ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

Bacteriophage-Antibiotic Synergy for ESKAPE Pathogens: Molecular Mechanisms, Therapeutic Applications and Clinical Translation Challenges

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Publication Date: 2026/06/30

Abstract: ***Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.*** **(collectively the ESKAPE pathogens) cause the majority of healthcare-associated infections worldwide and are the cause of most multidrug-resistant (MDR) bacterial deaths. Phage-antibiotic combination therapy takes advantage of phage-antibiotic synergy (PAS), a mechanistically interesting concept, to overcome drug resistance in such organisms. Methods and objectives: Here we describe the molecular mechanisms that constitute PAS, which include exploitation of efflux pumps, cell filamentation using the SOS response, alteration of the bacterial outer membrane to improve permeability, dissolution of biofilms, and selection of bacteriophage-resistant mutants which are paradoxically again sensitive to antibiotics due to associated fitness cost trade-offs. Data for PAS with ESKAPE pathogens, as determined in checkerboard assays, time kill experiments or in vivo model studies, will be presented and discussed in view of standard laboratory approaches and the few clinical case series reported. PAS represents a convergent therapeutic principle in which the actions of phages and antibiotics are synergistic by acting in an interdependent way on bacteria. However, the lack of a harmonized methodology to determine the PAS effect, immature regulations and an insufficient database on the clinical application of phage-antibiotic combinations, particularly with respect to rational and optimal sequencing and dosing in practice, still hamper its clinical development. The European Pharmacopoeia's agreement on standard quality methods for bacteriophages for medicinal use in the coming year, and the EMA Guideline on Phage therapy (expected 2025), show considerable progress in reducing regulatory barriers, but key data are still needed to establish PAS as a reliable and robust therapy.**

Keywords: *Phage-Antibiotic Synergy, ESKAPE Pathogens, Bacteriophage Therapy, Antimicrobial Resistance, Efflux Pump, Biofilm, SOS Response, Clinical Translation, Regulatory Framework.*

How to Cite: Siddharth Singh (2026) Bacteriophage-Antibiotic Synergy for ESKAPE Pathogens: Molecular Mechanisms, Therapeutic Applications and Clinical Translation Challenges. *International Journal of Innovative Science and Research Technology*, 11(6), 1723-1735. https://doi.org/10.38124/ijisrt/26jun1484

I. INTRODUCTION

Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp. – often referred to as the ESKAPE pathogens because of this acronym – comprise a group of the most important clinical bacterial pathogens in terms of incidence and impact in healthcare facilities. These organisms were first so named by Rice in 2008 due to these pathogens, which is a cause for major concern because of their broad multidrug resistance profile and their extensive ability to inhabit and proliferate in hospitals [1]. These six pathogens account for a disproportionately large percentage of healthcare-associated infections and also have extraordinary capabilities for resistance mechanisms that circumvents the efficacy of antibiotic therapeutics. The reason that these pathogens have come to be of such global importance as clinical challenges relates to the combination of innate (natural) resistances that these pathogens have evolved as well as acquired and adaptive resistance traits.

Some of the innate resistances includes a less penetrant outer membrane (that limits the concentration of antibiotics within the bacterial cells, also aided by pumps which remove antibiotics from within the bacterial cell – referred to as multi-drug efflux pumps and a poor cell wallpermeabilitywhich includes native -lactamaseenzymes which contribute to antibiotic resistance), others of these are acquired resistances which further support the bacteria's ability to persist in a setting with antibiotic pressures that include the production of carbapenemases, extended-spectrum-lactamases(ESBLs) and Aminoglycoside–modifying enzymes as well as changes in the targets to which

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

these antibiotics will bind [2-5]. In addition, the virulence characteristics possessed by ESKAPE pathogens are very likely instrumental in the colonization, persistence and disease caused by ESKAPE species – this include their ability to form biofilms on inanimate object, such as hospital devices as well as in host tissue; biofilms are protected from penetratingantibioticsand also help to hide the bacteria from host innate immune response [6]. AMR continue to be a growing and increasing problem throughout the world. There are approximately 4.71 million lives affected globally by bacterial AMR each year and 1.14 million deaths are associated with drug-resistant bacterial infections as a direct result (Global Burden of Disease)[7]. The biggest clinical contributors to the burden of AMR include, *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa* (Global Burden of Disease)[7].

