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Examination of the Problem of Antibiotic Resistance, Its Implications For Healthcare and Investigation of Novel Antimicrobial

(Systematic Review)

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ABSTRACT

The formation of biofilms resulting in life-threatening chronic infections in lung disease such as cystic fibrosisis well-established. These multicellular surface-associated form of microbial growth conferring antibiotic resistance are almost impossible to eradicate. Therefore, to examine the evidence for antibiofilm alternative therapies, a systematic review of alternative therapies for the treatment of adults with respiratory infections was undertaken. A search of PubMed, Cochrane Database and references from relevant articles published in the last 10 years was performed using. Following the screening of 582 clinical studies, a total of 6 studies were identified that reported on therapeutic effects of alternative therapies on pathogens involved in biofilm development in adults with respiratory disease. Also, none of the trials reported any significant treatment-related adverse side effects. The identified trials reported on therapies that targeted either bacterial virulence factors or anti-resistance mechanisms. Within limitations of this study, there is reasonable evidence to confirm effectiveness and safety of half of the identified alternative therapies primarily targeted at pathogen virulence factors. These approaches may support antibiotic therapy as an adjunct or preventive therapy. Novel alternative strategies should aim to be translational and based on preclinical models that focus on biofilm-forming pathogens. Large-scale, double-blind randomised trials of adequate duration with placebo controls are required to establish the efficacy and safety of any translational alternative therapies in adult populations.

Keywords:

Biofilm, infection, resistance, non-antibiotic treatment and biofilm, non-antibiotics strategy, biofilm and respiratory tract infection.

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الملخص:

تشكيل (البيوفيلم) الأغشية الحيوية يؤدي إلى التهابات مزمنة تهدد الحياة مثل التليف الكيسي في الرئة. يصعب جداً القضاء على النمو الميكروبي (الأغشية الحيوية) الذي يمنح مقاومة للمضادات الحيوية، لذلك ، لفحص طرق علاجي بديلة للتغلب على هذه البكتيريا عملنا مراجعة منهجية للطرق العلاجية البديلة للبالغين فيما يخص امراض الجهاز التنفسي. بحثنا في قاعدة بيانات جامعة دبلن للعلوم والتكنولوجيا بقاعدة بيانات (PubMed)، وقاعدة بيانات مؤسسة كوكرين والمراجع من المقالات ذات الصلة المنشورة في السنوات العشر الماضية. بعد فحص 582 دراسة سريرية تم تحديد 6 من الدراسات التي ذكرت الآثار العلاجية للعلاجات البديلة للقضاء على البكتيريا المسببة للأمراض والتي تشمل ايضا تشكيل الأغشية الحيوية في البالغين الذين يعانون من أمراض الجهاز التنفسي. كذلك ، لم تذكر أي من التجارب أي علاج ذي مغزى له آثار جانبية ضارة. ذكرت التجارب المحددة على العلاجات التي استهدفت أما عوامل لتكوين العدوى البكتيرية مثل المناعة ، واستشعار النصاب ، أو آليات مضادة للمقاومة مثل العلاج البكتيري أو مضاد للأكسدة. هناك أدلة معقولة لتأكيد فعالية وسلامة نصف العلاجات البديلة المحددة التي تستهدف أساسًا عوامل المقاومة المسببة للأمراض. هذه الأساليب قد تدعم العلاج بالمضادات الحيوية كعلاج مساعد أو وقائي. يجب أن تهدف الاستراتيجيات البديلة الجديدة إلى أن تكون مترجمة وتستند إلى نماذج ما قبل السريرية التي تركز على مسببات الأمراض المكونة للأغشية الحيوية. مطلوب عمل دراسات على نطاق واسع، وعشوائية ولمدة كافية لتأسيس فعالية وسلامة أي علاجات بديلة للبالغين.

الكلمات المفتاحية:

بيوفيلم ، أغشية حيوية، عدوى ، مقاومة ، العلاج بغير مضاد حيوي وبيوفيلم ، إستراتيجية غير مضادات حيوية، بيوفيلم وعدوى بالجهاز التنفسيز

1. INTRODUCTION

The advent of antibiotics more than five decades ago transformed the healthcare delivery and not only dramatically reduced serious and fatal human infections but also significantly impacted pediatric medicine, invasive surgical treatments, transplants and chemotherapy{Spellberg, 2011 ;Gould, 2013}as well as farming and agriculture industries.{Mazel, 1999 }This unprecedented dominance of antibiotics as the key antimicrobial agent was transient and became rapidly under threat by the upsurge in antibiotic resistance. Today, the increasing prevalence of antimicrobial resistance complicated by the lack of novel agents penetrating the pharmaceutical market has become a major global public health challenge.

In the current global disease epidemic, microorganisms continue to develop resistance to antimicrobial agents at a rapid rate and with novel resistance patterns that render commercially available agents considerably less effective, threatening to propel us back to the 'pre-antibiotic era' sooner rather than later.{Li, 2009}A recent systematic review found robust evidence of antibiotic resistance associated with being prescribed in primary care setting. This effect was particularly noted in skin, respiratory and urinary tracts. The authors concluded community prevalence of high endemic of antibiotic resistance is possibly due to presence of antibiotic effects up to a year post treatment.{Costelloe, 2010}

Every year in the U.S, an alarming estimated 2 million people have been found to become infected with antibiotic-resistant pathogens resulting in 23,000 deaths per year.{Hampton, 2013; Blair, 2015} In Europe, a staggering €1.5 billion is the annual cost to the economy from the death of an estimated 25,000 individuals due to infections resulting from drug-resistant bacteria pathogens.{Walker, 2011;Blair, 2015}

Adding fuel to the antibiotic resistance crisis is the increased resistance of some bacterial strains such as Enterobacteriaceae and Pseudomonas to multiple antimicrobial agents known as the multidrug-resistant (MDR)bacteria which refers to the bacterial ability to withstand toxic doses of structurally distinct antibiotics that would normally eradicate non-resistant bacterial strains.{Sun, 2014}The concern with MDR pathogens is echoed by numerous centres around the globe including

World Health Organisation (WHO), Center for Disease Control and Prevention (CDC) in the U.S.A, Antibiotic Resistance Center (AMR) in the UK, and the European Center for Disease Prevention and Control (ECDC). All these disease-control centres endeavour to research and implement novel strategies in order to prevent and eradicate emerging global infections that is rapidly becoming a major public health problem.{Roca, 2015}

The recent recommendations of WHO to develop a strategic action plan for challenging antimicrobial resistance focused on: 1) increasing understanding and recognition of antimicrobial resistance as a global problem; 2) enhancing awareness through research and monitoring; 3) diminishing incidence of infectious disease; 4) encouraging optimal use of antimicrobial agents by health care professionals; and 5) maintaining continuous interest and providing support in development of countermeasures against antimicrobial resistance.{Organization, 2015;Bell, 2014}

The underlying mechanisms of resistance in bacteria are consistently being researched and reported with novel genes that code for antibiotic resistance and facilitate transmission of genetic material between bacteria continuously being identified. Bacteria are either naturally resistant to some antibiotics or have acquired resistance through certain mechanisms. Natural mechanism of resistance is owing to structural or functional properties of the pathogen. Two of the widely studied mechanisms by which bacteria acquire resistance are either through gene mutations or horizontal gene transfer.{Blair, 2015}Similarly, MDR may occur by upregulation of the gene expression for multidrug efflux pumps capable of expelling a spectrum of drugs. Another possible mechanism attributed to MDR of bacteria has been associated with accumulation of multiple genes encoding resistance to a particular drug.{Nikaido, 2009}

Hence, the urgency to develop and implement innovative approaches to challenge antibacterial resistance requires an understanding of the underlying biochemical and genetic mechanisms of microorganisms in order to design and research broad-spectrum drugs that can target MDR bacteria more effectively and safely long-term.

This literature review is divided into two distinct sections. The first part of the review will focus on the literature discussing different types of antibiotic resistance, the underlying mechanism of

bacterial antibiotic resistance and MDR, importance of biofilm in antibiotic resistance, classification and mode of action of antibiotics, clinical implications together with novel therapeutic agents for antimicrobial resistance. The second section of the review will be a systematic review of the evidence on the effectiveness of non-antibiotics therapies against biofilm pathogens which are known to cause the most serious and life threatening infections.{Kostakioti, 2013} The systematic review will include methodology, results and discussion of the findings. Finally, future direction and recommendations will be discussed.

1.1 Emergence of Antibiotic resistance

Most antibiotics were discovered by humans during the 1945-1960 period.{Wright, 2007}The antibiotic resistance genes (ARGs) similarly have been around as long as antibiotics with reports that have identified a number of genes with clinical resistance against antibiotics from either ancient permafrost core{Perron, 2015}or microorganisms located in caves.{Bhullar, 2012}Furthermore, a recent study traced diversity and abundance of antibiotic resistance elements to more than 30,000 ago.{D'Costa, 2011}The extended and progressive overuse of antibiotics in clinical setting and extensive agricultural use to improve the health of both human and animals in recent decades has seen an unfortunate dramatic increase in resistance strains of bacterial pathogens.{von Wintersdorff, 2016}Pretty soon, if a proactive strategy does not commence to prevent or reduce the rapid development of resistant microbes, the morbidity and mortality from infections will overtake diabetes and dementia epidemic.

