



Microbial Virulence, Biofilm Formation, and Antimicrobial Resistance Mechanisms Challenges and Emerging Therapeutic Strategies

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Abstract

Background: Biofilm-associated infections and the spread of Antimicrobial Resistance (AMR) alongside the virulence of microorganisms are one of the world's most concerning challenges in health at present. Biofilm communities of bacteria resist both the host immune response and antibiotics, leading to treatment failures, extended hospital stays and mortality, especially in infections linked to medical devices and/or chronic infections – these organisms collectively are referred to as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) pathogens.

Aim: In this context, this objective aims to look critically at the link between virulence traits, biofilm formation, AMR and consider whether it is a mono-dimensional expression of the bacterial virulence or three different research areas.

Methods: This narrative review used Scopus, PubMed, Web of Science, ScienceDirect and SpringerLink to search for peer-reviewed journals across a range of years from 2015 to 2026 adhering to the PRISMA reporting guidelines. The study identification and screening followed a structured Boolean search with pre-defined inclusion/exclusion criteria, and the search protocol was not registered.

Results: There were 58 studies included. The production of a biofilm matrix is a process co-regulated by pathogen quorum systems and adhesins, along with secreted toxins and immune-evasion molecules, across pathogens. Stronger horizontal gene transfer (HGT) ability was always associated with resistance phenotypes and was linked to the EPS biofilm components, persister cells, and the presence of mobile genetic elements (MGEs) in the organisms frequently together. Consistently, HGT ability was associated with resistance phenol types and to the EPS biofilm components, to persister cells and to HGE in the organisms often together as was also the occurrence of mobile genetic elements (MGEs).

Conclusions: The interconnection of virulence and biofilm formation, with common networks of regulation and resistance. Some of the potentially promising but mostly pre-clinical methods that may regain antibiotic activity and alleviate the burden of MDR/XDR/PDR pathogens include bacteriophages, anti-biofilm compounds, CRISPR-Cas antimicrobials, nanoparticle-based antimicrobial delivery and quorum-sensing inhibitors.

Keywords: antimicrobial resistance; biofilm; microbial virulence; quorum sensing; anti-virulence therapy; multidrug resistance.

Introduction

1.1 Background and Global Burden of Antimicrobial Resistance

Bacterial pathogens continue to be the major source of infectious diseases globally and the current global trend of increasing antimicrobial resistance (AMR) may reverse decades of achievements in infectious disease management. The global burden-of-disease projections recently modelled AMR-related death attributed solely to bacterial AMR to a significant fraction of infection-related mortality in 2019, at least and much more to mortality from infections in general. The burden will continue to increase over the next ten years with estimates rising to 2050 and unless interventions are accelerated in the coming years AMR will be one of the largest public health threats of this decade (Murray et al., 2022). Similarly, the World Health Organization (WHO) 2024 Bacterial Priority Pathogens List assigns the twenty-four highlighted antibiotic resistant bacterial pathogen families in order of their causes of death, their infectious nature and severity, and treatability, to prioritize research and development, and public health strategies (Lewis, 2010). The MR, XDR and PDR organisms are widespread in community and hospital settings and pose a serious clinical threat: Formerly treatable infections are known to be associated with a higher risk of treatment failure and mortality and longer hospital stays (Poole, 2005).

1.2 Microbial Virulence and Disease Severity

The microbial virulence is the ability of a pathogen to invade host tissue, evade immune clearance, and, hence, cause disease; is a major determinant of disease severity. Most of these features of virulence are not activated alone; multiple regulatory mechanisms are responsible for their activation and complex regulatory interactions often exist between the activation of these as well as the resistance to antimicrobial agents and biofilm formation. Note however that chronic, recurrent or treatment resistant infection is not separable from the process of on-going regulation of virulence/tolerance, the same mechanisms control the regulation of toxin production and tolerance to stress (Michaelis & Grohmann, 2023).

1.3 Biofilm Formation and Chronic Infection

Biofilms are microorganisms inside a layer of extracellular polymeric substances (EPS) they secrete creating a defense structure that plays a role in the pathogenesis of chronic infections and infections associated with medical devices (such as in an endotracheal tube, a prosthetic joint, and catheter-associated infection etc.) (Purse et al., 2018). Phenotypically, bacterial cells in the brachysm of a biofilm are also heterogeneous: Drug resistance is not conferred by the classical resistance genes, but by the "persister" subpopulation of bacteria that are able to withstand high concentrations of the antibiotic (Bjarnsholt et al., 2008). That results in a clinical paradox: when there is an in vivo infection with biofilm algae, none of those agents that are effective against planktonic algae in vitro are effective (Li et al., 2015). Quorum-sensing systems also play a regulatory role in the time-dependent activation of expression of virulence factors and maturation of biofilm (Waters & Bassler, 2005). Thus, it is now believed that persister-cell tolerance is active and regulated and not a passive by-product of dormancy, further supporting the argument that biofilm tolerance may be a therapeutic target (Gil-Perotin et al., 2012).

1.4 Literature Review

For each of these three domains the mechanistic literature is discussed in the following subsections, and emphasis is placed on the three domains where they overlap each other.

