

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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# The Effect of Telithromycin in Acute Exacerbations of Asthma: the TELICAST Study

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## Supplementary web appendix

### In-clinic pulmonary function tests

#### Methods

Secondary efficacy assessments included peak expiratory flow (PEF), forced expiratory volume in one second (FEV<sub>1</sub>), forced vital capacity (FVC), and forced expiratory flow at 25–75% of forced vital capacity (FEF<sub>25–75%</sub>), recorded at Visits 1 and 2 and performed by appropriately trained clinical staff according to American Thoracic Society standards.<sup>1</sup>

#### Results

**Mean Change from Baseline to End of Treatment and Mean Change from Baseline to End of Study for Each Treatment Group in In-clinic Pulmonary Function Tests (Intent to Treat Population).**

	<b>Telithromycin (800 mg/day) (N=126)</b>	<b>Placebo (N=129)</b>	<b>Difference Between Treatments [95% CI]</b>	<b>Significance</b>
<b>Mean Change from Baseline to End of Treatment</b>				
FEV <sub>1</sub> , L	0.63	0.34	0.29 [0.12, 0.46]	P=0.001
FEV <sub>1</sub> % predicted, %	16.9	9.6	7.3 [3.2, 11.4]	P=0.001
PEF, L/min	115.8	88.9	26.9 [1.8, 52.1]	P=0.036
PEF % predicted, %	21.8	17.0	4.8 [0.5, 9.1]	P=0.028
FVC, L	0.58	0.31	0.27 [0.08, 0.45]	P=0.006

FEF <sub>25-75%</sub> , L/sec	0.85	0.45	0.40 [0.13, 0.67]	P=0.004
<b>Mean Change from Baseline to End of Study</b>				
FEV <sub>1</sub> , L	0.54	0.51	0.03 [-0.15, 0.20]	P=0.745
FEV <sub>1</sub> % predicted, %	14.4	14.0	0.4 [-3.8, 4.5]	P=0.872
PEF, L/min	112.1	112.0	0.1 [-28.3, 28.4]	P=0.997
PEF % predicted, %	20.6	21.4	0.8 [-5.6, 4.1]	P=0.763
FVC, L	0.47	0.44	0.03 [-0.14, 0.20]	P=0.739
FEF <sub>25-75%</sub> , L/sec	0.80	0.66	0.14 [-0.11, 0.38]	P=0.281

FEF<sub>25-75%</sub>, forced expiratory flow at 25-75% of forced vital capacity; FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity; PEF, peak expiratory flow.

## **Detection of *C. pneumoniae* and *M. pneumoniae***

Sputum samples and nasopharyngeal swabs were obtained prior to initiation of study treatment at Visit 1. If patients were unable spontaneously to produce a sputum sample, sputum was induced as follows: an FEV<sub>1</sub> measurement was performed prior to and 10 minutes after administration of 200 µg inhaled salbutamol. Sputum induction was then initiated using 0.9% sterile saline solution and, if necessary, using 3% and then 4.5% saline solution.

After collection, specimens were stored -20°C until ready for transport to the reference laboratory (G.R. Micro, London, UK); samples were transported on dry ice in two batches (one in the middle of the study and one at the end of the study). Samples were tested for *C. pneumoniae* and *M. pneumoniae* by polymerase chain reaction (PCR) and culture at the reference laboratory as follows: for *C. pneumoniae*, in-house TaqMan<sup>®</sup>-based quantitative PCR used the *ompA* and 16S rRNA genes as targets. A previously published semi-nested assay was also performed in parallel on all specimens.<sup>2</sup> This assay was one of four recognized by the Centers for Disease Control and Prevention (CDC) and the Canadian Laboratory Centre for Disease Control (LCDC) that met proposed validation criteria for diagnosis.<sup>3</sup> For *M. pneumoniae*, in-house TaqMan<sup>®</sup>-based quantitative PCR and an enzyme-linked immunoassay (both targeting the P1 cytoadhesin gene) were run in parallel with a previously published nested method.<sup>4</sup> A PCR assay to detect PCR inhibition was included. The presence of human DNA in samples was determined by PCR amplification and detection of the human β-actin gene. Culture was as recommended by the CDC/LCDC.<sup>3</sup>

Acute and convalescent serum samples were obtained for determination of titers of antibodies to *M. pneumoniae* and *C. pneumoniae*. IgM, IgG, and IgA antibodies against *C. pneumoniae* were detected by both microimmunofluorescence (MIF; Focus Technologies, Cypress, CA, USA) and the Medac *C. pneumoniae* sandwich enzyme-linked immunosorbent assay (ELISA; Medac, Hamburg, Germany). Serologic diagnosis of *M. pneumoniae* infection was performed using particle agglutination titers (Serodia-Myco II, Fujirebio Inc., Japan) and IgM ELISA (Serion, Germany).