Despite efforts to contain this rapid resistance evolution, antibiotic resistance is now highly prevalent in all common antibiotics.{Control; Ventola, 2015}Worse still, some strains such as methicillin-resistant *S. aureus* (MRSA) not only impart resistance to multiple antibiotics such as methicillin, tetracycline, aminoglycosides and chloramphenicol but also disinfectants becoming one of the major sources of acquired infections in hospital setting.{Nikaido, 2009 ;de Lencastre, 2007}

One of the most serious threats from microorganisms is the emergence of pathogens such as *Enterobacteriaceae* that virtually confer resistance to all antibiotics.{Livermore, 2004} More recently, gram negative species such as *Pseudomonas* and Acinetobacter have developed the so called 'pan resistant' strains with the latter being extensively resistant largely due to lack of availability of new antimicrobial agents in the market.{Nikaido, 2009} During 1980-1990's another species *A. baumanii* known to be penicillin-resistant, developed resistance to both cephalosporin and fluoroquinolones drugs.{Livermore, 2004}

In multidrug resistant bacteria accumulative resistance is present on transposons of genes that code for different antimicrobial agents and or multidrug efflux pumps releasing multiple drugs.{Nikaido, 2009}

1.2 Types of Antibiotic Resistance: Intrinsic, Acquired and Adaptive

Antibiotic resistance in bacteria is classified into three main types namely, intrinsic or inherent, acquired and adaptive (*Fig 1.1*). Intrinsic resistance basically refers to inherent characteristics of a microorganism responsible for limiting antimicrobial activity.{Fernández, 2012}The efflux pumps present in numerous bacteria and semipermeable outer membrane of gram negative pathogens such as *P. aeruginosa* are good examples of these intrinsic properties.{Fernández, 2012; Nikaido, 2009; Livermore, 2004} At molecular level, antimicrobial resistance genes confer innate resistance as an adaptive intrinsic protective mechanism for the bacteria,{Schroeder, 2017}such as in *streptomycetes* well known to produce a plethora of β-lactamases enzymes that represent a superfamily of genes from which source of clinical β-lactamase resistance develops.{Ogawara, 1999}

In acquired resistance type, normally new genetic material from transposons or plasmids becomes integrated in the bacteria or mutations occur to confer resistance in a susceptible microbe.{Fernández, 2012; Nikaido, 2009} Such acquired resistance under antibiotic selective pressure presumes that the resistance bacterial genotype may be sustained and induced at certain concentration of antibiotics.{Schroeder, 2017} There is also some suggestion that when the resistance occurs as a result of a single mutation the bacteria confers low level resistance, whereas

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accumulation of such mutations may result in a large increase in resistance. For instance, several mutations in the genes coding for *P. aeruginosa* were demonstrated to exert additive effect of 16-fold resistance on aminoglycoside tobramycin as opposed to individual mutational effect conferring only a two-fold resistance.{El'Garch, 2007}

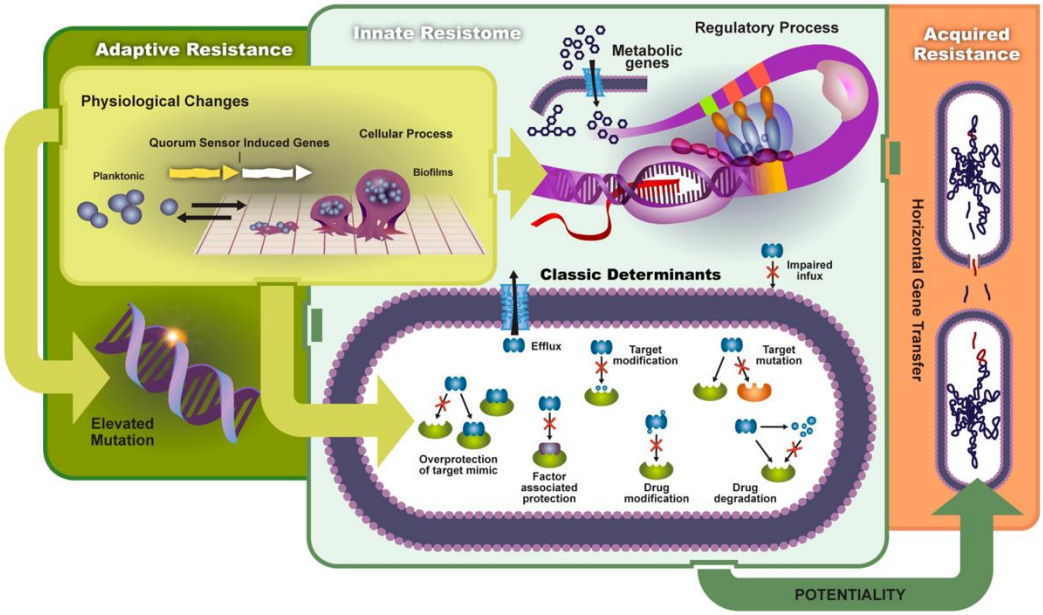

Figure 1.1 Diagrammatic representation of different types of bacterial antibiotic resistance. Environmental cues can 1) cause increased mutation rates; 2) alterations in metabolic genes and regulatory pathways, and 3) multitude development of resistance mechanism and antibiotic inactivation. These increased resistance mechanism can ultimately lead to acquired resistance.{Schroeder, 2017}

Adaptive resistance is transient and has been described as a result of environmental pressures which tend to reverse once the inducing condition is removed.{Schroeder, 2017; Fernández, 2012} The process involves the bacteria survival mechanism to temporarily intensify in order to survive trauma from antibiotics by provisional alteration in levels of gene or protein experession. The trigger is normally exposure to an environmental insult such as change in nutrient availability, ecological stress and high concentration of certain antibiotics.{Fernández, 2012} This highlights

the fact that availability of rich source of antibiotics in the environment elevates the number of antibiotic resistant pathogens.{Andersson, 2010; Brooks, 2014} Examples of adaptive resistance include genetic changes due to adaptation to environment conditions such as biofilm development or inactivation of antibiotics by enzyme stimuli, cell permeability alterations and control of efflux pump.{Schroeder, 2017; Nikaido, 2009; Nikaido, 2012}

1.3 Novel therapeutic antibacterial agents

With the rapidly emerging new antibiotic-resistant bacterial strains the next step is to introduce antibiotics that target such resistant mutants. However, there are very few successful novel antibacterial agents introduced to the market during the past two decades. While genomic strategy has proven to have abundant molecular target, never the less no antibiotics have reached the market place. The rapid rise of resistance among bacterial pathogens is surpassing replacement of old antibiotics that have lost efficacy with newer ones for a number of pathogenic bacteria especially in case of gram-negative bacteria.{Coates, 2007} Presently, most novel antibiotics are likely to be structural equivalent of existing antibiotic families and to date the evidence for efficacy of these antibacterial agents have not been reported.

Other novel alternative agents to antibiotics are therefore much needed to at least slow down the growth of microorganisms. In recent years, attention has been directed towards inhibitors of quorum sensing (cell to cell signaling) that obstruct bacteria from exporting antibiotics outside their cells, biological mediators such as bacteriophages that lead to lysis and breakdown of bacteria, as well as agents such as fucose and galactose sugars that interfere with biofilm formation (*Table 1.2*). While there are numerous studies in vitro and animal models, only in recent years the clinical evidence for these alternative or adjunctive therapies to antibiotics is accumulating and showing promise.

1.4 Clinical relevance and implications

Since the formation of biofilm is considered the main mechanism for bacterial growth in clinical setting and natural environments, therefore biofilm dispersal plays a significant role in transmission

of pathogenic bacteria to human hosts as well as spread of infection intra-host.{Parsek, 2003} In addition, communicable transmission of vast number of bacterial pathogens is facilitated by biofilm dispersal.{Morris, 2007; Nielsen, 2006} It becomes then crucial to understand the biofilm dispersal mechanism in order to develop clinically beneficial antimicrobial agents targeted at preventing biofilm formation or triggering biofilm detachment.{Kaplan, 2010; Lebeaux, 2014}*Table 1.3* lists the most common biofilm associated pathogens associated with medical conditions.{Römling, 2012}

1.5 Aims and Objectives

The primary objective of systematic reviews in general is to address the extend that current research has progressed towards clarification of a particular question or problem. It involves identifying, assessing and assimilating all high-quality studies, finding gaps and inconsistencies in published literature as well as indicating clinical relevance and implications that translate into practice. Although currently antibiotics are still the preferred treatment modality for bacterial infections, accumulating evidence indicate that antibiotic resistance is developed as a result of increased selective pressure.{Kostakioti, 2013}

In human, more than 80% of bacterial infections are associated with biofilm{Harro, 2010} (see *Table 1.3*) and conventional antibiotics are largely ineffective at eradicating biofilm related infections.{Wu, 2015} In particular, biofilm bacteria are refractory to antibiotic treatments due to the presence of extracellular matrix preventing access to the bacteria cell, inactivation of antibiotics by low pH and high number of resistant markers dispersed within the biofilm colony.{Kostakioti, 2013} Collectively, these unique metabolic features of biofilms can potentially increase tolerance to antibiotics by up to a thousand fold compared to planktonic counterparts.{Hoiby, 2010}Thus, more effective strategies to the traditional antibiotic treatment is urgently needed to thwart biofilm formation especially in serious and common infections such as respiratory related infections.