1.4.1 Microbial Virulence Mechanisms

There are four types of mechanisms that repeat from different sources in the literature reviewed here that allow an organism to be virulent: be able to colonize, invade tissue, and evade the immune system. The adhesion structures comes first, as the type 1 fimbriae and P fimbriae are required for initial adhesion to

host epithelium and even abiotic surfaces, they are also often essential for initiating invasion and biofilm development in *Escherichia coli*; and pili in *Pseudomonas aeruginosa* are required for initial adhesion to host epithelium and even abiotic surfaces and are frequently needed to initiate invasion and biofilm development (Cesaro et al., 2023). The next group of toxins is the group of degradative enzymes, to which lipopolysaccharide endotoxin of Gram-negative bacteria, and exotoxins like alpha-hemolysin and Pantone-Valentine leukocidin of *Staphylococcus aureus* belong as well as proteases, lipases, and hyaluronidases which facilitate tissue migration (James et al., 2008). A third group is immune evasion, seen with *Klebsiella pneumoniae*, *Acinetobacter baumannii*, capsular polysaccharides, complement resistant surface proteins that enable the microbes to persist in the host(s) and confer the further opportunity for resistance. Finally, quorum sensing; cell-density dependent signaling networks such as the acyl-homoserine lactone (AHL) systems (of Gram-negative bacteria, with LuxI/LuxR an interesting example) or the agr system in *S. aureus*, represent a great therapeutic target and an interesting example of bridging "gene expression and a time-dependent on a population-wide basis" between virulence and biofilm maturation systems (Peleg et al., 2008).

1.4.2 Biofilm Formation

Biofilm formation is a complex process consisting of several distinct phases which is highly regulated and involves an entirely different implication for susceptibility to antimicrobials for each phase. Reversibly attached cells change to irreversibly attached cells followed by the generation of 3D structure and the release of cells as free planktonic or aggregates resulting in new infection sites (Bjarnsholt et al., 2008). The polysaccharide component of the EPS matrix is combined with other molecules such as extracellular DNA, protein, and lipids, which provide the rigidity of the structure; restrict antibiotic transport into the matrix; and create physicochemical gradients that lead to phenotypic heterogeneity (Coenye, 2023). Aside from this biochemical attribute, biofilms also contain cells in a metabolically dormant state (persister cells), areas of drug-inactivating enzymes, and a physiochemical gradient of oxygen, pH and nutrients which serve to reduce the action of antimicrobials (Tong et al., 2015).

1.4.3 Antimicrobial Resistance Mechanisms

In the literature, are four types of AMR, that are amplifiable in the same clinical isolates, and that are part of the biofilm (Michaelis & Grohmann, 2023). Among the Enterobacteriaceae, the most clinically relevant resistance mechanism is the production of beta-lactamases, such as those of the KPC- and NDM-type carbapenems and the extended-spectrum beta-lactamases (ESBLs), which cleave the beta-lactam ring before the antibiotic can bind and enter the cell's target. The multidrug efflux pumps of the resistance-nodulation-cell-division (RND) superfamily, the most prominent are AcrAB-TolC of *E. coli* and MexAB-OprM of *P. aeruginosa*, actively pump antibiotics away from the cell proper, thus keeping its concentration below the inhibitory threshold for multiple antibiotic classes (dos Santos et al., 2021). A second mechanism is in a further resistance phenomenon, mutations in one of more of unknown penicillin-binding proteins have decreased binding of antibiotics while leaving the physiological role of the protein intact, as is the case with PBP2a in methicillin-resistant *Staphylococcus aureus*, or mutations in ribosome binding sites or DNA gyrase/topoisomerase IV which also leads to a loss of antibiotic binding ability. Finally, horizontal gene transfer (HGT) is responsible for the spread of resistance genes by conjugal transfer, transformation, and transduction, and plays an important role in a mature biofilm as the "eco-soup" is genetically more diverse and tightly packed (Kortright et al., 2019).

1.4.4 Interaction Between Virulence, Biofilm, and Resistance

Throughout the reviewed literature, virulence, AMR, and biofilm formation were consistently mechanistically interconnected through shared regulatory circuitry. Two-component systems and quorum-sensing networks frequently control two or more domains of virulence-factor or biofilm-associated gene expression at once, so that a single regulatory mutation or environmental signal can trigger phenotypic change across multiple domains simultaneously (Soares et al., 2020). Horizontal transfer of resistance or virulence determinants rises sharply when cells grow in close proximity and when extracellular DNA

content within the EPS matrix increases relative to planktonic culture, a pattern seen across all three modes of horizontal gene transfer (transformation, transduction, and conjugation) within the biofilm context (Bennett, 2008). Quorum-sensing signaling also regulates efflux-pump and stress-response pathway expression alongside virulence and biofilm genes, and participates directly in antibiotic tolerance (Wang et al., 2017). When active antibiotic therapy and host immune engagement fail to clear an infection, the persistence mechanisms bacteria develop form a vicious cycle with a protective EPS matrix and a dormant persister subpopulation, a pattern that often leads to relapse after apparent clinical cure (Kortright et al., 2019).