*C. pneumoniae* and *M. pneumoniae* infection was diagnosed by the presence of IgM serum antibodies and/or a fourfold rise between baseline and convalescent samples in IgG (*C. pneumoniae*) or particle agglutination titer (*M. pneumoniae*), and/or a positive sputum or nasopharyngeal PCR, or culture.

## **Additional methodology**

### **1 Specimen collection, transportation, and processing**

#### ***1.1 Sputum samples for PCR and culture***

Samples consisted of expectorated sputum produced from a deep cough and were collected as follows: patients first rinsed their mouth and gargled with water, the deep cough specimen was collected in a sterile screw-cap container, and patients were instructed not to expectorate saliva or postnasal discharge into the container. The

specimen was then transported immediately without refrigeration to the microbiology laboratory for bacteriologic processing.

A fresh Dacron<sup>®</sup>-tipped plastic shaft swab was dragged through the sputum sample for 30 seconds. Next, a small amount of purulent material (or mucus if nonpurulent) was placed in a vial of M4-3 atypical bacteria transport medium (Remel, Lenexa, USA). The swab was swirled in the medium, excess fluid was removed, and the swab discarded. The vial (containing glass beads to help break up the specimen) was shaken for 30 seconds and stored at -20°C. The samples (both original sputum and sputum in transport medium) were shipped on dry ice to the reference laboratory, where they were stored at -70°C.

All processing was performed following Category 3 procedures. Mucolysis was performed as previously described<sup>5</sup> on the original sputum samples and samples in transport medium if they were mucoid or purulent. DNA extraction was performed using the High Pure PCR Template Preparation Kit (Roche, Lewes, UK).<sup>6</sup> Eluted DNA could be stored at 2–8°C for up to 72 hours or at -15 to -25°C for longer periods.

### ***1.2 Nasopharyngeal (NP) swab for PCR and culture***

The nasopharynx was swabbed using a standard protocol and a Dacron-tipped wire NP swab. The swab was then placed in the transport medium provided with the swab and transported immediately without refrigeration to the microbiology laboratory for bacteriologic processing.

The swab was removed from the transport medium and swirled in a vial of M4-3 atypical bacteria transport medium (Remel, Lenexa, USA), excess fluid was removed, and the swab discarded. The vial (containing glass beads to help break up the specimen) was shaken for 30 seconds and stored at -20°C. The samples were shipped on dry ice to the reference laboratory, where they were stored at -70°C.

All processing was performed following Category 3 procedures. DNA extraction was performed using the High Pure PCR Template Preparation Kit (Roche, Lewes, UK).<sup>3</sup> Eluted DNA could be stored at 2–8°C for up to 72 hours or at -15 to -25°C for longer periods.

### ***1.3 Blood samples for serologic testing***

Blood samples were collected into sterile tubes without anticoagulants or preservatives and centrifuged according to specifications (re: rpms and time) provided by the central reference laboratory. The serum was separated into tubes and stored at -20°C. Samples were then shipped on dry ice to the reference laboratory.

## **2 Culture**

### ***2.1 Chlamydomphila pneumoniae***

Stock cultures of McCoy and Hep 2 cells (CAMR, Salisbury, UK) were maintained by continuous passage every 3–5 days. 75 cm<sup>3</sup> tissue culture flasks were seeded at a

concentration of  $3.5 \times 10^6$  cells/mL in 15–20 mL McCoy growth medium and incubated at  $35 \pm 1^\circ\text{C}$ . The cells were confluent after 4 days. Aliquots of prepared specimen were added to monolayers of both McCoy and Hep 2 cell lines, with gentamicin and vancomycin added at a final concentration of 10 mg/L to reduce the growth of bacterial oral flora present in the specimens. After 3 days' incubation, specimens were passaged to a new tissue culture tube. After a further 3 days' incubation, monolayers were stained with the *C. pneumoniae* immunofluorescence staining method as per the manufacturer's instructions (Imagen<sup>TM</sup> Chlamydia, Dako, Germany) and examined under a fluorescent microscope for the presence or absence of inclusions. Positive and negative control cultures were run in parallel with each batch.