Therefore, the purpose of this review was to investigate the effectiveness of therapeutic alternatives to antibiotics against bacterial biofilm pathogens present in respiratory diseases. The specific aims of this study include:

1. Systematic evaluation of the quality of the evidence concerning effectiveness of non-antibiotics therapies for preventing or reducing the number of bacterial pathogens,
2. To identify all human clinical trials involving non-antibiotic therapies against biofilm pathogens associated with respiratory infections,
3. To assess the quality of the methodology of these studies,
4. To analyse outcomes of these trials and provide indications of overall efficacy of non-antibiotics in treatment of biofilm-related respiratory infections.

Table 1.2 Alternative agents to antibiotics for treatment of biofilm associated respiratory infections.{Römling, 2012; Czaplewski, 2016}

Alternative agent/therapy	**Target pathogen**	**Regulatory mechanism of pathogen**	**Examples of antibiofilm agent**
Inhibitors of signaling molecule c-di-GMP	*P. aeruginosa*	Key activator in biofilm formation and leads to virulence phenotype Controls matrix expression	LP-3145
Quorum sensing Inhibitors (antagonists)	*P aeruginosa*	Quorum sensing manages cell activity based on bacterial density & increases bacterial virulence	Modified furanones (C30 & C56) Ajoene (from garlic)
Antioxidants	*Staph aureus* *S. pneumonia* *H. influenza*	Promotes disruption of macro biofilm colonies, Reduce extracellular matrix production	N-Acetylcysteine (NAC)
Bacteriophages	*P aeruginosa*	infiltrate extracellular biofilm matrix to eliminate target bacterial cells	AmpliPhage-001
mucolytic agents	*P. aeruginosa, H. influenza* *Staph. aureus*	Interfere with different stages of biofilm formation: reversible & irreversible attachment and maturation	metabolite VIII of Bromhexine (Ambroxol)
Lysins	*Staph aureus*	Weaken biofilm by destroying cell wall	SAL200
Vaccines	*P aeruginosa*	Against outer membrane protein based	Ic43,

Blocks virulence mechanism	
Staph aureus	SA4Ag

Table 1.3 Biofilm-associated respiratory infections subsequent to bacterial pathogen colonisation that have been unresponsive to antibiotic therapy. {Blasi, 2016; Koch, 1993}

Airway diseases	Biofilm Infection	Main disease characteristics	Bacterial pathogen	Prevalence of pathogen	Clinical evidence for non-antibiotic antibiofilm pharmacotherapies
Lower respiratory infections	Chronic bronchitis{Kim, 2013}	recurrent infection chronic cough&sputum production, pathologic airway dilation	Non-capsulated *H. influenza* *S. pneumonia*	30-70% cases	-Bacterial lysates -immuno-stimulatory therapy (eg Buccalin*) -mucolytic agents (eg Ambroxol)
	Cystic Fibrosis (CF){Safdar, 2009}	Reduced airway volume due to lack of epithelial chloride channel leading to impaired ciliary clearance	*P. aeruginosa* *H. influenza* *Staph. aureus* *B. cepacia*	80% adults with chronic infection 10% cases	-Hypertonic saline solution aerosols (increase mucociliary clearance lead to less chance of bacteria colonisation, eg atypical mycobacterial infection) - mucolytic agents -Bacterial lysates
	Chronic Obstructive Pulmonary Disease	Limited airway with chronic inflammation of	Non-capsulated	25-30%	-antioxidants (N-acetylcysteine) -immuno-stimulatory therapy (eg

	(COPD){Braido, 2007} Severe COPD	the airways, Oxidative stress, Mucus hypersecretion Requires mechanical ventilation	*H. influenza* *M. catarrhalis* *S. pneumonia* *P. aeruginosa*	cases 10-15% cases 10-15% cases 5-10% cases	Buccalin*) -Mucolytics (carbocisteine) -vaccines
Upper respiratory infections	Chronic rhinosinusitis{Adriaensen, 2013}	Persistent inflammation of the nose & paranasal mucosa 10% prevalence in Uk	*Staph. aureus* Non-capsulated *H. influenza*	50% of cases 28% of cases	-0.05%sodium chloride with saline solution -Xylitol -Bacterial lysates -antioxidants (N-acetylcysteine)
	Otitis media{Costerton, 1999}	Chronic inflammation of middle ear with discharge Acute inflammation of	*Staph. aureus* *P. aeruginosa*	4-30% cases 30-50% cases	-Bacteriophage

		middle ear	*S. pneumonia* *H. influenza*	25-40% cases	-Vaccines (Pneumovax 23)

2. MATERIALS & METHODS

2.1General protocol for systematic review on antibiofilm therapies

The purpose of this systematic review was to identify and appraise all relevant published randomised clinical trials on non-antibiotic interventions for common biofilm associated respiratory infections by discussing the study methodology qualities and comparing conclusions. Also, to discuss the limitations and strengths of these conclusions which will invariably facilitate translation of best evidence into optimal clinical practice. The approach to data collection for this study was mainly qualitative and descriptive in nature.

A systematic review of all human randomised clinical trials of non-antibiotics antibacterial therapies in the treatment of any respiratory disease due to biofilm associated infection was conducted. The focus of this systematic review was to identify clinical trials involving adults attending mainly primary care or community centres. The interventions for the patients included any non-antibiotic or alternative antibiofilm therapeutic treatments for common bacterial infections normally associated with upper respiratory and urinary systems. The outcomes commonly reported in the literature were selected based on bacteria count and clinical outcomes. The outcome measures of each study were described as reported by the study authors.

The general approach of the methodology involved searching Google first to obtain additional search terms followed by searching the common electronic databases as well as Cochrane library from January 2007 to January 2017 inclusive. This strategy is to ensure no relevant search words are missed and all common databases have been searched. Also, manual searches of the references from relevant articles was conducted. The search terms were clearly described ensuring they were relevant to the research question (see **Appendix A** for a full list). Moreover, the scope of search terms was focused to capture all relevant data without capturing literature that was irrelevant to the research study. This was achieved by performing the searches according to the inclusion and exclusion criteria formulated to locate eligible and relevant studies for this review.

The search results were then presented in the format of PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow chart of the literature search process.{Moher, 2009}Once search was completed and full text articles obtained for this study, the characteristics of the trials found were tabulated and analysed. This followed the SPICO formula as described above (*Table 2.1*). The results were then evaluated for methodological quality using Jadad scoring method. appraised in terms of the quality of included studies, inconsistencies in the literature, and main findings summarised in discussion which should present a balance between theory, evidence found and translation to practice. Finally, conclusions were drawn and recommendations for future practice made. The future directions in terms of theory, evidence and practice will be described and any unresolved issues pointed out.

Table 2.1 Different components of SPICO approach for this systematic review

SPICO	**Explanation**
S: Study Design	Randomised Clinical Trial
P: Population	Patients affected bybiofilm associated with respiratory infections
I: Intervention	any non-antibiotic treatment defined for this review
C: Control	any, placebo
O: Outcome	at least one microbiological or inflammatory outcome

2.2Search Methodology

2.2.1 Search strategy & search terms

The search strategy used was based on the Boolean search methodology for screening of the University electronic search engine (includes Science direct, PubMed and other databases) and Cochrane library for clinical trials. The key search terms were either a combination of key words alone or combined that described *biofilm infection, non-antibiotics, antibiotic resistance, bacteria, pathogen, therapeutic strategies, alternative strategies or antibiofilm therapies, 'biofilm' AND 'respiratory tract infection' OR 'cystic fibrosis', 'COPD', 'otitis', OR 'bronchitis'*with relevant medical subject headings (MeSH) terms in titles and abstracts(see appendix A for more examples

of search terms). Other search terms used alone or combined with above terms were related to specific therapies such as *'N-acetylcysteine'*, *'bacteriophage', 'inhalation'* or *'Quorum sensing inhibitors'*.In addition, manual search of relevant references from included papers and other systematic reviews including Cochrane reviews was conducted. A detailed list of search terms and search strategies used is presented in *Appendix A and B.*

2.2.2 Eligibility criteria

Inclusion Criteria

The criteria for eligible studies were based on the scope of the research question, interventions, key variables, participants, timeframe, and study design. The inclusion criteria specified for this study were randomised clinical trials with non-antibiotic or alternative pharmacological or natural interventions to reduce or eliminate biofilm associated respiratory infections that were resistant to antibiotics, published in English Language, studies published last decade (January 2007- January 2017), adults 18 years or older and in any clinical setting.

Types of interventions

Any study that compared non-antibiotic or alternative therapies (see definition) to placebo or antibiotics or other controls were acceptable.

