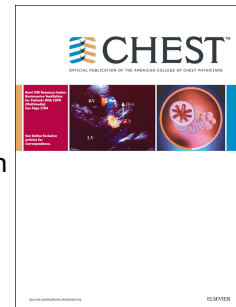


# Accepted Manuscript

A pilot feasibility study in establishing the role of ultrasound-guided pleural biopsies in pleural infection (The AUDIO study)

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**TITLE PAGE****CHEST Submission – Original Article****Title:**

**A pilot feasibility study in establishing the role of ultrasound-guided pleural biopsies in pleural infection (The AUDIO study)**

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**Short title:**

US guided pleural biopsies in pleural infection.

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**Key Words:** empyema; pleural disease; pleural infection; bacterial infection

**Abbreviations:**

16S rRNA= 16S ribosomal ribonucleic acid; CT = computed tomography; ICC = interclass correlation coefficient; NHS = National Health Service; qPCR= quantitative Polymerase Chain Reaction; ROC = receiver operating characteristic; UK = United Kingdom; US = thoracic ultrasound; USA= United States of America; VAS = visual analogue scale

### **Funding**

This study has been supported by an Oxfordshire Health Services Research Committee grant (AH2016/1225).

### **Competing interests**

The authors have declared no competing interest exist.

### **Abstract**

**Background** Pleural infection is a common complication of pneumonia associated with high mortality and poor clinical outcome. Treatment of pleural infection relies on the use of broad-spectrum antibiotics, since reliable pathogen identification occurs infrequently. We performed a feasibility interventional clinical study assessing the safety and significance of ultrasound (US)-guided pleural biopsy culture to increase microbiological yield. In an exploratory investigation, the 16S rRNA technique was applied to assess its utility on increasing speed and accuracy versus standard microbiological diagnosis.

**Methods** 20 patients with clinically established pleural infection were recruited. Participants underwent a detailed US scan and US-guided pleural biopsies before chest drain insertion, alongside standard clinical management. Pleural biopsies and routine clinical samples (pleural fluid and blood) were submitted for microbiological analysis.

**Results** US-guided pleural biopsies were safe with no adverse events. US-guided pleural biopsies increased microbiological yield by 25% in addition to pleural fluid and blood samples. The technique provided a substantially higher microbiological yield compared to pleural fluid and blood culture samples (45% compared to 20% and 10% respectively). The 16S rRNA technique was successfully applied to pleural biopsy samples, demonstrating high sensitivity (93%) and specificity (89.5%).

**Conclusions** Our findings demonstrate the safety of US-guided pleural biopsies in patients with pleural infection and a substantial increase in microbiological diagnosis, suggesting potential niche of infection in this disease. qPCR primer assessment of pleural fluid and biopsy appears to have excellent sensitivity and specificity.

## Clinical trial registration

This study is registered with ClinicalTrials.gov, number NCT02608814

## Introduction

Pleural infection is a common complication of pneumonia with a high mortality, affecting 80,000 patients per year in the USA and UK combined, translating to 220 new cases per day<sup>1</sup>. Epidemiological data from Europe and the USA suggest the incidence is increasing year on year and most of all in the elderly<sup>2-5</sup>. Mortality of the disease is considerable at approximately 20% in the six months following initial presentation<sup>6,7</sup>.

Treatment for pleural infection requires fluid drainage and antibiotic therapy, which is initially necessarily broad-spectrum until culture results become available<sup>1</sup>. In up to 40% of cases of pleural infection, a microbiological diagnosis cannot be made using standard (pleural fluid and blood culture) techniques and antibiotic treatment is empirical based on local knowledge and clinical judgment<sup>4,5,8</sup>. This lack of a specific microbiological diagnosis leads to non-specific and broad antibiotic treatment, potentially risking inaccurate management and contributing to poor outcomes, including the development of resistance and complications of antibiotic therapies (e.g. increasing incidence of methicillin-resistant *Staphylococcus aureus*<sup>9</sup> and *Clostridium difficile*<sup>10</sup> infections).