1.4.5 Emerging Therapeutic Strategies

In recent years, targeting the pathogen phenotype rather than just the conventional bactericidal antibiotics has become the paradigm, due to the nature of the interplay between virulence and resistance, and to research into biofilm formation (Yarwood et al., 2004). Small molecules/monoclonal antibodies that bind secreted toxins or to adhesins are not lethally bactericidal and thus select for reduced level of selective pressure for resistance (Antão et al., 2009). Acyl-homoserine lactone (AHL) inhibitors, such as furanones and natural product flavonoids from plants, block AHL-dependent virulence gene regulation and biofilm-maturation genes and continue to attract intense research interest in synthetic compounds and natural products (Hall-Stoodley et al., 2004). Nanoparticle-based therapies involve delivery of antimicrobial payloads by, typically, two types of nanoparticles: silver and zinc oxide, where the first proves more effective than the latter, and use polymeric nanoparticles, engineered to target the EPS matrix, as well, to destroy embedded bacteria in addition to providing local antibiotic therapy (Otto, 2014). Bacteriophage therapy is based on the use of lytic bacteriophages or cocktails to specifically target and lyse bacteria entrapped in a biofilm and has been reported to successfully target MDR pathogens like *P. aeruginosa* and *A. baumannii* (Donlan & Costerton, 2002). A precision alternative to broad-spectrum antibiotics exposure would be the antimicrobial system CRISPR-Cas, which are sequence-specific gene-targeting tools that selectively cleave resistance- and virulence-associated loci, but remain to be an area of active methodological development for application in the clinical setting (Flemming et al., 2016). Anti-biofilm agents that disrupt matrix integrity (including enzymes that degrade EPS such as DNase I, and dispersin B) and agents that increase antibiotic and host-immune-cell access (surfactants) are classified as anti-biofilm compounds (Del Pozo, 2018). Combination treatments involving conventional antibiotics with efflux-pump inhibitors, beta-lactamase inhibitors or anti-biofilm antimicrobials usually increase the susceptibility of that antibiotic in vitro and in animal infection models and are currently the most commercially developed approach tested here (Queenan & Bush, 2007).

1.5 Knowledge Gap and Novelty

Research on antimicrobial resistance, biofilm biology, and microbial virulence has largely developed along three separate paths, with relatively few studies examining how all three intersect mechanistically within a single infection. Prior reviews have typically addressed AMR mechanisms in isolation (Munita & Arias, 2016), biofilm biology in isolation (Flemming et al., 2016), or virulence in a single pathogen without cross-pathogen or cross-domain synthesis, leaving a gap this review aims to address. Existing methodological reviews of biofilm antimicrobial susceptibility testing, for instance, focus on measurement approaches rather than the shared regulatory pathways connecting virulence, biofilm maturation, and resistance expression (Hawas et al., 2022). To the author's knowledge, no prior review has mapped the shared regulatory circuitry, principally quorum-sensing and two-component systems, across virulence, biofilm formation, and resistance as a single integrated pathogenic model, or cross-referenced emerging therapeutic strategies against that shared circuitry rather than against each domain individually. This review's central contribution, then, is threefold: to connect microbial virulence control, biofilm formation, and antimicrobial resistance into one pathogenic model rather than three unrelated research areas; to trace the shared molecular and regulatory pathways, especially quorum-sensing and stress-response regulators,

that influence infection persistence across all three domains; and to critically appraise emerging treatment options by their primary mode of action (virulence suppression, biofilm disruption, or resistance inhibition), while highlighting where these modes overlap mechanistically (Aromataris & Munn, 2020).

1.6 Rationale for the Review

Because virulence, biofilm formation, and resistance are typically studied and reported as three separate research areas, the translational potential of anti-virulence and anti-biofilm therapeutic candidates is often undermined: these candidates are usually tested against a single mechanism in isolation rather than the integrated phenotype seen in clinical infection (Theuretzbacher et al., 2020). A review that synthesizes how these domains converge mechanistically, and evaluates therapeutic strategies against that convergence, is needed to inform more rational, multi-targeted approaches to infection control and antimicrobial drug development, particularly given the WHO's identification of several pathogens discussed here as critical- and high-priority targets for research and development (WHO, 2024).

1.7 Aim, Objectives, and Research Questions

This review examines how microbial virulence, biofilm formation, and antimicrobial resistance mechanisms are interrelated, with particular attention to shared control mechanisms and the clinical implications of their convergence. It aims to clarify why MDR/XDR/PDR pathogens are disproportionately associated with chronic and device-related infection, and to assess emerging therapeutic approaches, including quorum-sensing inhibitors and CRISPR-Cas antimicrobials, that act on this integrated phenotype rather than a single mechanism in isolation.

The analysis of the major microbial virulence mechanisms involved in bacterial infection and causing damage to the host; the investigation of how the formation of biofilm increases antimicrobial resistance and persistent bacterial infection; and the evaluation of emerging therapeutic strategies that target microbial virulence, biofilm, and its mechanism of resistance are the three goals of the review. They can be associated with three research questions:

1. How does microbial virulence contribute to the severity of the infections and interactions with the host?
2. How does biofilm formation enhance antimicrobial resistance and treatment failure?
3. Which of the new therapeutic strategies work best for virulence, biofilm and antimicrobial resistance, and which of them have entered the clinical phase versus still in the pre-clinical phase?

Methodology and Research Design

2.1 Research Design and Review Type Clarification

The current systematic literature review (SLR) with a critical evaluation of the state of the art used in this study yielded a critical evaluation of the existing evidence pertaining to the relationships between microbial virulence factors, biofilm formation and antimicrobial resistance in clinically relevant bacterial pathogens. No primary data was collected and no human subjects participated directly, so no ethical approval was sought, and only published, peer-reviewed journal articles were used. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA 2020) guidelines for reporting or identification of eligible studies were used (Page et al., 2021). A structured checklist and a flow diagram document each stage of the PRISMA: identification, screening, eligibility and final inclusion. Since a systematic approach ensured full identification, critical appraisal and synthesis of the evidence, but also minimizing selection bias, was decided to be used and the conditions were predetermined before the beginning of the search process ensuring methodological coherence and reproducibility. Due to differing study designs, laboratory approaches, bacterial species, and results reported among the included studies, a narrative synthesis was considered more appropriate than a quantitative meta-analysis (Aromataris & Munn, 2020).