## **2.2 *Mycoplasma pneumoniae***

Mycoplasma broth (MB) was prepared fresh — containing Mycoplasma Broth Base and 0.05% yeast extract (Oxoid Ltd, Basingstoke, UK), 0.005% w/v phenol red indicator (Sigma-Aldrich Company Ltd, Poole, UK), 20% horse serum (heat-inactivated) (TCS Biosciences Ltd, Buckingham, UK), and 1.0% glucose — and pH adjusted to  $7.6 \pm 0.5$ . Vancomycin was added at a concentration of 10 mg/L to reduce the growth of bacterial oral flora present in the specimens. Positive and negative control cultures were run in parallel with each batch.

An aliquot of prepared specimen was added to 2 mL of MB in plastic bijoux and incubated at  $35 \pm 1^\circ\text{C}$  in air for a maximum of 10 days. Presumptive positive cultures can be identified by a typical 3- to 5-day incubation period and metabolism of glucose to produce acid, thus lowering the pH (indicated by a color change in the medium from pink/red to yellow).

Positive cultures of *C. pneumoniae* and *M. pneumoniae* were to be confirmed by PCR; however, as no positive cultures were obtained, the methodology is not provided here.

## **3 PCR methodology**

### **3.1 Controls and standards**

All assays include positive and negative controls. The following control strains were used in all assays and served as positive or negative controls dependent on the assay:

*C. pneumoniae* — IOL 207/TW183  
*M. pneumoniae* — ATCC 15531 (NCTC 10119)  
*Legionella pneumophila* — NCTC 11191  
*Bordetella pertussis* — Tohama I

Nucleic acids were extracted from suspensions of pure culture in water (PCR grade) using the High Pure PCR Template Preparation Kit (Roche, Lewes, UK)<sup>6</sup> and included in the assays at two dilutions (predetermined positive). Diluted controls were aliquoted, stored at  $-20^\circ\text{C}$ , and used once only.

For quantitative assays, standards with known DNA content were used in order to construct a standard curve. Commercially synthesized oligonucleotides of the target DNA of known concentration were used for accuracy (Thermo Electron GmbH, Ulm, Germany). Standards were included in assays at  $4 \times \log_{10}$  dilutions (the lowest two predetermined to cross the detection limit of the assay). Diluted standards were aliquoted, stored at  $-20^{\circ}\text{C}$ , and used once only.

### 3.2 Internal control construction

An assay-specific internal control was constructed for the *C. pneumoniae* assay as described previously.<sup>7</sup>

The resulting amplicon was of the same size as the amplicon for the *C. pneumoniae* assay and had the same sequences at either end; however, it contained mouse DNA in the internal region. The internal control was then amplified by the diagnostic PCR method to produce a large amount of internal control. The internal control was then serially diluted and amplified in the standard *C. pneumoniae* assay, and the cutoff for positivity was determined. The concentration used in the master mixture was one dilution higher than the cut-off concentration. If PCR-inhibitory substances were present in the specimen, they would be detected by failure to amplify the internal control, which was detected in a separate microwell using an oligonucleotide probe (DFMBAP) specific for mouse actin DNA.

### 3.3 *Chlamydomyphila pneumoniae* assays

(1) Reference (nested method) as previously described.<sup>2</sup>

(2) Real-time quantitative PCR methods

ABI Prism<sup>®</sup> 7000 Sequence Detection System (Applied Biosystems, Warrington, UK).

For the amplification of genetic material, 5  $\mu\text{L}$  of template DNA was added to 20  $\mu\text{L}$  of a master mix containing 12.5  $\mu\text{L}$  of 2X TaqMan<sup>®</sup> Master mix (Applied Biosystems, Warrington, UK), 2.5 pmol of each primer (designed using Primer Express Software, Applied Biosystems — Table 2.1), and 25 pmol probe in an ABI optical 96-well microtiter plate. The specific analyte assay and the internal control assay were performed in the different wells (with different reporter dyes).