Lack of guidance due to negative blood or pleural fluid microbiology may lead to medical treatment failure, which then often means surgical intervention is required in those fit enough to undergo such management, with all the associated risks inherent to such an approach<sup>1,11</sup>. Methods demonstrated to increase the microbiological yield include the use of nucleic acid amplification techniques (targeting 16S ribosomal RNA sequence -16S rRNA) which have been proposed to potentially increase overall microbiology sensitivity<sup>1,11</sup>.

Important questions regarding the disease microbiology remain unanswered, which may in part account for the lack of recent therapeutic advances<sup>12</sup>. Although infected pleural fluid is usually sampled in clinical practice, as this is available for analysis, there is no direct evidence that microbes infecting the pleural preferentially inhabit the fluid. A recent animal study of pleural infection identified the presence of *Streptococcus pneumoniae* in the pleural tissue, raising questions as to whether sampling of the pleural tissue may improve diagnostics<sup>13</sup>. In conditions such as malignant pleural effusion and tuberculous pleuritis, it is well recognized that pleural biopsy has a much

higher yield than that obtained from fluid alone <sup>14</sup>. It is hypothesized that due to a rich blood supply in pleural tissue, bacteria may anchor in pleural tissue with the minority of organisms existing in pleural fluid, but this theory has not been tested.

We hypothesised that US-guided pleural biopsies would be safe and improve microbiological yield in addition to conventional methods in patients presenting with pleural infection. We aimed to assess the use of the 16S rRNA technique in combined pleural fluid and biopsy samples, using specifically designed primers for common microbes causing pleural infection.

## **Methods**

### **Study design**

This was a pilot feasibility interventional study performed in two centres in the UK.

### **Subjects enrolled**

Study enrolment was offered to all subjects fulfilling the entry criteria at the Oxford University Hospitals NHS Foundation Trust (Oxford, UK) and Southmead Hospital (Bristol, UK). Subjects were screened during normal clinical practice and enrolled at the point of initial diagnostic pleural aspiration, which diagnosed pleural infection. Specific details about inclusion and exclusion criteria can be found in the online supplementary material.

### **Ultrasound imaging**

All patients underwent ultrasound assessment prior to intervention by two respiratory physicians of Royal College of Radiology Thoracic Ultrasound level I or II competence <sup>15</sup>. The size of effusion (small = one rib space, moderate = two to three rib spaces, large  $\geq$  four rib spaces), echogenicity and average number of septations per image were recorded.

### **Study intervention**

All patients underwent real-time US-guided pleural biopsies performed at the same procedure as chest drain insertion, using an 18-gauge Temno cutting needle with a throw of 2 cm (Temno BD) <sup>16</sup>. The site of biopsies was determined during the US assessment by targeting the rib space with evidence of more than 3 cm of pleural fluid and no underlying vessels on Doppler investigation. Evidence of pleural thickening was not prerequisite for US-guided pleural biopsies. Between six to eight biopsies were performed until macroscopically satisfactory material was obtained. US guided pleural biopsies were taken only from one site for safety and time reasons, as AUDIO study participants required urgent chest drain insertion for the infection. No other techniques or needles were tested in light of the results of a previous

study published by our group <sup>16</sup>. The material was sent for microbiological examination in bottles with normal saline separately to the pleural fluid and blood for microbiological analysis in the local laboratory.

Alongside with the study interventions all patients had pleural fluid (inoculation of fluid in BACTEC bottles and normal microbiology sample containers) and blood culture at enrolment as per standard treatment <sup>17</sup>. The same laboratory on each site tested blood and pleural fluid and biopsies for all patients. Pleural fluid and biopsy samples were stored for further investigation in the exploratory phase of this study.

### **Study outcomes**

#### *Primary end-point*

The primary outcome was the frequency of positive microbiological results using pleural biopsy in addition to conventional microbiological culture and gram stain.

#### *Secondary end-point*

The secondary outcome was the association of 16S rRNA assessment of pleural biopsy tissue.

