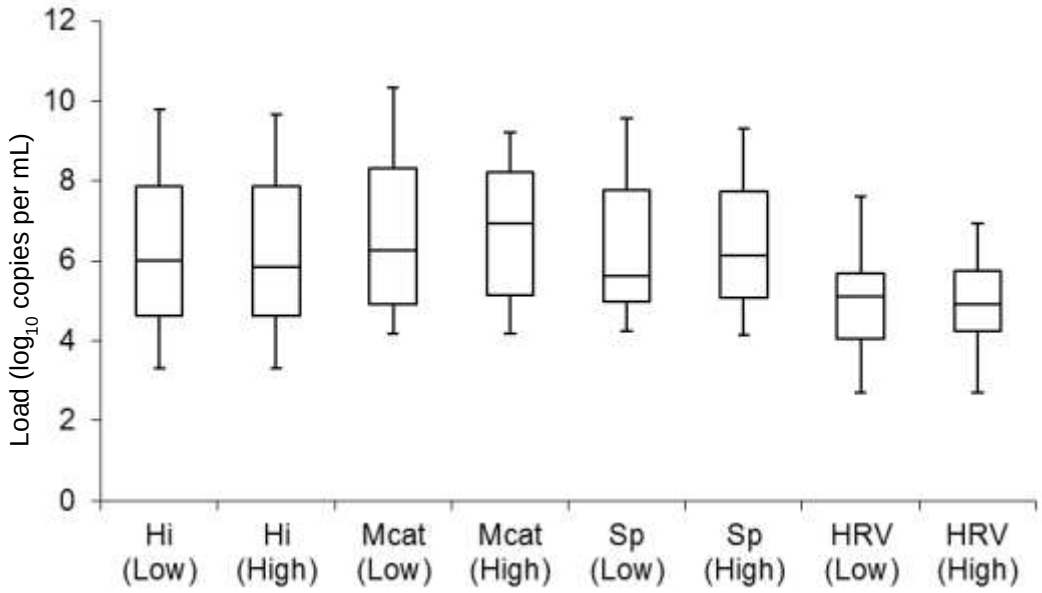


Figure S2. Bacterial and viral load by PCR in sputum at low and high season at exacerbation and stable visits (full cohort year 1). Box and whisker plots show median, interquartile range, and minimum and maximum values. Mann-Whitney test was used to test for significant differences between low and high season.

A. Exacerbation visits. Difference between low and high season was statistically significant for NTHi (p=0·015) and *M. catarrhalis* (p=0·048) only.



B. Stable visits. No statistically significant differences were detected between low and high season.



Hi = non-typeable *Haemophilus influenzae*. Mcat = *Moraxella catarrhalis.* Sp = *Streptococcus pneumoniae.* HRV = human rhinovirus. Low = low season (April to September). High = high season (October to March).