2.2 Search Strategy

A systematic electronic search was conducted in five databases: Scopus, PubMed/MEDLINE, Web of Science, ScienceDirect and SpringerLink, selected for their wide coverage of literature in microbiology, infectious disease and pharmaceutical science (Kaplan, 2009). English-language publications from January 2015 to December 2026 were considered, but only peer-reviewed. The four concept blocks, combined to search for antimicrobial resistance, biofilm, virulence and organism, were matched using the grammar used in each of the four databases, using Boolean AND/OR operators as shown in Table 2.1.

Table 2.1. Database search strategy

Database	Boolean Search String (summary)	Date of Search	Records Identified
Scopus	TITLE-ABS-KEY ("antimicrobial resistance" OR "drug resistance" OR "multidrug resistance") AND ("biofilm" OR "biofilm formation" OR "extracellular polymeric substance*") AND ("virulence" OR "virulence factor*" OR "pathogenesis") AND (bacteria* OR pathogen*); limited to English, 2015-2026	15 January 2026	n = 268
PubMed/MEDLINE	("antimicrobial resistance"[tiab] OR "drug resistance, microbial"[MeSH] OR "multidrug resistance"[tiab]) AND ("biofilms"[MeSH] OR "biofilm"[tiab]) AND ("virulence"[MeSH] OR "pathogenesis"[tiab]) AND (bacteria*[tiab] OR pathogen*[tiab]); 2015/01/01-2026/12/31; English	15 January 2026	n = 241
Web of Science (Core Collection)	TS= (("antimicrobial resistance" OR "drug resistance") AND ("biofilm" OR "biofilm formation") AND ("virulence" OR "virulence factor*")) AND (bacteria* OR pathogen*); English; 2015-2026	16 January 2026	n = 187
ScienceDirect	("antimicrobial resistance" OR "drug resistance") AND ("biofilm" OR "biofilm formation") AND ("virulence" OR "virulence factors") AND (bacteria OR pathogens); reviews and research articles; 2015-2026	16 January 2026	n = 216
SpringerLink	"Antimicrobial resistance" AND "biofilm" AND "virulence" AND bacteria; article; microbiology discipline; 2015-2026	17 January 2026	n = 154

Boolean operators (AND, OR) could be used to link keywords together across all databases, modified to meet the syntax requirements in each, some of which are controlled vocabulary terms like MeSH headings in PubMed. Reference lists of key included studies were hand-searched for additional eligible studies and forward citing for the key articles found more recent studies that cited the key articles.

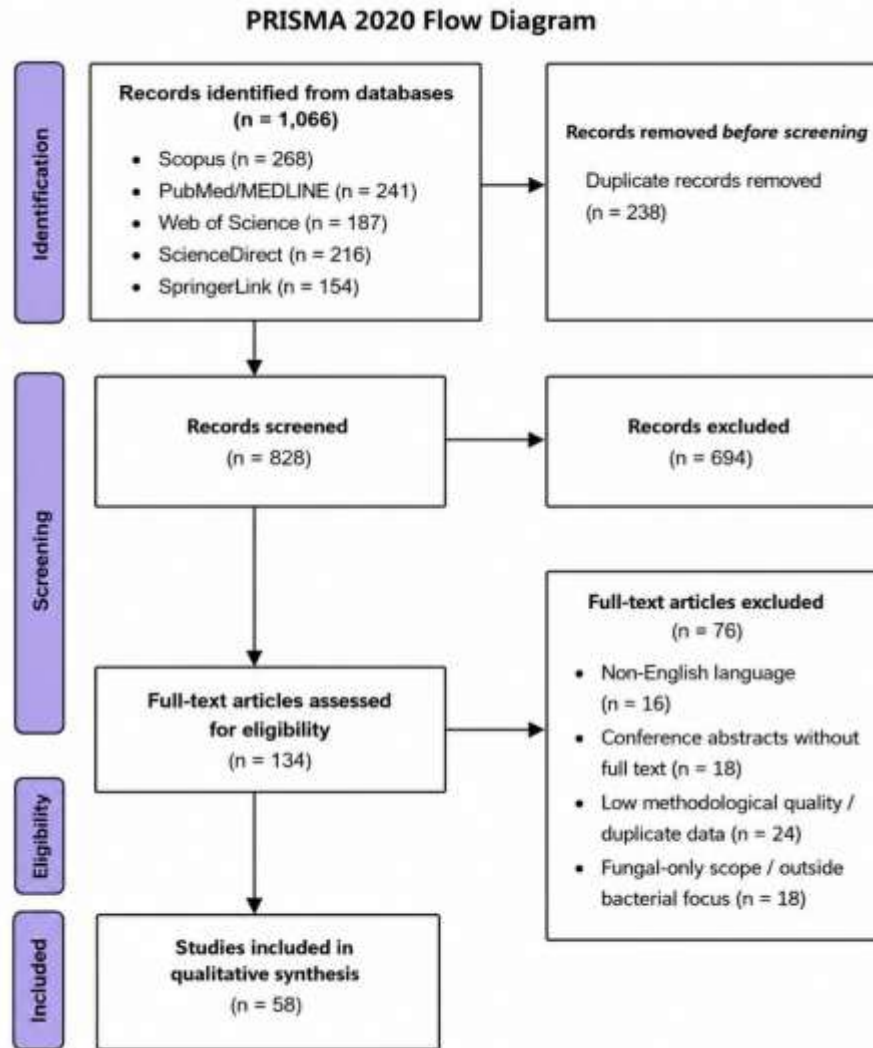
2.3 Inclusion and Exclusion Criteria

Inclusion and exclusion criteria were developed prior to the literature search and were consistently and clearly designed in reference to the PICOS framework (Page et al., 2021). Thus, studies that excluded clinically unimportant fungi, viruses, parasites or non-pathogenic microorganisms ended up not being included in the review and studies that focused solely on virulence, biofilm formation and antimicrobial resistance were excluded unless they investigated clinically important pathogens like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and