The high level of clinical concern surrounding resistance development and its impact can be demonstrated by the identification of these as WHO "high-priority" pathogenic bacteria requiring innovative treatments [8]. It is against this backdrop of increasing global antibiotic resistance that there has been a resurgence in the use of the bacteriophage (phage) therapies, both as alternatives or as complements to existing treatment regimens of antibiotic usage. Bacteriophages, which are viruses that exclusively target bacteria, had previously been a topic of considerable interest prior to the age of antibiotics but is regaining interest as potential tools to combat antibiotic resistant microorganisms [9,10].

It is established by multiple decades of in vivo and in vitro clinical and experimental evidence that usingphages in combination with antibiotics enhances efficacy as opposed to utilizing only one of the therapeutic agents [11]. This enhancement effect is known as phage-antibiotic synergy (PAS) was formally described by Comeau et al. Who showed that the administration of sublethal concentrations of some antibiotics could inducephage production as well as bacterial lysis [12]. Subsequently research revealed thatPAS is more than additive; it represents complex biologic phenomena that impact multiple levels of the bacteria and phage infection processes including antibiotic effects on bacterial morphology and biofilm production, changes in the phage binding and adsorption steps, modification of the bacteria stress response, and development of evolved strategies of bacteria which increase their susceptibility to antibiotic treatment when challenged with phage infection [11-13]. In order to rationally design efficient and effective phage-antibiotic combinations targeting multidrug-resistant ESKAPE pathogens it is necessary to thoroughly understand the mechanisms of PAS.

Thus, the aim of this review is to describe mechanisms by which theseESKAPEpathogens resistantibiotics and explain some of the major pathways that are implicated in PAS, such asefflux pump targeting, SOS-induced filamentation of bacteria, outer membrane disruption, biofilm disintegration and evolutionary tradeoffs. Special focus is placed on Examples in particular pathogen's specific resistance mechanism as well as models to determine PAS and evidence for efficacy of PAS in the clinic and experimental setting.

II. THE ESKAPE PATHOGENS: RESISTANCE MECHANISMS AND CLINICAL BURDEN

Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and *Enterobacter spp* (ESKAPE) pathogens comprise a significant and prevalent group of multidrug-resistant (MDR) pathogens that cause the majority of healthcare-associated infections worldwide. Intrinsic and acquired resistance mechanisms, such as enzymatic inactivation of antimicrobials, modification or protection of the antibiotic target site, active efflux of drugs, reduced permeability across the cell membrane, bio-film formation, and metabolic adaptive states supporting persistence, enable survival in the presence of antimicrobials. Inherent resistance coupled with acquisition of genetic elements has resulted in widespread therapeutical restrictions and the overall global burden is increasing (14).

- *Gram-Positive ESKAPE Pathogens*

Enterococcus faecium is one of the most notorious nosocomial pathogens because of its ability to acquire and transmit determinants that confers antimicrobial resistance. The high-level vancomycin resistance to EF is associated with the vanA and vanB loci which express an enzyme that cleaves the peptidoglycans precursors into two pieces and forms the unique D-Ala-D-Lac sequence which is incompatible with the recognition by Vancomycin (15). The high resistance of EF against-lactam antibiotics is mainly due to a low-affinity penicillin-binding protein PBP5, that reduces EF sensitivity toward -lactam, as the reduced interaction with the cell-wall synthesizing Machinery leads to a lower affinities of -lactam antibiotics towards its targets. Structural features of resistance to beta-lactams induced by mutations leading to PBP5 hyperactivity may explain some mutations involved in the mechanism of hyper resistance found in clinically isolates (16).

Staphylococcus aureus, in particular methicillin resistant Staphylococcus aureus (MRSA), is among the leading Gram-Positive ESKAPE organism clinically significant pathogens. In the case of resistance against-lactam antibiotics MRSA carries the mecA gene that encodes the MRSA-PBP2 (PBP2a), a modified penicilln-bindlng protegn with low affinity towards-lactam antibiotics, that allow cell wall synthesis to continue even with exposure of the cell to antimicrobial compounds (17). In addition to antimcrolbiol resistance MRSA frequently develops biofilms when implanted in medtcal devices or in damaged tissue. Biofilms comprise communities of inactive, metabolicaly dormant bacteria immersed within an Extracellular matrlX which allows for better penetratlon resistance by antimicrobials. Also, the development of vancomycin intermediate Staphylococcus aureus (VISA) or heterogeneous VISA (hVISA) may complicate the therapeutic strategies used for the treatments of patients infected by that microbe (18).