### **DNA Extraction and quantitative polymerase chain reaction (qPCR)**

DNA from both pleural fluid and biopsy samples was extracted using QIAamp UCP Pathogen Mini Kit (Qiagen, Cat No./ID: 50214). For the DNA extraction either two mls of pleural fluid or the whole pleural biopsy tissue were used. Before DNA extraction pleural fluid samples were centrifuged at 21,000 g for 15 minutes. DNA quantity and quality was measured by NanoDrop 2000/2000c. All our samples passed the quantity and quality cut-off, which was ten times the minimum amount of DNA per microliter needed for each reaction. Equal volumes of DNA were used for each qPCR reaction. qPCR was performed using either Power SYBR® Green PCR Master Mix (ThermoFisher Scientific, Cat No 368706) or TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific, Cat No 4444963) in a LightCycler® 480 Instrument II (Roche, Cat No 05015243001). For the Power SYBR® Green PCR Master Mix assays, bacterial universal primers were used targeting the 16S rRNA gene. (Supplementary Table 1) This primer set amplifies a 467 nucleotide sequence of the gene, incorporating hypervariable regions V3 and V4 <sup>18</sup>. For the pathogen identification qPCR assays, aiming to increase the specificity of the technique, we chose the Taqman qPCR method and designed primers for *Streptococcus pneumoniae*, *Staphylococcus aureus* methicillin-sensitive (MSSA) and *Staphylococcus aureus* methicillin-resistant (MRSA). (Supplementary Table 2) For the detection of anaerobic bacteria a previously published primer set (Supplementary Table 1) was used which amplifies a 1,500 nucleotide sequence of the 16S-23S rRNA intergenic spacer

19. Threshold cycle (Ct) values from triplicate qPCR reactions were used for the analysis.

### **Study databases used for exploratory phase**

Samples from the AUDIO study were used to assess the 16S rRNA technique in pleural fluid and biopsies. Samples (pleural fluid and biopsies) taken from patients without pleural infection stored in the Oxford Radcliffe Pleural Biobank were used as negative controls. The development of specific primers was based on samples from a previously published study in pleural infection (MIST2 database)<sup>5</sup>.

### **Statistics**

All analyses were performed using GraphPad Prism (Version 7.0; GraphPad Software, La Jolla, CA, USA). Data are presented as mean±sd or median with interquartile range (IQR) as appropriate. Descriptive statistics were used to summarise patient characteristics. T-tests were used to examine differences between groups with parametric data. Mann–Whitney U-test and Dunn's multiple comparisons were used for nonparametric data. Fisher's exact test was used for categorical variables. Statistical significance was taken at the 5% level.

### **Study approval**

Ethical and regulatory approval for the study was obtained before recruitment commenced (UK research ethics committee reference 15/SC/0171). Ethics approval for sample analysis was obtained before the laboratory investigations (Oxford Radcliffe Biobank ethics committee reference 15/A252).

### **Study registration**

This study is registered with ClinicalTrials.gov, number NCT02608814.

## **Results**

### **Patients**

A flowchart showing enrolment, intervention and ultrasound findings of patients until primary analysis is presented in Figure 1. A total of 25 participants were screened and 20 were recruited between June 2015 and December 2016 (17 patients from Oxford University Hospitals NHS Foundation Trust, Oxford, UK and 3 patients from Southmead Hospital, Bristol, UK). Five patients were not recruited due to no established diagnosis of pleural infection or the presence of exclusion criteria. Twenty participants underwent imaging with thoracic ultrasound and US-guided pleural biopsies prior to chest drain insertion. None of the patients had treatment with



fibrinolytics prior to study enrolment. Baseline demographics are provided in Table 1.

### **Data quality**

The primary outcome measure (assessment of microbiological yield) was available in all (20/20) participants. There were no losses to follow-up, with three-month post-recruitment data available in all participants who were alive.

### **Primary end-point**

The addition of pleural biopsies to routine blood culture and pleural fluid significantly increased microbiological yield (Figure 2A). Of the 20 patients recruited, all had results from blood and pleural fluid samples either at or prior to enrolment during the same treatment period. Blood samples were positive in 10% of cases, pleural fluid in 20% and pleural biopsies in 45% (Figure 2). Addition of pleural biopsies to routine (blood and pleural fluid) microbiological analysis increased the diagnostic yield by 25%. Detailed microbiological results are presented in Table 2.

A total of 15/20 (75%) patients were established on antibiotic therapy prior to study recruitment. Of these patients, 1/15 (7%) blood cultures, 2/15 (13%) pleural fluid cultures and 6/15 (40%) pleural biopsy cultures were positive. Our results suggest that in patients previously treated with antibiotics culturing of pleural biopsies samples is more likely to provide positive results compared to current standard practice (pleural fluid and blood samples).