Outcome measures

The important outcomes included were:

1. Change in bacteria count isolated from respiratory tract culture as assessed by clinicians following intervention
2. Change in inflammatory biomarkers in sputum (cytokines or leukotrienes)
3. Proportion of participants that showed improvement in microbiological outcomes following intervention at follow-up visits
4. Isolation of micro-organisms or other pathogens associated with respiratory disease with or without antibiotic resistance
5. Any adverse effects

Exclusion Criteria

Generally, studies were excluded when not a randomised clinical study, not in English, could not be accessed, the intervention was device-related infection, the outcome was not related to biofilm prevention or elimination, animal studies or survey based. The reason interventions for device related infections were excluded is because there are several systematic reviews already completed. In addition, oral or eye infections were excluded due to large body of literature and reviews already published.

Other excluded studies were trials of anti-inflammatory agents such as ibuprofen, agents that alter host environment such as immunotherapy, and physical therapies such as acupuncture or exercise, and changes in the environment such as infection control-related policies. The main eligibility criteria are summarised in *Table 2.2.*

2.2.3 Study selection process

Relevant studies were selected by the author (usually two researchers would independently select the studies in this step). Once selected based on relevant abstract, the collected articles were screened and selected papers were then entered in Endnote, a useful referencing management software that assists in removal of duplicates and keeps a record of all searched relevant articles. The full texts of articles were further limited according to the predefined inclusion and exclusion criteria. Excluded articles were tabulated with reasons for exclusion described.

2.2.4 Data extraction

The data extracted from eligible studies included details of study population, intervention types and outcome measures which were entered on a standard form designed to enter data extraction details. The following items were included on the form:

1. Information such as lead author, publication date
2. Characteristics of the clinical trial such as study design, method of randomisation and blinding, duration of intervention as well as follow up periods.
3. Intervention(s) including the dosage and route of administration, use of comparator (placebo or other control)
4. Participants: total number in both intervention and control groups, mean age of adults, any losses due to withdrawal to follow-up.

5. Outcome measures: above mentioned outcomes, other effects, quality of the outcomes reported.

Table 2.2 Generalcriteria for inclusion and exclusion of studies

	Inclusion	**Exclusion**
Population	General population Adults ≥ 18 years old Respiratory infections any clinical setting	Children or teenagers (aged under 18 years) Adults with 0ral, dental and eye infections
Interventions	Antibiofilm strategies that do not involve use of antibiotics rather single intervention of non-antibiotic therapies Interventions aimed at common respiratory infections Only antibiotic resistant biofilm pathogens causing respiratory infections Alternative or non-antibiotic therapies administered via oral route or inhalation	Any antibiotic or drug related treatment Any antibiotic combined with other therapies Device related infections Any physical therapies alone or combined with acupuncture, PDT or other devices Any surgeries or injections
Comparators	Any	Not Applicable
Outcomes	At least one outcome related to bacterial count, change in pathogen rate and or microbiological outcomes	Not clear outcomes or no relevant outcome Related to bacteria or inflammation

Study design and quality	Randomised clinical trials (RCTs) Peer reviewed original research studies, Systematic reviews with or without meta-analysis	Population-based surveys Grey literature (non-peer reviewed) Policy reports, white papers case studies and reviews
Other criteria	Papers in English language Papers with full-access Papers with relevance to the topic Studies from January 2007 to January 2017	Non-English language Non-full access papers Studies older than 2007

2.4.5 Quality assessment of included studies

The quality of the methodology of each included trial was assessed using the Jadad scoring system.{Jadad, 1996} The system is based on five point reporting on three methodology qualities. Scores of 2 points or less represent poor quality with 0 the lowest quality score and 5 indicating a high-quality score. Any study with score of 3 or more is considered good quality article. Any study described as randomised, double blind and documented reason for participant dropping out of the study is given 1 point for each of the three criteria. Other points are granted if the randomisation (1 point) and the double blinding (1 point) procedure is described in the study. However, if the randomisation or double-blinding were not sufficiently explained, one point may be deducted for each process.

A detailed step by step systematic approach to this review is presented in *Appendix C.*

3. RESULTS

3.1 Results of the search

There was a total of 582 potentially relevant studies screened from electronic databases and manual search of references published after January 2007 to January 2017 inclusive. Following duplication removal and exclusions based on eligibility criteria for the study, 35full text articles were retrieved (*Figure 3.1*). Review of full text articles led to exclusion of 29 studies mainly because the articles were inaccessible, inclusion criteria was not met or the outcome was not relevant to the topic of biofilm associated pathogens. The reasons for exclusion of all studies is detailed in Appendix C. The relevant characteristics of the6 included trials as well as patient populations are summarised in *Table 3.1*, while *Table 3*.2 represents the details of methodological quality of the randomised clinical trials based on Jadad score. Since the number of RCTs were small together with presence of wide heterogeneity among the trials including different interventions and outcome measures it was not possible to conduct statistical analysis to compare treatment effects of clinical trials.

Six trials with a total of 271 patients were included with studies on glutamine

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supplementation,{Forrester, 2016} glutathione (GSH) inhalation,{Griese, 2013} garlic extract supplementation,{Smyth, 2010} N-acetylcysteine (NAC) oral supplementation, Biophage,{Wright, 2009}and fucose/galactose inhalation.{Hauber, 2008}All six trials were randomised; five trials were double blinded, four trials placebo controlled while the other two were parallel controlled design. Three trials were based in UK; the glutamine, garlic and Biophage study.{Forrester, 2016; Smyth, 2010; Wright, 2009} The GSH and fucose/galactose inhalation as well as NAC oral intervention study were based in Germany.{Griese, 2013; Dauletbaev, 2009; Hauber, 2008}Five of the trials were quite small and one trial had intermediate participant numbers with all trials ranging from 11-153and mean ages of 18 to 58 years. Interventions varied among studies and included oral consumption of glutamine, garlic capsules, Biophage cocktail, and N-acetylcysteine (NAC)as well as inhalation administration of glutathione (GSH) and fucose/galactose.The intervention durations of trials ranged from 4 to 24 weeks with various doses of each intervention detailed in *Table 3.1*. All studies but one described *P. aeruginosa* pathogens associated with respiratory infections.

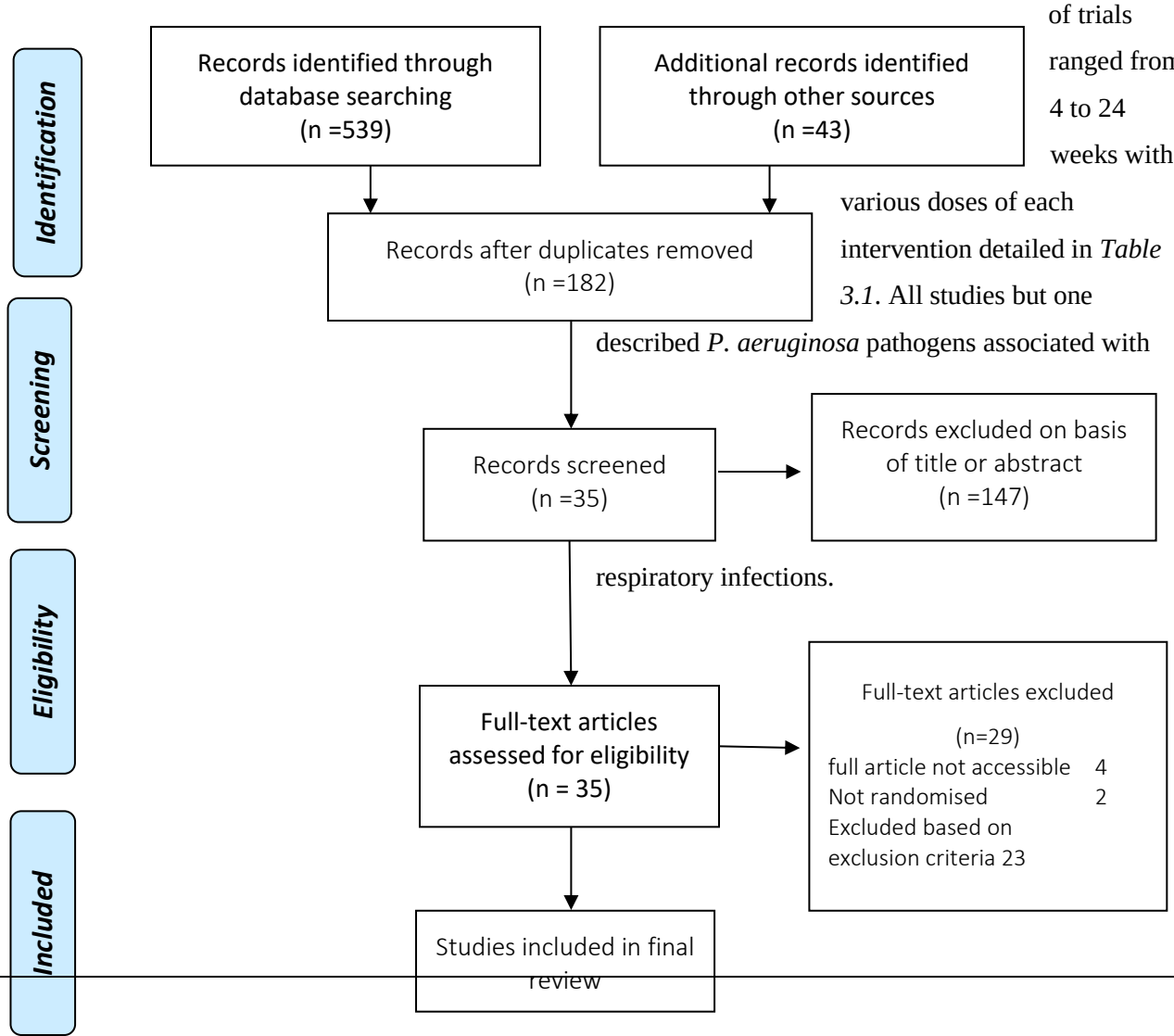

Figure 3.1 Prisma flow chart of search methodology (source of template Moher).{Moher, 2009}