Amplification was performed on an ABI Prism<sup>®</sup> 7000 Sequence Detection System (Applied Biosystems, Warrington, UK) using the parameters:

Step	Temperature/time	No. of cycles
1	94 <sup>o</sup> C for 2 min	1

2	94°C for 10 secs 55°C for 10 secs 72°C for 15 secs	43
3	72°C for 5 min	1
4	4°C forever	

### 3.4 *Mycoplasma pneumoniae* assays

(1) Reference (nested method) as previously described.<sup>4</sup>

(2) EIA probe based method as previously described.<sup>8</sup>

(3) Real-time quantitative PCR

ABI Prism<sup>®</sup> 7000 Sequence Detection System (Applied Biosystems, Warrington, UK).

Amplification was performed as for *C. pneumoniae*, but using the oligonucleotides detailed in Table 2.2.

The human  $\beta$ -actin gene was used as a specimen control to detect adequate human DNA in the specimens. Amplification of Human B-Actin (7000) was performed using primers HBA2544F and HBA2594R, with SYBR green master mix, according to the manufacturer's instructions (Applied Biosystems, Warrington, UK).

HBA2594R: GGT AGT TTC GTG GAT GCC ACA

HBA2544F: GCC CTC ATT TCC CTC TCA GG

**Table 2.1 *C. pneumoniae***

Assay name	Target	Designation	5' – 3' sequence	Modifications
CPGRompR	<i>ompA</i>	Forward primer “CPOMPAF”	GCTTTCCCCTTGCCAACAG	
		Reverse primer “CPOMPAR”	TCGCAGACTTTGTTCCAGTAGCT	
		Probe “CPOMPAP”	CGCTGGCGTAGCAA	5'FAM, 3'MGB
		Internal control sequence “CPOMPAIC”	GCTTTCCCCTTGCCAACAGACGCTGegtcAGCAACAGCTACTGGAACA AAGTCTGCGA	
		Internal control probe “CPOMPICP”	CGCTGegtcAGCAA	5'VIC, 3'MGB
		Positive control standard sequence “CpompAstd”	GCTTTCCCCTTGCCAACAGACGCTGGCGTAGCAACA	
CPGR16SR	16S rRNA	Forward primer “CP16SF”	TCAGCCATAACGCCGTGAA	
		Reverse primer “CP16SR”	TGTGACGGGCGGTGTGTA	
		Probe “CP16SP”	ACGTTCTCGGGCCTT	5'VIC, 3'MGB

**Table 2.2 *M. pneumoniae***

Assay name	Target	Designation	5' – 3' sequence	Modifications
MPGRp1R	P1 adhesin	Forward primer “MPNMGBF”	CCCACCCAGTCACCGATCT	
		Reverse primer	ACACTTGCTGGGATATCAATAAACAG	

		“MPNMGBR”		
		Probe “MPNMGBP”	TTGATCCGGTAACTATG	5’FAM, 3’MGB
		Positive control standard “MPNP1std”	CCCACCCAGTCACCGATCTGTTTGATCCGGTAACTA	

## 4 Serology

### 4.1 *Chlamydia pneumoniae*

The following serologic tests for *C. pneumoniae* were performed:

- MIF (Focus Technologies, Cypress USA) for IgG, IgA, and IgM against *C. pneumoniae*. Carried out and interpreted according to the manufacturer's instructions.
- Medac sandwich-ELISA (Medac, Hamburg, Germany) for IgG, IgA, and IgM against *C. pneumoniae*. Carried out and interpreted according to the manufacturer's instructions.

### 4.2 *Mycoplasma pneumoniae*

The following serologic tests for *M. pneumoniae* were performed:

- Fujirebio Serodia Myco II (Fujirebio, Tokyo, Japan) particle agglutination for class nonspecific antibodies against *M. pneumoniae*. Carried out and interpreted according to the manufacturers' instructions.
- Serion ELISA (Institut Virion/Serion, Würzburg, Germany) for IgM against *M. pneumoniae*. Carried out and interpreted according to the manufacturer's instructions.

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