Pathological investigation of US-guided pleural biopsies identified microbes on the pleural surface. An example is shown in Figure 2C with gram-positive cocci in the acute inflammatory exudate on the pleural surface (Figure 2C).

### **Population with positive US guided pleural biopsies**

In order to improve patient selection the baseline characteristics of individuals with positive and negative US-guided pleural biopsy are illustrated in Table 3. The data revealed no difference in terms of duration of antibiotics, severity of pneumonia with CURB-65 score, CRP, pleural fluid LDH, blood platelets and white bloods cell count that could improve patients' selection with a potential positive pleural biopsy.

### **Procedure side effects and safety data**

There were no technical difficulties or adverse events in any patient, specifically including bleeding, vasovagal reaction, pain requiring intervention or serious adverse events.

### **Use of 16S rRNA in pleural fluid and biopsies**



In the exploratory phase we investigated the utility of molecular biology methods on increasing the sensitivity versus conventional procedures in AUDIO samples. 16S rRNA amplification in pleural infection positive biopsies significantly differed compared to negative samples ( $p < 0.0001$ ), mean positive samples 24.8 cycle threshold (Ct), (95% CI: 23.74 to 26.68) and mean negative samples 32.1 Ct (95% CI: 30.65 to 31.79) (Figure 3A). Pleural fluid DNA method exhibited greater mean qPCR cycle difference ( $p < 0.0001$ ), mean positive samples 13.1 Ct, (95% CI 12.4 to 14.9), mean negative samples 34.7 Ct (95% CI 33.8 to, 35.7) compared to pleural biopsy DNA method (difference of 7.25 cycles) (Figure 3B).

#### *Development of specific primers for pleural infection*

Pleural fluid samples were used from a previously published study of pleural infection (the MIST-2 study) as a platform to design specific primers for microbes to improve diagnostics<sup>5</sup>. All Taqman qPCR reactions for *Streptococcus pneumoniae*, *Staphylococcus aureus* (MSSA) and *Staphylococcus aureus* (MRSA), exhibited 100% specificity and 100% sensitivity (total 42 samples) suggesting that qPCR based pathogen detection is feasible in pleural infection (Supplementary Table 3). By using universal primers for anaerobes (total 39 samples) we were able to confirm the microbiological pleural fluid culture in 5/8 (62%) samples and detected positive anaerobes in 13/31 (42%) samples with negative pleural fluid culture (Supplementary table 3).

Taqman qPCR reactions identified two positive samples for *Streptococcus pneumoniae* with negative pleural fluid samples. The two samples with positive reactions had positive blood cultures for *Streptococcus pneumoniae* which confirms our results. No increase in the yield was detected with *Staphylococcus aureus* (MSSA and MRSA) in our database.

## **Discussion**

The results of this study suggest that US-guided pleural biopsies are safe and significantly improve diagnostic microbiological yield compared to routine techniques in patients with pleural infection. Pleural biopsies had the highest diagnostic yield of all techniques assessed (45% positivity compared with 20% for pleural fluid and 10% for blood cultures). Furthermore, addition of pleural biopsies to blood and pleural fluid microbiological analysis increased the diagnostic yield by 25% and in these cases the pleural biopsy was the only microbiologically positive sample obtained. Moreover our results suggest that pleural biopsy samples are less likely to be negative due to prior antibiotics. The addition of biopsy results directly altered antibiotic treatment in

2/20 (10%). In these two patients, biopsy results demonstrated *Klebsiella pneumoniae* and *Streptococcus intermedius*, which permitted the use of focused antibiotic treatment based on sensitivities (altered Piperacillin/tazobactam to meropenem and co-amoxiclav to Piperacillin/tazobactam respectively).

Given the safety, relative ease of learning and the increased yield of this technique, as well as the fact that it can be performed in the same procedure as chest tube insertion under US guidance and using a common local anaesthetic tract, we suggest that the technique is safe, meriting further evaluation as part of a wider study. It has the potential to become part of the standard of care in cases of suspected pleural infection.