Only two trials had equal number of males and females, the other four trials consisted of more than two thirds males.Three trials consisted of mixed population of adults and children while the other three trials comprised of only adult participants. While all populations had chronic respiratory disease, all but one trial consisted of CF patients with chronic infection associated with *P. aeruginosa*{Forrester, 2016; Griese, 2013; Smyth, 2010; Dauletbaev, 2009; Hauber, 2008} and one trial was infection associated with upper respiratory tract (otitis).{Wright, 2009}Four of the trials included placebo as comparator while the other two had parallel groups.

3.2 Study & Population Characteristics

Two studies involved nutritional supplementation of glutamine and garlic extract as interventions.{Forrester, 2016;Smyth, 2010} A recent clinical trial examined potential benefits of glutamine supplementation in CF patients. The study randomised 36 patients with CF and mean age of 30 years to an intervention arm to receive 21g L-glutamine daily (7g x3 times) and included a placebo arm. While there was no difference between the two groups for clinical biomarkers of pulmonary inflammation, however after 8 weeks of oral glutamine intervention there was a significant increase in sputum neutrophil and Pseudomonas isolation agar cell forming units (CFUs) as well as total cell numbers in the intervention compared to the control group.{Forrester, 2016}

The garlic extract study involved 26 patients with CF and 18 years as median age administered daily garlic capsules (656 mg) for duration of 8 weeks. Although half of the patient were receiving IV antibiotics during the intervention, it was decided to include this study as most trials had patients with some form of existing ongoing treatment. There were some improvements in clinical outcomes, due to the small number of participants no statistical significance was noted between the two groups. The microbiological outcomes measured in this study were based on detection of signaling molecules N-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL) and N-but anoylhomoserine lactone (C4-HSL) from *P. aeruginosa* quorum sensing (QS) systems. These molecules have been known to contribute to the pathogenic mechanism of *P. aeruginosa* in biofilm maturation and production of virulence factors.{Smyth, 2010}Although due to insufficient sampling the levels of C4-HSL were unmeasurable, however the sputum from some of the

participants revealed a significant correlation of measured 3-oxo-C12HSL signaling molecule between the plasma and sputum of CF patients in the garlic intervention group.{Smyth, 2010}The intervention group displayed minor gastro related adverse event during the intervention.

Further trials evaluated effectiveness of antioxidants in CF patients with one involving inhalation administration{Griese, 2013} and the other oral intake{Dauletbaev, 2009} of intervention. A recent clinical trial reported inhalation of glutathione (GSH) at pharmacological dosage in 153 CF patients of mean age 23 years old with half demonstrating chronic infection with *P. aeruginosa* and about 36% harboring *Staph aureus* infections.{Griese, 2013} Glutathione is found to be depleted in CF patients and occurs as a major antioxidant present in the extracellular lining of lungs promoting anti-inflammation.{Jacquot, 2008}In this study, there was significant increase in extracellular GSH, however, over the 24 weeks duration of intervention there was no evidence of improvement in clinical outcomes (lung function or related symptoms), oxidative and inflammatory biomarkers of sputum.{Griese, 2013}A previous trial on precursor of GSH, N-acetyl cysteine (NAC) also showed increased extracellular GSH concentration in sputum as compared to plasma. The trial compared high (2800mg/day) and low (700mg/day) dose of oral NAC administered to21 CF patients with an average of 27.8 years over 12 weeks and found high doses to be well-tolerated.{Dauletbaev, 2009}There was no difference observed in airway inflammatory markers cytokines and IL-8 between the two groups.

The systematic search revealed only one published trial in English using an alternative strategy to treat biofilm associated upper respiratory infection that fitted inclusion criteria.{Wright, 2009} The trial included asingle small dose (2.4ng) Bacteriophage treatment of 24 older patients (mean age 56.7 years) diagnosed with chronic ear infection due to *P. aeruginosa*that was resistant to antibiotics. Bacteriophages selectively destroy target bacteria and have been shown to be effective in clinical improvement of refractory ear infections in animal models.{Soothill, 2004} There was significant improvement in clinical outcomes reported for the phage-treated group over 6-week follow-up period. Furthermore, the bacterial count of *p. aeruginosa* were markedly diminished in Biophage-treated group while it remained the same in the placebo group.{Wright, 2009}

Table 3.1 Summary of the included intervention clinical studies on clinical biofilm non-antibiotic treatment strategies

Study (year) [country]	**Study Design**	**Rationale For the trial**	**Participants**			**Respiratory disease characteristic**	**Interventions**			**Comparator (control)**	**Main Outcomes**
			Sample Size	**Age mean**	**Male (%)**	Biofilm-associated infection	**Intervention**	**Follow-up duration**	**Dose**	**Control group**	
Forrester, 2016} [UK]	Randomised Double-blind Placebo group controlled	Possible selective activity against *P. aeruginosa*	36	30 years	62%	CF patients Chronic infection *P. aeruginosa*	Glutamine-L oral solution	8 weeks	7g sachet 3x daily	Placebo Iso nitrogenous [1]	Significant Increase in Pseudomonas Isolation Agar CFU
{Griese, 2013} [Germany]	Randomised Double-blind Controlled multicentre	Glutathione has been shown to disrupt biofilms of clinical *P. aeruginosa*	153	23 years	52%	CF patients 50% with Chronic infection *P. aeruginosa*	Glutathione inhalation	24 weeks	One pharmacological doseof 646mg/ 12 hours nebulised	Placebo (lactose & cellulose)	Increased extracellular glutathione in sputum No change in inflammatory or oxidative

											markers
{Smyth, 2010} [UK]	Randomised Double-blind Placebo controlled pilot trial	Garlic may act as QS inhibitor by lowering virulence & increase susceptibility of *P. aeruginosa* to phagocytosis	26	18 years	54%	CF patients Chronic infection *P. aeruginosa*	Garlic oil macerate + cardamom oil	8 weeks	656mg garlic oil + 10mg cardamom oil daily	Placebo Olive oil +cardamom oil	Significant correlation between plasma and sputum for the QS molecule 3-oxo-C12-HSL

Table 3.1 continued…

Study (year)	Study Design	Rationale for the trial	Participants			Respiratory disease characteristic	Interventions			Comparator (control)	Main Outcomes
			Sample Size	Age group mean	Male (%)	Biofilm-associated infection	Intervention	Follow-up duration	Dose	Control group	
Dauletbaev, 2009} [Germany]	Randomised Double-blind parallel group	Overexposure of CF airwaysto bacteria-derived oxidants increases OS	21	27.8 years	76%	CF patients	N-acetylcysteine (NAC) oral	12 weeks	700mg /day	Parallel group 2800mg /day	Increased extracellular glutathione in induced sputum (high dose NAC)

Wright, 2009} [UK]	Randomised Double-blind placebo-controlled	Bacteria in biofilm are prone to phage lysis	24	57.6 years	82%	Human chronic otitis multi-resistant *P. aeruginosa*	Biophage-PA cocktail[2] Therapeutic bacteriophage preparation	7,21,42 days	One dose 200 uL	Placebo (glycerol-PBS)	Significant reduction in bacteria count; Improved clinical scores
{Hauber, 2008} [Germany]	Randomised open trial parallel group	Colonisation by bacteria is prevented by blocking lectin binding	11	27 years	73%	CF patients Chronic infection *P. aeruginosa*	Fuose/ galactose solution inhalation	3 weeks	10ml inhaled/2x day	Parallel IV AB+ inhalation	Significant decrease of bacteria in sputum No side effects

[1] Placebo is a mix of 82% asparagine & 18% glycine

[2]combination of different phages effective against *P. aeruginosa*

Abbreviations CF: Cystic fibrosis; CFU: colony forming Units;COPD: Cardio Obstructive Pulmonary Disease; GSH: Glutathione; IV: Intravenous; NAC: N-acetylcysteine, OS: oxidative stress; PBS: Phosphate Buffered Saline

Table 3.2 Methodology quality assessment. Detailed Jadad score assessment profiling of each clinical trial