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➢ *Gram-Negative ESKAPE Pathogens*

Klebsiella pneumoniae is a common opportunlstc, pathogen causnng various infection ssuch as bloodste am infectiort, pneumonta,urhnarytract infeotlons and, outbreaks inthe hospital. Its pathogenicity is inhiinforced by It's thick capsule, a polysaccharide that prevents complementarity activation and.inhibites phagocytosiss. There are over 130 serotypes of capsules in K.pneumoniae (K-types) that contribute to considerable strain diversity (19). Most of the K pneumoniae resistance mechanism towards antimicrobials has to be associated with the presence of, extended spectrum-lactamase- or carbapenemases production like KPC, NDM and OXA-48-like -lactamases. Besides this type of mechanismspornins defects and efflux pumps hyperexpression like AcrAB-Tolc system canalso contributed to resistance mechanisms of K pneumoniae (19). According to recently published' Surveillance'studies a marked increase in. Carbapenem-resistant, K. Pneumoniae harbourin, multiple, carbapenemase -genes such as NDM and. OXA-48 like ones have beendescribed, in clinical isolate (20).

Acinetobacter baumannii is recognized as one of the. Most clinically. Significant, challenging Gram. Negative MDR pathogens especially in ill patient, and the organism's endurance in the environment such as survival to dessicaton and prolong survival in surface of, the. Hospitals allows for Its persistence. In general, resistance to carbapenems inA baumannii is driven mostly by. Class D--lactamases, including OXA-type carbapenemases (e.g. OXA-23); but further mechamims involving efflux, 'permeability reduction and. Target modifications also contributed to this' resistance (21). These mechanisms of endurance confer A baumannii enhanced fitness to persist in long-term, transmission within. Hospital (especially intensive care units) environments (22).

Pseudomonas aeruginosais a, major pathogen whose resistance profile is complicated; by inherent and acquired mechanisms. Limited penetration of antimicrobial agents into the cell's periplasmic space. The bacteria is equipped with. Various efflux. Systems, MexAB-OprM, MexCD-OprJ, MexEF-OprN andMexXY thatactively pumpedout the Drugs. Moreover inducible chromosomal AmpC beta -lactamases production could also be associated with resistance toward various-lactam antibiotics. (23) *P.aeruginosahas* good aptitude for chromosome mutations. That along with the ability toform, a biofilm and, acquire plasmadrivenresistant genes lead to resistance against various drugs. Resistance rates toward carbapenems, extended-spectrum-lactams and fluroquinolones in, clinical P. Aeruginosa have been reporting by international surveillance systems (24).

In the case of *Enterobacter ssp* the inherent resistance is linked to inducible chromosomal AmpC, as upon introduction of, -lactam antibiotics a, mutations Derepress the inducble promoter resulting to hyper production. Of the AmpC -lactamase leading to resistance against third and fourth generation of cephalosporins etc. (25) Additionally, resistance of plasmid borne carbapenemases such as NDM, KPC, and OXA-type -lactamase enzymes have resulted to high level-resistant Enterobacter isolates (25).

III. MOLECULAR MECHANISMS OF PHAGE-ANTIBIOTIC SYNERGY

Phage-antibiotic synergy (PAS) is a complex mechanism involving multifactorial interactions between bacteriophages and antibiotics resulting in enhanced anti-bacterial activity beyond additive effects. The underlying mechanisms of PAS are highly specific and combinatorial depending on the infecting phage, target bacteria and therapeutic antibiotics used. Examples include interactions with phage adsorption factors, bacterial stress responses, antibiotic susceptibility mechanisms, and biofilm dynamics, as well as evolutionary considerations involving phage resistance trade-offs [26].

➢ *Efflux Pump Exploitation and Antibiotic Resensitization*

One prominent, clinically relevant mechanism of PAS exploits bacterial resistance-associated surface features that may also play a role in antibiotic resistance. These structures include surface proteins, lipopolysaccharide (LPS), capsular polysaccharides, porins, and in some instances, cellular structures associated with multi-drug resistance (MDR) efflux systems [27].