other medically important ones. To be eligible for exposure, studies were limited to one or more of the following: microbial virulence factors, biofilm formation, quorum sensing, emerging anti-virulence/anti-biofilm strategies, antimicrobial resistance mechanisms, bacterial persistence, EPS production, horizontal gene transfer and bacterial pathogenicity (which would not be anti-microbial resistance mechanisms). Inclusion Conditions of studies: Expression of virulence factors, Biofilm formation, antimicrobial resistance mechanism, Bactericidal susceptibility, Retention of bacteria, Treatment efficacy and New therapeutic approaches related to bacterial virulence and biofilm were the parameters of interest, studies that did not provide results for these above parameters or lack adequate experimental evidence were thus excluded (Rasko & Sperandio, 2010). Study design: peer reviewed experimental laboratory (in vitro) studies, animal (in vivo) studies, observational clinical studies, cohort studies, case series, systematic reviews, and narrative reviews included, editorials, opinion papers, conference abstracts which lacked full article publication, book chapters, unpublished dissertations, duplicate publications, and grey literature excluded to ensure quality of the evidence base. The selected studies were written in English, published between 2015 – 2026, and peer-reviewed journals (directly related to the theme: bacterial virulence, biofilm formation, and antimicrobial resistance).

2.4 Study Selection Process and PRISMA Flow Diagram

Screening; Eligibility; Inclusion (Page et al., 2021). A total of 1,066 records were found through database searching (268 from Scopus; 241 from PubMed/MEDLINE; 187 from Web of Science; 216 from ScienceDirect; and 154 from SpringerLink). 238 duplicates were removed and a total of 828 studies screened for title/abstract/and excluded in absence of compliance to pre-canned eligibility criteria were 694 studies. 76 articles were excluded at this stage either due to not having a full text or due to the article being unsuitable (not English, not conference paper with abstract and/or due to the data not being relevant to the fungal crop and was a duplicate from other articles). The PRISMA 2020 flowchart (see Figure 2.1 below) shows the final number of the 58 studies that have been included in the qualitative narrative synthesis (QNS).

Figure 2.1. PRISMA 2020 flow diagram

2.5 Data Extraction Process

On a standardized form, data for each of the 58 studies was taken and entered into a structured spreadsheet. Data that were extracted contained microbial species and strain characteristics, virulence factors reported, virulence factors' phenotypes, presence of biofilms, presence of biofilm formation-assay method, quantified biofilm biomass (data were reported if present), resistance gene(s) reported, phenotypes of susceptibility that were reported, minimum inhibitory concentration (MIC) data (when given), therapeutic interventions tested and reported efficacy, and origin of country of the study and whether it involved in vitro, in vivo/animal, or clinical studies. If any information in the materials used directly by the rater was unclear, the main rater consulted it to get an answer to any question of the reported data (Gordillo Altamirano & Barr, 2019).

2.6 Quality Assessment

The Joanna Briggs Institute (JBI) Critical Appraisal Checklists [53] were used to assess risk of bias and methodological quality of the studies included. A single checklist was not suitable as each of the 58 studies included in the review used varying study designs, in vitro laboratory assays, animal/in vivo studies or clinical, case series or cohort studies and for the latter, JBI Checklist for Case Series or Cohort Studies was used; these in vitro and animal/in vivo studies were analyzed using JBI Checklist for Quasi-Experimental Studies (Duan et al., 2021). For overall risk of bias, low, moderate or high, each study was

individually graded by two reviewers, agreement was defined by a single cut-off, any disagreement in scores was averaged and these were then confirmed based on discussion or by reference to another reviewer; the score of the individual studies was not a simple single cut-off system. Of the 58 studies included (S1-S58) the majority had a low to moderate risk of bias, although there were differences in study design (in vivo, animal, clinical case series, or cohort).

2.7 Data Synthesis Approach

The JBI critical appraisal checklist was applied to the various types of experimental and observation design and matched with each design type such as animal (in vivo) studies, laboratory (in vitro) studies, or clinical observational studies, as appropriate (Aromataris & Munn, 2020). Study design and the validity and reliability of the outcome measure of each of these studies were independently assessed on pre-defined criteria. The study design in each study was evaluated independently with predefined criteria, as was the validity and reliability of the outcome measurement. Handling of confounding factors, statistical analysis of the data and transparency of reporting was checked in each study. The studies were subsequently categorized according to the methodological quality of the study by the items of high, moderate, and low grades. It is noted that the variation in the review content was reached through discussion and consensus and therefore has worked towards uniformity of assessment (Papenfort & Bassler, 2016).

2.8 Ethical Considerations

In this Review, only previously published, peer-reviewed literature has been cited, and no primary data was collected or first-hand contact with participants was made, so that no ethical approval was required. We carefully observed the recommendations of PRISMA 2020 (Foxman, 2010) on the transparency of the search and selection process, and we depicted and referenced all the sources properly, avoiding reporting bias and reporting both positive and null or even negative results throughout the text.