Study No.	First author (year)	Study is randomised controlled trial (RCT)	Randomisation process explained in the study	Study double blinded	Treatment allocation is described adequately	If drop-out reasons explained	Jadad Score
1	Forrester et al.{Forrester, 2016} (2016)	Yes	Yes	Yes	Yes	Yes	**5**
2	Griese et al.{Griese, 2013} (2013)	Yes	Yes	Yes	Yes	Yes	**5**
3	Smyth et al.{Smyth, 2010} (2010)	Yes	No	No	Yes	Yes	**3**
4	Dauletbaev et al.{Dauletbaev, 2009} (2009)	Yes	Yes	No	Yes	Yes	**4**
5	Wright et al.{Wright, 2009}	Yes	Yes	Yes	Yes	Yes	**5**

	(2009)						
6	Hauber et al.{Hauber, 2008} (2008)	Yes	Yes	No	No	NA	2

The final included trial examined the effect of an inhaled sugar solution in CF patients diagnosed with chronic *P. aeruginosa* infections. Colonisation of airways by this pathogen is facilitated through two specific carbohydrate binding moieties that attach to the airway epithelium to prevent cilia beating. This activity has been shown previously to be blocked by addition of simple sugars. The clinical study included 11 CF patients with mean age of 27 years that were randomised to an intervention arm receiving twice daily 10ml of fucose/galactose solution (0.1M in 0.9% NaCl) alone or combined with intravenous (IV) antibiotic therapy (cephalosporin & aminoglycoside) for 3 weeks. The clinical and microbiological outcomes were clearly improved in the intervention group compared to the IV antibiotics combined group. Inflammatory marker (macrophages & lymphocytes) percentages in sputum were significantly increased in the inhalation group compared to no change in the IV plus inhalation group. The count of *P. aeruginosa* in the sputum collected from patients that were administered fucose/galactose solution alone was significantly reduced compared to the group that received combination IV & inhalation therapy.{Hauber, 2008}

3.3 Quality Assessment

The studies were classified as either Randomised Clinical Trial Placebo Controlled (RCT-PC 4) or Randomised Clinical Trials with parallel group (RCT-P 2). Only one study was rated as low quality,{Hauber, 2008} with the other 5 trials rated as high quality studies with 3-5 points as Jadad score.{Forrester, 2016; Griese, 2013; Smyth, 2010; Dauletbaev, 2009; Wright, 2009} The Jadad score and details of quality assessment criteria for each trial is presented in *Table 3.2.*

3.4 Adverse Events

Three of the trials reported no treatment-related adverse side effects,{Forrester, 2016; Wright, 2009; Hauber, 2008} while one reported mild gastro symptoms such as diarrhea and abdominal pain as well as halitosis (bad breath) during the intervention.{Smyth, 2010} Two trials reported a similar incidence of adverse events in both intervention and placebo groups.{Griese, 2013} One trial reported mild events such as pyrexia, abnormal sputum and upper respiratory infection,{Griese, 2013} while the other trial adverse events were mostly due to exacerbations of CF lung disease.{Dauletbaev, 2009}

4 DISCUSSION

One of the most common causes of mortality and morbidity in humansby infection is represented by respiratory tract infections (RTIs).[85] Although viruses are the source of original disease, several bacterial species are responsible for recurrent infections among which *Enterobacteriaceae*, *H. influenza*, *P. aeruginosa*, *S. pneumonia* and *Staph aureus* are relatively frequent.{Braido, 2007} The formation of biofilms has been shown to account for the fact that respiratory infections presenting an ever formidable challenge for clinical management.{Lebeaux, 2014} To complicate the matter further, systemic antibiotics have not been effective in eliminating biofilms. Consequently, research has increased in studying the role of non-antibiotic therapies in the treatment of airway infections.

Despite publications of several strategies to explore treatment avenues for reducing or eliminating infections with antibiotic resistant pathogens, a scan of available literature however provided very few clinical trials on respiratory tract biofilm associated infections with the majority of studies limited to observational or cohort studies. Other more rigorous trials did not measure microbiological outcomes or were not randomised to be included in this systematic review. In the past ten years, only one systematic review has been published on alternative therapies for pulmonary infections that reported on microbiological outcomes. This Cochrane review included only four trials with different interventions as quality-evidence for non-antibiotic therapies.{Hurley, 2013} Three of the studies could not be included in this systematic review due to reporting on children population only (2 trials) and vaccine as an intervention (1 trial). Similarly, this systematic review identified only a small number (6) of clinical trials with five reporting high quality evidence.

In the systematic review for the current study, quality evidence for the effectiveness of non-antibiotic the rapies in eliminating biofilm formation in respiratory tract infections was investigated. The focus of the study was on oral and inhalation intervention with the main outcome as microbiological measures and inflammatory markers. First, a summary of main systematic search findings is presented followed by separate sections on evidence for alternative therapies

identified in the study. Finally, strengths & limitations of the study at the end of the chapter will be followed by conclusions and future recommendations.

4.1 Summary of main results

All studies identified through a systematic approach in this study reported infections associated with *P. aeruginosa* mainly in patients with cystic fibrosis (CF){Forrester, 2016;Griese, 2013;Smyth, 2010;Hauber, 2008;Dauletbaev, 2009} with only one additional study reporting on chronic ear infection.{Wright, 2009} As there are currently no antibiotics available to eradicate *P. aeruginosa* infections that are already established, alternative therapeutic approaches are clearly much needed. This opportunistic pathogen is versatile as it can utilise aerobic as well as anaerobic metabolism with innate antibiotic resistance by growing as biofilms in vulnerable respiratory tracts. The CF lung is known to result from a defect in the CF transmembrane regulator leading to dehydrated epithelial surfaces as well as thick secretions that provide an ideal niche for the colonisation of *P. aeruginosa* and subsequent biofilm formation.{Hurley, 2012}

The six studies of this systematic review more or less fall into two broad non-antibiotic approaches providing opportunities for inhibiting bacterial infections: anti-virulence and anti-resistance strategies. Three studies reported on anti-virulence strategies including immunomodulation therapy, quorum sensing inhibitors and outer membrane protein inhibitors (Lectin).{Forrester, 2016;Smyth, 2010;Hauber, 2008}The other three studies reported on anti-resistance approaches including bacteriophage and potentially antioxidant therapies although the role of antioxidants as antibiofilm agents is largely unclear.

4.2 Anti-virulence strategies

Complex signaling mechanisms of microorganisms are involved in regulation of the host immune interaction, virulence as well as antibiotic resistance. Such bacterial chemical mechanisms specially provide the opportunity for exploring potential therapeutic targets for developing antibacterial non-antibiotic agents.{Hurley, 2012}

4.2.1 Immunomodulation therapy

A recent randomised clinical trial with placebo found dietary supplementation with oral glutamine for 8 weeks in patients with CF did not show any changes in pulmonary inflammatory biomarkers and authors concluded addition of oral glutamine did not offer any clear clinical benefits to CF patients.{Forrester, 2016} Glutamine is known to be involved as part of neutrophil metabolic processes in several cellular immune responses such as motility andsecretion of various enzymes active in the initiation of bacterial phagocytosis. A recent meta-analysis examined the association of glutamine supplementation with reduced infections and mortality in critically ill and surgical patients. The authors identified 44 RCTs, 16 of which were published between 2007-2013. However, none of these RCTs could be included in the current systematic review because there were no populations with respiratory tract infections examined and glutamine supplementation was via parenteral administrations. The meta-analysis found a significant reduction in risk of infection and shortening of hospital stay following administration of intravenous dietary glutamine.{Bollhalder, 2013}

Furthermore, all previous meta-analyses have only examined glutamine use in critically ill patient populations only and as this was the first glutamine trial in CF patients, it was not possible to compare to any previous studies. In addition to the different study population, the route of administration, glutamine dose, duration of intervention as well as the methodological design may account for the differences between the study identified in this review and previous glutamine studies. Further well designed and longer randomised trials in CF patients comparing different routes of administration will elucidate the clinical importance of this abundant amino acid.

4.2.2 Quorum Sensing Inhibitors

The virulence factors for *P. aeruginosa* in biofilm formation are regulated via a cell-cell signaling mechanism known as quorum sensing (QS).{Hurley, 2012 #184} This involves the production of signal molecules responsible for virulence, expression of which is activated depending on the number of bacteria within the biofilm. Multiple QS signaling pathways are used by *P. aeruginosa* and have a significant role in its resistance and subsequent biofilm formation. The main pathways

include Las, RhI and the alkyl quinoline pathway or the Pseudomonas quinolone signal (PQS).{Hurley, 2012}

Garlic has been reported to act as an anti-biofilm agent by inhibiting quorum sensing *in vitro* and facilitating susceptibility to antibiotics.{Hurley, 2012} Furthermore, animal studies show that addition of garlic significantly reduced pulmonary*P. aeruginosa* pathogens compared to placebo group.{Bjarnsholt, 2005} Although the human trial identified in this review using garlic extract as an intervention did not find any statistical significance in clinical outcomes between the treated and placebo group due to the small population size, however the authors showed that QS molecules were detected in both plasma and sputum of CF patients.{Smyth, 2010} Therefore, this pilot trial is a promising start to investigate strategies for translating potential benefits of garlic treatment as well as other natural QS inhibitors to clinical practice.