The microbiology results from pleural biopsies potentially further our understanding of the pathobiology of this difficult to treat condition. The high biopsy yield may suggest that microbes are topographically more likely to be located on the parietal pleural surface (perhaps due to blood supply and better nutrition) rather than being “planktonic” within the pleural fluid, which is known to be acidic, hypoxic and lacking in nutrition. If this theory is correct, it may help to explain the results seen with treatments that not only divide septations but have effects on biofilms in pleural infection<sup>5</sup>.

The majority of patients (75% of study population) were on antibiotics before study recruitment which might affected the percentage of positive pleural fluid culture that expected to be approximately 60% based on previous literature. The fact that high number of patients had positive pleural biopsy culture results despite previous antibiotics treatment highlights the limited antibiotics penetration and efficacy to the pleural space. Our results suggest that negative microbiology in this situation is more likely from pleural fluid and blood samples than from biopsy samples Culture of pleural biopsies appears more robust in the presence of previous antibiotics and this may again suggest features of pathogenesis (such as biofilm formation) in this disease.

The exploratory section of this study demonstrated the potential utility of the 16S rRNA technique using pleural biopsies. We have here identified four primer sets for this assay with extremely high (93% and 89.5%) specificity and sensitivity for the detection of the four most common pathogens in pleural infection. Given that a 16S rRNA technique can be completed within a few hours of receiving samples, the practical clinical utility of this technique should now be explored further.

Our results indicate that 16S rRNA is more sensitive in pleural fluid compared to biopsy; this could be related to contamination of tissue samples during processing. The specific primers tested in the AUDIO samples and in samples

from a previous large scale multicentre published study suggests the possibility of rapid diagnosis of the underlying cause of common causes of pleural infection with excellent results <sup>5</sup>. These four underlying aetiologies (*Streptococcus pneumoniae*, anaerobes, *Staphylococcus aureus* MSSA and *Staphylococcus aureus* MRSA) can be combined and provide results within two hours with excellent specificity and thus potential clinical impact for patients.

There are several limitations to this study. Due to the small sample size it was not possible to establish specific primers for microbes which have not been previously cultured in our studies. Our results show that 16S rRNA technique can be negative on patients with pleural infection and signifies the limitation of using the technique on everyday clinical practice. Additionally only two sites recruited for the AUDIO study with experienced respiratory physicians and radiologist in the technique.

US-guided pleural biopsies are safe in pleural infection and improve microbiological yield when combined with blood and pleural fluid samples. The results of this feasibility study highlight that among currently standard practice tests (pleural fluid and blood culture) US-guided pleural biopsies is the most probable method to identify the underlying pathogen causing pleural infection. qPCR primer assessment of pleural fluid and biopsy appears to have excellent sensitivity and specificity. There is now a compelling need for large clinical studies using pleural biopsy technique as additional test in pleural infection and the exploration of qPCR use to improve diagnosis and management.

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### **Guarantor**

IP takes responsibility of the content of the manuscript including data and analysis.

### **Author Contributions**

IP, HEGP, and NMR designed the study. IP, NIK, and RR performed the exploratory analysis. IP, RB, AY, AJE, RMM, RA, JPC, RPH, SP, TD, CC, NM, NMR collected data. IP, NMR analysed and interpreted the data. IP, NIK, CC, NMR created the figures. IP, NIK, RB, RR, AY, AJE, RMM, JPC, RPH, TD, SP, HEGP, CC, NM, NMR reviewed the manuscript. All authors approved the final manuscript.

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**Table 1.** Patients' demographics for AUDIO study

<b>Patients demographics recruited in AUDIO study</b>	
Number	20
Age (mean, SD)	64 (15.9)
Sex (% male/% female)	20% / 80%
Type of infection (%community/%hospital)	85% / 15%
Pleural fluid appearances	
• pus	25%
• turbid	35%
• blood stained	25%
• straw coloured	15%
Pleural fluid pH (mean, SD)	7.02 (0.24)
C-reactive protein mg·L <sup>-1</sup> (mean, SD)	425.2 (167)
Pleural fluid protein g·L <sup>-1</sup> (mean, SD)	39.4 (10.2)
Pleural fluid LDH IU·L <sup>-1</sup> (median, IQR)	2260 (1883)
Platelets x10 <sup>9</sup> (mean, SD)	425.2 (167.5)
Already on antibiotics prior samples taken (%)	15/20 (75%)