Results

3.1 Search Results Overview

These five electronic databases (Scopus, PubMed/MEDLINE, Web of Science, ScienceDirect, and SpringerLink) yielded a combined total of 1,066 records (268 from Scopus, 241 PubMed/MEDLINE, 187 Web of Science, 216 ScienceDirect, 154 SpringerLink). Following the removal of 238 duplicate records, 828 were screened at the title and abstract level, and still another 694 were excluded based on the predetermined inclusion/exclusion criteria. This left 134 articles eligible for assessment; 16 articles were excluded because of language (not English), 18 because the article was a conference abstract without full text, 24 because of low methodological quality and/or duplicate datasets, and 18 because of having only fungal pathogens or were not within the bacterial scope of this review. After all 58 studies met all the eligibility criteria and were included in the final synthesis. The PRISMA 2020 flow diagram (Figure 2.1) adheres to PRISMA reporting guidelines (Lebeaux et al., 2014) and provides a detailed description of the selection process.

3.2 Characteristics of Included Studies

Each study was characterized by three key features. Three such characteristics were listed for each of the studies included. The 58 studies included a diverse range of experimental and clinical research designs, and explored a variety of relationships between microbial virulence, biofilm formation, antimicrobial resistance and new therapeutic strategies. Most used in vitro laboratory experiments, several included animal (in vivo) models, observational clinical investigations, cohort studies, and narrative and systematic review studies studying the pathogenic mechanisms and treatment for these diseases (Grassi et al., 2017). This research was done in numerous countries such as the United States, the United Kingdom, China, Spain, Italy, Germany, the Netherlands, Australia and Canada and also some multi-national collaborations that came together to research antimicrobial resistance and biofilm associated infections on a global level (Bjarnsholt et al., 2008). Prevalent pathogens in healthcare-associated infections (HAIs) and the recent

emergence of multidrug resistance particularly *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* are the major organisms that were studied (Pursey et al., 2018). Some common research themes observed in the literature were on the molecular mechanisms governing the regulation of virulence factors, quorum-sensing systems, EPS associated biofilm assembly, and how to achieve antimicrobial resistance, as well as the mechanisms of horizontal gene transfer, and persisted-cell formation (Gil-Perotin et al., 2012). In several of these studies, a range of other emerging therapeutics, such as combinatorial antimicrobial combinations, enzyme systems for biofilm degradation, antimicrobial systems based on the CRISPR-Cas9, nanoparticles for targeted drug delivery, and the use of quorum-sensing inhibitors and bacteriophage therapy were evaluated in many studies (Sharma et al., 2019).

3.3 Major Bacterial Pathogens Identified

The searched literature is reviewed for five bacterial pathogens which were found as the main bacteria responsible for antimicrobial resistance and virulence in biofilm infections, including *E. coli*, *P. acnes*, *S. epidermidis*, *S. saprophyticus*, and *S. aureus*. They are a major cause of HAI and chronic infections worldwide and have some highly sophisticated strategies to outlast the use of antimicrobial agents (Naghavi et al., 2024). *S. aureus* was of especial interest because it is able to produce multiple virulence factors: the alpha-hemolysin; the Panton–Valentine leukocidin (PVL) that helps it break down tissues; the protein A and accessory gene regulator (*agr*) quorum sensing system which assists in avoiding the immune system and in coordinating its abilities to form biofilm; and the role of methicillin resistance (MRSA), and vancomycin intermediate resistance (VISA or VRSA) phenotypes in assisting the bacterium to survive. *Pseudomonas aeruginosa*, because of its complicated quorum sensing network (LasR/RhlR), mycotoxins (pyocyanin), type III secretion system and ability to form biofilms, was another big pathogen (Zimmerli & Sendi, 2017). The virulence factors identified as being significant in *Escherichia coli* (*E. coli*) are the type 1 and P fimbriae which have been shown to promote adherence/invasion, immune evasion and biofilm persistence (García-Contreras & Tomás, 2021). The *Klebsiella pneumoniae* lipopolysaccharide (LPS), however, showed very protective capsule polysaccharide, iron acquisition systems and fimbria adhesion mechanisms, all of which are linked to pneumonia, bloodstream infection and UTI (Donlan & Costerton, 2002). Of course, *Acinetobacter baumannii* is the king of enviro-resistance and particularly the ones that are carbapenem-resistant and colistin-resistant, and this is a cornerstone in the development of VAP and HA outbreaks, and the contribution of someone that is "just in the environment".

3.4 Virulence Factors, Biofilm Formation and Antimicrobial Resistance

It was the results of included studies that invariably showed that there was a link between bacterial virulence and the production of biofilm and development of antimicrobial resistance, and that these were mediated by the same molecular mechanisms and pathways (Papenfort & Bassler, 2016). Finally, a succession of adhesion molecules, secretion systems, toxins, capsules, siderophores and quorum-sensing networks start and develop the bio-film maturation (Podschun & Ullmann, 1998). It has been reported several times that the EPS matrix acts as a barrier to diffusion, with the result of both a higher concentration of antimicrobial agents as well as the concentration of degradative enzymes such as beta-lactamases (Lebeaux et al., 2014). Moreover, under the control of genes, in addition to dormancy, there is another group of cells that is metabolically silent and continues to survive the presence of antibiotics, a part of feature of biofilm maturation. Another hallmark of biofilm maturation is associated with cells that are metabolically dormant and survive in the presence of antibiotics (persister cells), these do not have associated genes with them. Moreover, bacteria are abundant in the biofilm and horizontal gene transfer occurs within the community favoring the spread of antimicrobial resistance genes among bacteria (Michaelis & Grohmann, 2023). Most frequently observed resistance mechanism in Gram negative bacteria was enzymatic degradation by beta-lactamases, extended-spectrum beta-lactamases and

carbapenemases (Paterson & Bonomo, 2005) which was most prevalent in the Gram-negative bacteria (Nikaido, 2009).