4.2.3 Lectin inhibitors and airway infections

There was only one clinical trial identified by this review that involved the use of lectin inhibitors but the evidence is robust and promising.{Hauber, 2008} The trial randomised one arm to receive inhaled sugars (inhaled fucose and galactose) as treatment while the parallel arm received inhaled sugars together with intravenous antibiotics for the duration of 3 weeks.{Hauber, 2008} Lectins are extracellular membrane proteins known to facilitate bacterial cell aggregation to form biofilms and may contribute to abnormal ciliary beating in the human respiratory tract. Specific lectins are identified as LecA and LecB that have binding sites for fucose and galactose sugars. Recent *in vitro* studies have demonstrated lectin inhibitors capable of preventing bacterial aggregations and assisting in *P. aeruginosa* biofilm dispersal.{Hurley, 2012} In the clinical trial, both parallel groups exhibited a significant decrease in sputum *P. aeruginosa* CFUs as well as inflammatory markers.{Hauber, 2008} These findings are promising in the use of inhalation sugars alone or as an adjunct to antibiotics as potential therapeutic agents for preventing or eliminating Pseudomonas biofilms associated with respiratory infections. While the fucose/galactose inhaled solution was reported to be well-tolerated within the short time frame of this trial (3 weeks), therefore any potential side effect with longer duration of inhalation remains to be determined in larger clinical trials.

4.3 Anti-resistance strategies

4.3.1 Bacteriophages

The only randomised phage therapy trial for respiratory disease identified by this systematic review was performed in patients with chronic otitis media.{Wright, 2009} The trial involved 24 patients withotitis media due to persistent *P. aeruginosa* infection who were treated with a single dose of bacteriophage cocktail prepared from six phage strains. Complete resolution of the chronic disease was noted in almost25% of the phage treated patients within 6 weeks. The bacterial count was significantly reduced in this group of patients. Although promising this is the only trial published on respiratory disease, other ongoing trials are mainly on wound treatment. The phage formulation was shown to be effective and safe to be used in refractory *P. aeruginosa* infections. Further larger trials are required to test the sensitivity of individuals to phage mixes and efficacy of the dose, since single phage dose to treat bacterial infections requires various mix of phages to be effective against multiple pathogen strains.

4.3.2 Antioxidants and airway infections

The presence of several oxidative stress markers in the airways of CF patients has prompted researchers to explore different antioxidant therapies. Supplementation with antioxidants may assist in reducing the oxidative damage caused from persistent infections in the lungs. Various types of antioxidants exist including, Beta-carotene, Vitamin E, Vitamin C and Glutathione.{Ciofu, 2014}While a number of trials on the effects of antioxidants (oral or inhaled) in CF patients have been reported and analysed in a recent Cochrane systematic review, however the authors concluded that the outcomes from trials identified were too inconsistent to draw any conclusions about the use of antioxidants for this population. This is because it was not possible to distinguish what effects were due to antibiotic or antioxidant therapy.{Ciofu, 2014} Only one trial{Griese, 2013} from the Cochrane review was identified for this systematic review while the other 8 trials were either published before 2007 or were conducted on young children.{Ciofu, 2014}

Although GSH depletion is a characteristic of CF disease, due to its lack of bioavailability and restricted access to the cell, GSH administration is found to be not the best practice. Consequently, other means of delivery such as the use of mucolytic agents to reduce viscosity has been

considered. The precursor of GSH, N-acetyl-cysteine (NAC) has been found to be rapidly absorbed and acts by delivering cysteine residues for the disruption of the sulfhydryl bridges in mucus{Rushworth, 2014}. NAC has also been demonstrated to elevate sputum penetration by the DNA nanoparticle preparations.{Suk, 2011}

Glutathione antioxidant

A recent trial reported that inhaled GSH significantly improved clinical outcome in CF patients treated with inhaled GSH over 3months but there was no statistical significance compared to the placebo group by the end of 6-month duration.{Griese, 2013} This study was border line with respect to inclusion criteria as high number of the treated group were on existing medication including oral NAC (53%), inhaled fluticasone (40%) and oral ibuprofen (14%) which may have skewed the results.{Griese, 2013} The trial was included due to the difficulty in identifying trials with patients that are not using some form of medicine beforehand and the patients in this trial were already on existing medication but had not started any new concomitant drugs.

The included trial did not report any changes in the inflammatory or oxidative markers in either of the studied groups.{Griese, 2013} One plausible explanation is that the interaction between free sulfhydryl groupand reactive oxidative species (ROS) already present in CF airways contributed to the increased oxidative burden over long-term use.{Galli, 2012}GSH is a major antioxidant present in abundance in the fluid-filled epithelial lining of the lungs; at a concentration of 0.25-0.8mM GSH level is 400 x higher than in plasma.{Klare, 2016}The GSH levels are found to be considerably diminished in CF patients. GSH is known to exert its antioxidant effect by using free sulfhydryl groups to reduce the impact of oxidative stress while providing antioxidant protection of cellular components. Another possibility for no significant change in the markers between the intervention and control group, could be due to the insufficient levels of inhaled GSH to affect *P. aeruginosa* biofilm formation with the free GSH levels only slightly higher in the intervention sputum compared to placebo group.

Despite these findings, a recent in vitro study demonstrated GSH addition alone was sufficient to disrupt the development of P. aeruginosa clinical biofilms although at much higher concentrations than the included trial in this review.{Klare, 2016} Also, GSH was found to improve antibiotic

activity (ciprofloxacin) against *P. aeruginosa* in a dose-dependent manner with complete arrest at maximum (10mM) GSH concentrations. This study findings therefore suggested that GSH activity facilitates increased permeability of the biofilm structure to its external environment.{Klare, 2016} Whether these higher concentrations used for longer duration can be translated to in vivo and clinical settings without toxicity remains to be determined.

N-Acetyl-Cysteine antioxidant

One of the identified clinical trials compared different oral doses of GSH precursor, N-acetyl-cysteine (NAC) in 21 CF patients and found that after 12 weeks there were no changes in the inflammatory markers of induced sputum or in the clinical outcomes between the low and high dose NAC used.{Dauletbaev, 2009}Nevertheless, the study reported increased extracellular GSH in induced sputum in the high dose NAC group. Similarly, a more recent randomised clinical trial of 70 CF patients administered a high dose (900mg tablets 3 times a day for 24 days) of oral NAC did not find any difference in the inflammatory biomarkers tested between the two cohorts. Despite this, the study showed that high dose of oral NAC benefited CF patients by stabilising their lung function over a 6 month intervention period compared to the control group.{Conrad, 2015}The study also reported that high-doses of NAC were well-tolerated and safe for long-term therapy suggesting it may potentially assist as an adjunct to antibiotics in long- term survival. Although the study population consisted of 75% adults, the proportion of adults with measured clinical outcome was not reported.

While the available evidence on GSH and NAC suggests some improvement in the clinical outcome and oxidative stress, however determining the benefits of antioxidants in the company of intensive therapies that CF patients with chronic infection undergo is extremely challenging. Not only are longer-term studies required but also a larger population sample size would allow the role of antioxidant supplementation and involvement in oxidative stress as well as its function as an anti-biofilm mechanism to be determined.

4.4 Limitations & strengths of the study

This systematic review which evaluated all human clinical trials published in the English language during the past ten years on the effectiveness and safety of alternative therapies to antibiotics for biofilm-induced respiratory infections had several limitations. While the review applied an independent and systematic search approach, bias may have been introduced by limiting publications to the last ten years, the study design and the English language search only. There may be other studies published in local journals and languages posing difficulty to access or search. In addition, there may be some unpublished studies with negative outcomes reported.{Ernst, 1997}Most of the trials identified did not have a positive or changed clinical outcome (although they showed microbiological changes) possibly due to the study design, patients being on other medications making the specified effect of the alternative therapy difficult to assess, the study population included adults and children (the assessment however only took into account adult results), the dose of the intervention and duration of the study. The clinical trials were all conducted in Europe and results might have been influenced by population effects linked to environmental, genetic, nutritional or other lifestyle activities. Additional multicentre worldwide randomised well-controlled trials will further clarify the influence of these effects. Finally, the degree of severity of respiratory disease and clinical implications may differ significantly among the study population with different patient characteristics such as baseline nutrition, stress levels as well as other conditions. Notwithstanding these limitations, the strength of this systematic review was that all the identified included studies were high quality, randomised and incorporated double-blinded strategy which enhanced the internal validity of the clinical trials.

In summary, the findings of this review within the limitations of the study indicate that the evidence for the use of alternative therapies to antibiotics as a single therapy is limited due to clinically insufficient reporting of the therapeutic benefits. Furthermore, most of the identified trials were performed on a limited number of participants and larger more robust trials with adequate controls and longer duration are necessary. The trials identified here for respiratory infections were focused on biofilms associated with persistent *P. aeruginosa* infections with the majority reporting a reduction in biofilm pathogens. Thus, alternative strategies aimed at bacterial virulence for the treatment of respiratory disease, specifically recurrent infection, though preliminary appear promising as an adjunct to antibiotics.