**Table 2.** Positive microbiology results for each AUDIO patient

<b>Positive Microbiology Results</b>			
<b>Patient</b>	<b>Blood</b>	<b>Pleural fluid</b>	<b>Pleural biopsies</b>
1	Fusobacterium necrophorum	Negative	Negative
2	Staphylococcus aureus (MSSA)	Staphylococcus aureus (MSSA)	Negative
3	Negative	Mixed anaerobes	Streptococcus (MMSA) & Streptococcus Milleri
4	Negative	Klebsiella pneumonia	Klebsiella pneumonia
5	Negative	Staphylococcus Lugdunensis & Anaerobes	Staphylococcus Lugdunensis & Anerobes
6	Negative	Negative	Streptococcus intermedius
7	Negative	Negative	Staphylococcus Aureus (MMSA)
8	Negative	Negative	Streptococcus Milleri & Staphylococcus aureus (MMSA)
9	Negative	Negative	Streptococcus Milleri
10	Negative	Negative	Streptococcus Milleri & Staphylococcus epidermidis
11	Negative	Negative	Streptococcus intermedius



**Table 3.** Patients' demographics with positive and negative US-guided pleural biopsy

Characteristics of study population with pleural biopsy		
	Microbiology Positive	Microbiology Negative
Age (mean, SD)	64.2 (14.5)	64.1 (17)
Pleural fluid LDH IU·L <sup>-1</sup> (median, IQR)	2200 (5745)	2440 (1328.75)
Prior antibiotics (%total population)	7/9 (87.5%)	8/11 (73%)
CURB-65 (mean, SD)	0.44 (0.35)	0.58 (0.41)
Platelets x10 <sup>9</sup> (mean, SD)	405 (168)	413 (172)
White blood cell x10 <sup>3</sup> ·L <sup>-3</sup> (mean, SD)	14.7 (7.5)	13.11 (3.9)
CRP mg·L <sup>-1</sup> (mean, SD)	210 (113)	184 (67)

**Figure legends:**

**Figure 1.** Flow chart diaphragm for AUDIO study.

**Figure 2.** A. % increase in positive culture samples in patients with pleural infection. B. Results of pleural biopsy culture. C. Gram stain of acute inflammatory exudate in a pleural biopsy showing small colonies of Gram positive cocci.

**Figure 3.** A. Ct number of positive and negative pleural biopsy samples. Mean positive samples 24.8 Ct, (95% CI: 23.74 to 26.68) and mean negative samples 32.1 Ct (95% CI: 30.65 to 31.79) B. CT number of positive and negative pleural fluid samples. Mean positive samples 13.1 Ct, (95% CI 12.4 to 14.9), mean negative samples 34.7 Ct (95% CI 33.8 to, 35.7).

FIGURE 1

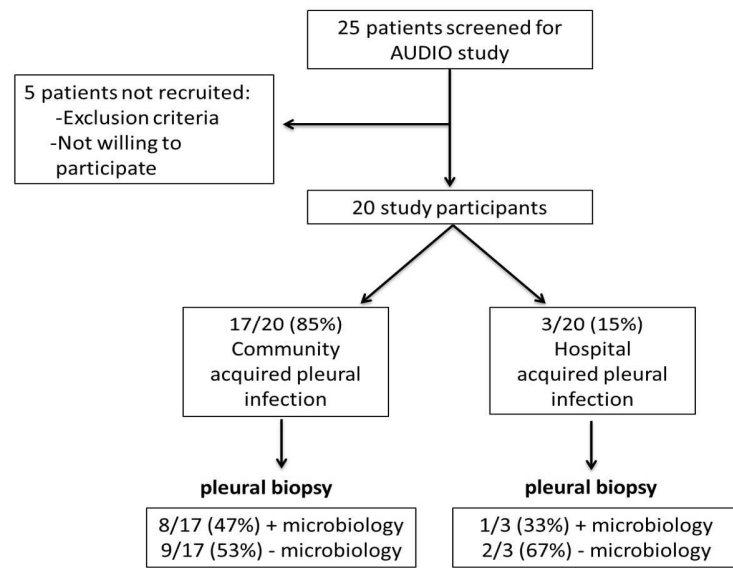


Figure 1. Flow chart diagram for AUDIO study.