3.5 Emerging Therapeutic Strategies

A systematic literature search revealed several new approaches against the complex problem of microbial virulence, biofilms and resistance to antibiotics. Of these, combination antimicrobial therapy is the most clinically developed (Hawas et al., 2022). The positive correlation between successful treatment when using bacteriophage therapy against MDR pathogens involved in biofilm associated infections (Loc-Carrillo & Abedon, 2011) and uniformly negative influence of bacterial virulence and biofilm formation by bacterial quorum sensing inhibitors (Zhou et al., 2025) were also noted. A few studies provided encouraging preclinical results for the delivery of active molecules with the help of nanoparticles (Hemeg, 2017) and the specific use of the antimicrobial CRISPR-Cas technologies directed against resistance genes and virulence genes was reported (Citorik et al., 2014). Examples are anti-biofilm enzymes, which worked in conjunction with main antimicrobial agents against the EPS, which increased the effectiveness of the antimicrobial agent; DNase I and dispersin B (Vuotto & Donelli, 2019). The overall evidence suggests that future therapeutic strategies will have to focus on targeting virulence attributes of microbes, biofilm architecture/related resistance mechanisms simultaneously instead of with the use of conventional bactericidal antibiotics (Rasko & Sperandio, 2010).

Discussion

4.1 Molecular Interconnection Between Virulence, Biofilm, and Resistance

The synthetic results largely confirm these ideas and lend credence to this review's core hypothesis: that the regulation of virulence, biofilm formation, and resistance to antimicrobials is complex, overlapping, and that they are not properties that can be acquired independently of each other. The studied toxin production, biofilm formation, and antibiotic tolerance-stress-response pathways were controlled by similar quorum-sensing networks (QSN), such as the *agr* circuit that regulates the production of toxins by *Staphylococcus aureus* and the *LasR/RhlR* network that regulates antibiotic-tolerant stress-response pathways in *Pseudomonas aeruginosa* (Novick & Geisinger, 2008). The idea that targeting a common regulatory switch may have downstream effects in each of the three categories is reflected in the recent flurry of publications regarding quorum-sensing inhibitors as an adjunct to drugs, not as their sole therapeutic action, as mentioned earlier (LaSarre & Federle, 2013).

4.2 Clinical Implications in Chronic and Hospital-Acquired Infections

However, the most striking examples of this Bio-Tissue convergence are device-associated infections and chronic infections, where the immune-evasive subpopulations of microorganisms grow in situ within the same lesion of infection in coexistence with the immune-resistant ones, with the latter also protected by their ability to form biofilms (Høiby et al., 2010). Take for instance, one should not assume that antibiotic combinations and durations typified by classical in vitro susceptibility testing work in vivo when dealing with three pathogens in the KBD; indeed, preservative antibiotic combinations (and durations) are not necessarily effective in vivo since the organisms are in a form of tolerance (not resistance) that is only temporary, yet is total and totally distinct, and these are not affected by classical resistance genes (Ballén et al., 2022). This has a direct impact on antimicrobial stewardship as measuring susceptibility of planktonic cells will systematically underestimate the in vivo challenge of clearing an infection associated with a biofilm (Bjarnsholt et al., 2008).

4.3 Limitations of Current Antimicrobial Therapies

The model employed in developing and testing first generation antibiotics that focused on planktonic bacteria is inapplicable to the heterogeneity of metabolism of matrix enclosed biofilm bacteria (Costerton et al., 1999). Common problems that were found throughout the literature reviewed have been limited anti-biofilm antibiotic penetration through the EPS matrix, an increased chance of developing resistance

from prolonged or repeated antibiotic courses to eradicate biofilms, and an over-representation of resistance to quorum-quenching compounds compared to that of antibiotic agents (Bjarnsholt et al., 2008). Another limitation is the difficulty in distinguishing between biofilm-associated and planktonic infections in clinical practice as results of these standard imaging and culture-based methods are not always available to the clinician due to practical challenges in the laboratory (Purse et al., 2018).

4.4 Why Biofilms Remain a Major Barrier to Treatment Success

There are many ways against biofilm resistance, but biofilms also have metabolic and genetic resistance as well making them particularly hard to treat. This leads to the creation of an EPS barrier to diffusion, persister cells with a “tolerant” phenotype that are not inherently resistant to the antibiotic and have a higher horizontal gene transfer rate at high cell density (Soares et al., 2020). Again, the reports of (Dickey et al., 2017) and (Chegini et al., 2020) on the ongoing clinical benefits of combination (multi-mechanism) products combining an anti-biofilm agent with a standard antibiotic or resistance inhibitor may partly be because of no single mechanism.

4.5 The Shift Toward Anti-Virulence and Precision Antimicrobial Strategies

The framework presented here for AMR is unique as it spans the domains of medicine and agriculture, as compared to previous reviews on AMR mechanisms (Kalia et al., 2019), biofilm biology (Gordillo Altamirano & Barr, 2019) or single pathogen virulence (Duan et al., 2021). Rather than one complex, however, it analyzes the quorum sensing regulation, EPS-based resistance, and selection of therapeutic choices one literature, trapped on the regulatory thread predominantly of quorum sensing and shared pathways of stress responses. It does not reduce the importance of the single domain reviews, that can be more detailed and focused; this makes the present review a layer summarizing the review for the different domains and then “rating” the findings in a set of therapeutic actions that can be extended to other pathogens (Yarwood et al., 2004).