In Gram-negative pathogens that contribute to the ESKAPE acronym-including Escherichia coli, Staphylococcus aureus (methicillin-resistant strains are excluded), Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterococcus faecium-resistance can be conferred by the modification or loss of surface features that serve as receptors for phage infection. This disruption may confer an evolutionary fitness cost, and the particular structures affected by such changes might also be important for antibiotic efflux. Disruption of resistance-associated structures in turn could lead to accumulation of intracellular antibiotics and restored susceptibility to previously ineffective therapies [28].

This phenomenon has been termed “phage-driven evolutionary steering” or “phage-mediated antibiotic resensitization” and can result in conversion of phage resistance from a fitness cost into a fitness benefit, exploiting bacteria’s need to compromise between phage immunity and drug susceptibility via evolutionary fitness trade-offs [29].

Studies involving experimental evolution of P. Aeruginosa found that phage-resistant strains that evolve through modification or deletion of receptors also develop collateral sensitivity to antibiotics, and this collateral sensitivity varies with bacterial species, phage receptor, and the specific mutation incurred [29,30].

➢ *SOS Response Activation and Cell Filamentation*

Another important mode of PAS arises from antibiotic-induced disruption of DNA integrity, leading to the activation of the bacterialSOSstress pathway. Fluoroquinolone antibiotics, which target DNA gyrase and topoisomerase IV, elicit a robust SOS response, but other agents such as certain ␣-lactams can also initiate bacterial stress signaling by perturbing cell envelope biogenesis and cell division processes [31]. A key consequence of SOS response

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

activation in many species is the induction of cell filamentation.

The SOS regulated protein SulA inhibits division the FtsZ ring, promoting elongation into filament-like structures in a process that allows the bacteria to continue to grow in biomass without subsequent septum formation [31].

Recent advances in single-cell analysis of phage-bacteria Interactions revealed that filamentation-inducing treatments can significantly boost infection by bacteriophages. It has been shown that sub-lethal concentrations of filamentation-inducing antibiotics increased susceptibility of E. Coli to phage infection via the development of longer bacterial cells and expanded surface areas that enhanced phagemultuplication[32]. It is worth note that not all SOS responses will translate to improved PA. The stress induced filamentation will increase phage multiplicity and can also facilitate bacterial mutation rates thus potentially further exacerbating antibiotic resistance [33].

- *Outer Membrane Permeabilization*

The Gram-negative outer membrane acts as a barrier to antibiotic action in theESKAPEpathogens, impeding their access to target intracellular sites of action [34]. Some phage-infection events have been shown to increase permeability of the Gram-negative outer envelope via changes in cell surface structures or integrity that are triggered by adsorption, genome transfer, phage replication associated membrane changes or lysis. This mechanism is of particular interest for those antibiotics (including polymyxins such as colistin) whose efficacy is limited by poor access to their intracellular targets[35].

Combinations of these membrane-disrupting drugs with colistin increased the overall potency in A. Baumannii models, potentially reflecting a synergistic disruption of bacterial membrane integrity by both agents.

Another class of antibacterial phages encodes depolymerase enzymes, which act to degrade capsular polysaccharides on the surfaces of host bacteria (e.g., inK. Pneumoniae) and thereby increasing phagemultiplication, improving resistance to antibacterial host immune systems, or even improving antibiotics exposure to the bacterial surface [36].

- *Biofilm Disruption and Matrix Degradation*

Bacterial biofilms, formed by most strains of ESKAPEpathogens, represent one of the greatest barriers to effective treatment of chronic infections.biofilm EPS matrix impede antibiotic diffusion into the biofilm structure and the growth of metabolically active, replicative cells, thus contributing to reduced sensitivity to most therapeutics [37]. The ability of bacteriophages to target, inscribe into, destroy, and even feed on the EPS matrix provides a complementary strategy toAntibiotic therapy to disrupt these biofilm communities and potentiate killing by both phage and Antibiotics [38]. Studies utilizingPseudomonas aeruginosa biofilm models found that phage treatment in combination with antibiotics can lead to better reduction in biofilm biomass than treatment with a single modality alone, as phages increase antibiotic infiltration into the biofilms [39].