FIGURE 2

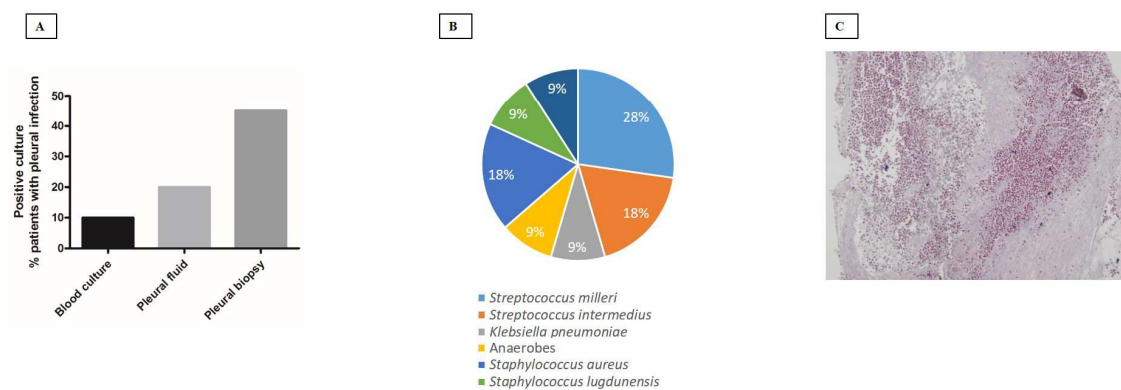


Figure 2. A % increase in positive culture samples in patients with pleural infection. B. Results of pleural biopsy culture. C. Gram stain of acute inflammatory exudate in a pleural biopsy showing small colonies of Gram positive cocci.

FIGURE 3

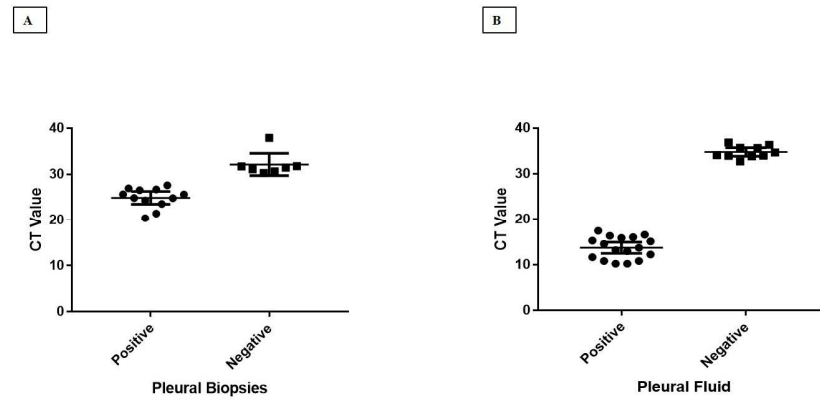


Figure 3. A. Ct number of positive and negative pleural biopsy samples. Mean positive samples 24.8 Ct, (95% CI: 23.74 to 26.68) and mean negative samples 32.1 Ct (95% CI: 30.65 to 31.79) B. CT number of positive and negative pleural fluid samples. Mean positive samples 13.1 Ct, (95% CI 12.4 to 14.9), mean negative samples 34.7 Ct (95% CI 33.8 to, 35.7).

## **e-Appendix 1. Supplemenatry Methods**

### **Study inclusion and exclusion criteria**

Inclusion criteria were both of the following criteria, as per our previous publications and based on current best practice guidelines <sup>1-3</sup>:

1. Clinical evidence of an infective illness (evidence of infection was assessed by the recruiting physician based on fever, an elevated white-cell count and elevated serum inflammatory markers and suitable clinical presentation)
2. Evidence of pleural infection defined as the presence of at least one of the following <sup>4</sup>
  - a. Macroscopically purulent pleural fluid;
  - b. Culture positive pleural fluid;
  - c. Gram stain positive pleural fluid
  - d. Acidic pleural fluid (pH<7.2, measured in a blood gas analyser)
  - e. Low pleural fluid glucose  $\leq 3\text{mmol/L}$  ( $\leq 55\text{mg/dL}$ )
  - f. Computer tomography (CT) evidence of pleural infection (consolidation of underlying lung with enhancing pleural collection on contrast-enhanced CT).