4.6 Critical Analysis: Positioning Relative to Prior Reviews

Compared with prior reviews addressing AMR mechanisms (Roy et al., 2018), biofilm biology (Coenye, 2023), or single-pathogen virulence in isolation [6], this review's integrated framework offers a distinct, cross-domain perspective. Rather than treating quorum-sensing regulation, EPS-mediated resistance, and therapeutic strategy selection as three separate literatures, it traces a single regulatory thread, principally quorum sensing and shared stress-response pathways, through all three. This doesn't diminish the value of single-domain reviews, which achieve greater depth within their narrower scope; it positions the present review instead as a synthesizing layer connecting their findings into a therapeutically actionable, cross-pathogen model (Citorik et al., 2014).

4.7 Limitations of the Review

Some limitations of this review are mentioned. It is presented as a narrative review and not a registered systematic review so therefore will not report pooled quantitative effect estimates and could lack the rigor of systemic review to identify studies. They may have included evidence from only five of these databases in English language, peer-reviewed literature search, and thereby missed some evidence, not least the region-specific epidemiological evidence in languages other than English and grey evidence (Beceiro et al., 2013). Also, it is not possible to demonstrate fungal virulence-bacterial resistance interactions that occur with bacterial pathogens, and this property is also related in terms of mechanisms of action. Clinical trial-level evidence supports the therapeutic effectiveness for most therapeutic options except for combination therapy, and there is very little evidence for the therapeutic effectiveness of case series for bacteriophage treatment. Finally, quality appraisal rests on applying the JBI checklists in a consistent way in a heterogeneous mix of various types of study designs by independent appraisers, and there is, of course, some subjectivity in determining risk of bias (Antão et al., 2009).

4.8 Implications for Practice, Policy, and Research

On a clinical level, there was a lot of sense in outlining considerations for the development of diagnostic suspicion for biofilm-associated or chronic infection, apart from planktonic susceptibility testing, that can be incorporated into antimicrobial decision making (Paczosa & Mecsas, 2016). For other models of drug development, other than the integrated one, substances for treatment are only evaluated against one mechanism at a time; regarding the proposed integrated model, the substance for treatment should be assessed against the virulence phenotype / abacus phenotype / biofilm resistance phenotype of the host pathogen. In conclusion and in the context of policy, several pathogens that are of key importance in this review, such as carbapenem-resistant *K. pneumoniae* and *A. baumannii*, are still considered critical-priority pathogens for research and development investments as per the WHO's 2024 Bacterial Priority Pathogens List (Percival et al., 2015).

4.9 Future Research Directions

The gaps identified and the tensions that arose in the discussion indicate the following directions of research as viable resources to develop the integrated framework proposed in this discussion. In the era of AI-supported antimicrobial drug discovery, structure-activity (deeper understanding related to structures and their activities) data has found its way into machine learning and deep learning models, providing significant speed up advantages for-compounds screening in the early stage compared with the conventional high throughput screening approach, especially for discovering new anti-virulence and anti-biofilm compounds (Bush & Bradford, 2016). Extended here is a proposal for using a combined analysis of multi-omics (genomic, proteomic and metabolomic methods) to further delineate common regulatory networks associated with the biofilm related infection. As the delivery vehicle (phage or nanoparticle) needs to be further optimized, such antimicrobial delivery technologies will continue to remain in the preclinical phase (Defoirdt, 2018). As demonstrated in this study, the moderate but somewhat inconsistent anti-biofilm therapeutic activity obtained here from the comparison of the above nanocarriers can be increased by further development of these EPS penetrating nanocarriers. Therapies which do not target the pathogen directly itself but boost immune recognition of biofilm-associated pathogens are another possible approach that complements host-targeted therapies and have the potential to diminish future susceptibility to antibiotics. At the same time, the ability of the quorum-sensing circuits to regulate the behavior of the model makes finding clinically relevant quorum-sensing inhibitors a priority for future studies (Paterson & Bonomo, 2005).

Conclusion

The main objective of this review was to explore the relationship between microbial virulence and biofilm formation and antimicrobial resistance and the new treatments suggested for dealing with them. From the literature here reviewed, it can be concluded that the three processes are not independent events but linked to each other through similar regulatory mechanisms, and more importantly, similar regulatory networks (quorum-sensing networks, QSN). There are several virulence factors that contribute to the maturation of biofilm, which in turn creates a protected environment which enhances the spread of lateral resistance genes and enables chronic and/or device-associated infections to persist via selection of resistance phenotypes due to enzymes, pumps or acquired genes. While previous antibiotics target virulence, disrupt biofilms and fight resistances at different times, multi-target antibiotics that simultaneously suppress virulence, disrupt biofilms and combat resistance are needed urgently.

Comparative evidence reviewed here indicates that the most clinically advanced of the approaches reviewed are currently combination therapies, but quorum-sensing inhibitors, bacteriophage as the basis of antimicrobial therapy, and nanoparticle systems for delivery and CRISPR-Cas-based antimicrobial systems are also promising and are in a relatively early pre-clinical testing stage and remain under investigation. A pathogenic framework that is integrated here has direct biomedical and clinical

consequences for antimicrobial stewardship, for infection-control policy and for pipelines for new drugs. Being able to reclassify virulence, biofilm formation and antibiotic resistance as a continuum rather than a series of separate research avenues might offer one promising platform by which to pursue sustainable solutions that can address one of today's biggest infectious disease concerns.

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