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5.1 CONCLUSIONS

This systematic review was performed to evaluate robust evidence from randomised double-blinded clinical trials for alternative therapies to antibiotics in patients with respiratory biofilm-related infections. The number of clinical trials was very limited in this systematic review to draw any definitive conclusions about the effectiveness of any specific non-antibiotic therapy in adults with respiratory infections. Previous systematic reviews that have evaluated individual therapies have found conflicting evidence with some trials showing improved clinical outcome while others did not show any changes. The high quality of current trials although small is promising indicating larger clinical trials with longer durations to assess effectiveness and safety of the alternative therapies are necessary to draw conclusions regarding the benefits of any natural dietary supplementation or other alternative therapeutic agents such as bacteriophages. Both systemic and inhalation delivery of the alternative therapies appears to be equally effective at high doses in reducing biofilm-associated pathogens, although safety needs to be further evaluated in larger adult cohorts with chronic respiratory infections for longer times.

While the identified clinical studies could be divided into two distinct therapeutic domains of anti-virulence and anti-resistance, there is insufficient evidence for any particular alternative strategy as a single treatment for any respiratory infection. Before well-defined recommendations can be made for the treatment of chronic respiratory infections with any antibiofilm alternative therapy, more rigorous studies with larger adult sample sizes, standardised doses and longer intervention durations are needed. This systematic review also highlights the necessity to find effective strategies for translational research of *in vitro* and animal models with positive outcomes to clinical practice. More laboratory research is required to determine the underlying biofilm pathogenesis involved in serious chronic respiratory infections to target specific bacterial mechanisms.

5.2 RECOMMENDATIONS&FUTURE DIRECTION

Over the more recent years, the prognosis for patients with chronic respiratory infections especially CF has improved considerably with the availability of several lines of selective therapies. While research is rapidly expanding, and progressing in the arena of developing novel therapeutic agents, however some prominent challenges remain such as optimisation of clinical outcome measures for this chronically ill population and improving access to such therapies that will be affordable to the at-risk population.

Although antibiotics remain the first line of therapy for respiratory tract infections especially when chronic infections are present, alternative therapies are emerging as a promising adjunct if not stand alone therapies targeting common pathogens involved in biofilm development. Clearly, the rise of antibiotic-resistant pathogens has challenged the effectiveness of antibiotic treatments especially for chronic conditions. Considering the findings of this systematic review that high quality trials, although few, reporting on alternative therapeutic agents is encouraging, further clinical trials with moderate to large size populations using alternative therapies alone or as an adjunct to existing antibiotics therefore should be implemented. It is important to reduce inflammation and biofilm formation in the early stages of the lung disease when the symptoms of the condition are more manageable and chronic bacterial infection avoidable. In future, this requires not only standardised and reliable diagnostic measures but also concerted efforts of multi-centre research on natural therapies that have shown promise in vitro and in animal models rather than an emphasis on development of new antibiotics for which bacterial pathogens eventually develop resistance.

Apart from *P. aeruginosa* other bacterial pathogens in recent years have been linked to higher mortality and decreased lung function. In the U.S as high as a quarter of the CF patients are found to be infected with the Methicillin-resistant *Staph aureus* (MSRA).{Edmondson, 2016}Therefore, in future multiapproach therapy combining multiple antibiotics with natural therapies then becomes even more crucial to eradicate and prevent biofilm-associated respiratory infections caused by these resistant pathogens.

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APPENDIX B- Search Strategies

PubMed initial title and abstract search:		**Scanned**	**Included**
Filters used included Languages (English), clinical trials, human and 10 years			
Biofilm infection		74	
biofilm non-antibiotic		31	
biofilm probiotics		8	
'Non-antibiotic' AND respiratory infection		6	
'biofilm' AND 'N-acetylcysteine'		4	
Glutathione		10	2 Griese, Dauletbaev
N-acetylcysteine AND infections		24	
N-acetylcysteine AND infections		3	1Dauletbaev
'bacteriophage' AND 'infection'		4	1 wright
'respiratory infection' AND 'N-acetylcysteine'	13		
cystic fibrosis pseudomonas aeruginosa		115	3Forrester, Smyth, Hauber
Total Scanned	**292**		
Systematic reviews scanned abstract and title:			
biofilm respiratory infection		16	
respiratory infections alternative therapy	133		
'probiotic' AND 'respiratory'		28	
'biofilm' AND 'N-acetylcysteine'		2	
'bacteriophage' AND 'infection'		4	
Total Scanned	**183**		
Cochrane library searched & scanned articles:			
Biofilm	5		
biofilm respiratory infection		16	
bacterial infections alternative therapy	26		
respiratory infections alternative therapy	12		
'probiotic' AND 'respiratory'		2	
'biofilm' AND 'N-acetylcysteine'		3	
Total Scanned	**64**		
References of articles scanned:	**43**		
FINAL SEARCHED		582	
Final total excluded studies		**29**	

Final total included studies	**6**

APPENDIX C- Detailed Systematic Approach

AC1. Introduction to Systematic reviews

A systematic review is commonly conducted to find answers to a well-defined research question by assembling empirical evidence that corresponds to specified eligibility criteria.{Aslam, 2010 #123;Jahan, 2016 #125} The main steps involved in performing this systematic review are summarised below:{Jahan, 2016 #125}

i) ***Research Question*** is the initial step in conducting a systematic review. In this case the question to be addressed was the effectiveness of antibiofilm therapeutic interventions other than antibiotics in preventing or eradicating the bacterial biofilm pathogens associated with common medical infections. The purpose of this systematic review was primarily to summarise a large amount of literature and evaluate the need for further large clinical trials and or surmise the effectiveness of all or some of the alternative therapeutics used against biofilm pathogens to date.

ii) ***Research Protocol*** was developed once the research question had been articulated. The goal here was to formulate questions and methods before embarking on literature searches. The methodology process of searching, screening, data collection as well as the analysis was documented first (see below). This was to reduce bias before starting the detailed literature search. Since the volume of literature on alternative therapies is quite large, this review was restricted to medical infections and all oral or dental related infections were excluded. In addition, a recent Cochrane review reported on alternative interventions for reducing central venous catheter (CVC)-related infections in adults and hence any studies on CVC infections was omitted.{Lai, 2016 #139}

iii) ***Literature Search*** was the next stage once research protocol had been documented. There are generally several sources to search studies including but not limited to the Cochrane Database of Systematic Reviews (using Cochrane Library), Medline, PubMed, Scopus, references of

primary sources, Foreign language literature, Grey literature (such as theses, non-peer reviewed journals).

For this systematic review the University Electronic Databases, Cochrane Database as well as references from relevant articles were searched. Normally a minimum of two reviewers are required to screen the titles, abstracts and the full text before submitting the selected studies for data extraction. However, as this was an individual assessment only the author of this review conducted the search.

i) ***Data Extraction*** followed once all relevant studies had been confirmed. A standardised form was developed to simplify the process of data extraction (see below for more information). The common items recorded included the author and year of publication, study design, population and demographic characteristics, type of interventions, any controls, outcomes measured, results and any other relevant details.

ii) ***Quality Appraisal*** was the next step to assess for any error or bias in the methodology of the studies. Each clinical study was evaluated for methodological quality to assess the extend the study design minimised errors (bias) using Jadad scoring.

iii) ***Data Analysis & Results*** followed once the included and excluded studies were recorded and quality of included studies appraised. First each study was evaluated and presented in a tabulated format following the SPICO Study design. Population Intervention Comparator and Outcome to ensure main characteristics of the studies are recorded. The PICO method is mainly used for comparison of different interventions. It assists in formulation of a research question linked to diagnosis, therapies or prognosis.{Jahan, 2016 #125}

iv) ***Interpretation of Results*** was the final step in the systematic review. Here the strengths and weaknesses of each included study was discussed in light of previous literature. Recommendations for future studies was made based on the gaps or inconsistencies found from the studies.


MECSJ

AC2. Main Definitions

Antibiotics refers to a substance or drug that kills or prevents growth of bacteria and used to treat infections.

Boolean search methodology Refers to the process by which combination of key words are connected by the NOT, AND, OR and NEAR known as the Boolean operators. These terms assist in limiting the search while increasing the focus on relevant articles. For this systematic review, these Boolean operator terms were utilised combined with key words to narrow the search.

biofilm-associated with respiratory infections will only be included in this review. The reason for this limitation is so that the findings from interventions for common bacterial infections can be generalised to the population at large. Also as there are numerous bacteria and numerous alternative strategies, the focus was narrowed.

Non-antibiotic interventions Any substance other than antibiotics or antibiotic derivative used to target common biofilm-associated infections such as biological agents (bacteriophages) and phytomedicines.{Czaplewski, 2016 #151} For the purpose of this systematic review devices or alternative therapies such as hemoperfusion device, Photodynamic Therapy (PDT) and acupuncture or other physical therapies were excluded since there is a great deal of literature and systematic reviews published already. Also, this review is restricted to alternative therapies that are administered via the oral route or inhalation only as again there is abundant literature on injections or vaccines.


MECSJ