Study exclusion criteria were: 1) Age <18 years; 2) Inability to obtain pleural fluid for analysis; 3) Previous pneumonectomy on the side of pleural infection; 4) Expected survival <3 months due to co-morbid disease; 5) Inability to give informed consent.

### **Chest-tube drainage and antibiotic therapy**

All patients underwent chest-tube drainage with a chest tube inserted under ultrasound guidance as per British Thoracic Society guidelines <sup>5</sup>. All received intravenous antibiotics according to local hospital guidelines and local microbiology advice. Antibiotic therapy was continued for between two and six weeks, according to clinical and radiological response in follow up.

### **Ultrasound imaging**

All patients underwent ultrasound assessment prior to intervention by two respiratory physicians of Royal College of Radiology Thoracic Ultrasound level I or II competence <sup>5</sup>. The size of effusion (small = one rib space, moderate = two to three rib spaces, large > four rib spaces), echogenicity and average number of septations per image were recorded. Each effusion was categorised based on the initial sonographic findings into one of the following groups: anechoic, complex non-septated, complex septated or homogenous echogenic <sup>6</sup>.

**Chest X Ray measurement of area of pleural collection**

The area of pleural opacity and the area of ipsilateral hemithorax were measured digitally by two separate assessors as per previous publications <sup>2</sup>.

**Power calculations**

As this was a pilot feasibility study, no formal power calculation was conducted. A total of 20 patients were recruited as an initial feasibility assessment.

**Role of the funding source**

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

**SUPPLEMENTARY REFERENCES**

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**e-Table 1.** Taqman qPCR primers

TaqMan® Fast Advanced Master Mix qPCR Assays					
Bacteria Target	Gene target	Name	Sequence	Tm	Product Length
<i>Streptococcus pneumonia</i>	lytA	Strep-lytA-For	5'-CGTTGACCCTTATCCATATCTTGCT-3'	65.32	73 bp
		Strep-lytA-Rev	5'-GCCGTTCTCAATATCATGCTTAAACTG-3'	65.29	
		Strep-lytA-Probe	5'-CTCACGGCTAATGCC-3'	55.98	
<i>Staphylococcus aureus</i> MSSA	nuc	MSSA-nuc-For	5'-CCTGAAGCAAGTGCATTTACGAAAA-3'	65.25	105 bp
		MSSA-nuc-Rev	5'-CGCTAAGCCACGTCCATATTTATCA-3'	65.55	
		MSSA-nuc-Probe	5'-CTCGACTTCAATTTTC-3'	49.33	
<i>Staphylococcus aureus</i> MRSA	mecA	MRSA-mecA-For	5'-GGCATGAGTAACGAAGAATATAATAAATTAACCG-3'	65.68	96 bp
		MRSA-mecA-Rev	5'-TTGAGTTGAACCTGGTGAAGTTGT-3'	65.77	
		MRSA-mecA-Probe	5'-CTGCTCAACAAGTTCC-3'	54.50	

**e-Table 2.** Power SYBR qPCR primers

Power SYBR® Green PCR Master Mix Assays			
Name	Sequence	Tm	Product length
16S-Uni-Forward	5'-TCCTACGGGAGGCAGCAGT-3'	59.4 °C	467 bp
16S-Uni-Reverse	5'-GGACTACCAGGGTATCTAATCCTGTT-3'	58.1 °C	
Anaer-ITS-Forward	5'-TTCTACACACCGCCCGTC-3'	63.51 °C	1,500 bp
Anaer-ITS-Reverse	5'-TTCGCCTTTCCCTCACGGTA-3'	66.26 °C	

**e-Table 3.** qPCR results on pleural fluid.

Streptococcus Pneumonia		
	Microbiology Positive	Microbiology Negative
qPCR Positive	9	0
qPCR Negative	0	33

Streptococcus Aureus MSSA		
	Microbiology Positive	Microbiology Negative
qPCR Positive	4	0
qPCR Negative	0	38

Streptococcus Aureus MRSA		
	Microbiology Positive	Microbiology Negative
qPCR Positive	3	0
qPCR Negative	0	39

Anaerobic Bacteria		
	Microbiology Positive	Microbiology Negative
qPCR Positive	5	13
qPCR Negative	3	18