Similarly, phageAntibiotic combinations were effective against methicillin-resistant S aureus (MRSA) biofilms, including against strains with reduced sensitivity to vancomycin (i.e. VISA)[40].

- *Stoichiometry and Antibiotic Class Dependence*

It is crucial to recognise that the PAS is not universally observable for every combination of phages and antibiotics but dependent on the specific combination, the drug mechanism of action, the status of the bacteria, the number of bacteriophages present, and treatment protocol [26]. Antibiotics that target cellular wall (e.g., -lactams) usually have synergistic effect on bacteriophages since they affect replication but not other functions required for phage synthesis, whereas translation inhibitors like aminoglycosides do not act synergistically withphages as they block the synthesis of phage proteins [41].fluoroquinolones interact with bacteriophages through the activation of the SOS-mediated filamentation response; they can also promote phages resistance and hence PA are complex [33, 42]. Some experiments have indicated that bacteriophage introduction prior to Antibiotics could leads to improved clearance of bacteria and enhancement of biofilms disintegration compared with Antibiotics only treatments but it is highly dependent on the bacterial strain [42].

Table 1 Principal Molecular Mechanisms of Phage-Antibiotic Synergy (PAS) and their Relationships to Antibiotic Class and ESKAPE Pathogen Relevance.

Mechanism of PAS	Antibiotic Classes	Primary ESKAPE Targets
Efflux pump exploitation / resensitization	Fluoroquinolones, aminoglycosides, beta-lactams	P. aeruginosa, K. pneumoniae, E. coli, A. baumannii
SOS induction / cell filamentation	Fluoroquinolones, beta-lactams (cell wall-active)	E. coli, Enterobacter spp., K. pneumoniae
Outer membrane permeabilization	Colistin, rifampicin, hydrophobic antibiotics	A. baumannii, P. aeruginosa, K. pneumoniae
Biofilm EPS matrix disruption	Beta-lactams, daptomycin, ceftaroline	MRSA, P. aeruginosa, K. pneumoniae, E. faecium
Fitness cost / antibiotic resensitization	Class-specific (depends on receptor lost)	All ESKAPE pathogens

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

Stoichiometry / antibiotic class selectivity	Cell-wall active > translation inhibitors	E. coli, S. aureus (mechanistic basis)

EPS = Extracellular Polymeric Substance; SOS = SOS Response; RND = Resistance-Nodulation-Division Efflux Family.

IV. PATHOGEN-SPECIFIC EVIDENCE FOR PHAGE-ANTIBIOTIC SYNERGY

➢ *Pseudomonas Aeruginosa*

Psedomonas aeruginosa is the best studied of the ESKAPE pathogens when it comes to phage-antibiotic synergy (PAS), largely due to its prevalence in hospitals, inherent drug resistance and tendency to form biofilms. There have been numerous studies in both laboratory conditions and in animal models showing that using a combination of lytic bacteriophages and an antibiotic results in better killing of bacteria than treatment with either monotherapy alone [43–45].PAS in Psedomonas aeruginosa can occur through disruption of biofilms by phages, increasing penetration of the antibiotic through the biofilm, or by an evolutionary trade-off with the development of resistance to the phage. Infections within biofilm matrices are problematic, not just due to physical restriction on antibiotic penetration but because dormant cells within the matrix can survive and persist following antibiotic treatment. De Soir and colleagues demonstrated that combinations of new phages and classical antibiotics were more efficient in destroying Pseudomonas aeruginosa PAO1 biofilm in an animal model of orthopedic infection compared to either single agent [43]. Destruction of biofilm components by the phage improved diffusion of the antibiotic to the entrapped bacteria and resulted in greater killing.

Another common mechanism of synergy involves phage mediated ‘selection steering’; Chan et al. Identified a bacterial strain which, in response to phage predation, rapidly evolved mutations that reduced binding of phage, but also coincidentally re-sensitised bacteria to previously inactive antibiotics [44]. In such a situation, phages may facilitate resensitisation of resistant bacterial cells to established antibiotic treatment regimes. In summary, the best available evidence of PAS in ESKAPE pathogens arises from studies into infections with P. Aeruginosa. [43–45]

➢ *Klebsiella Pneumoniae*

The global emergence of resistance to carbapenems in K. Pneumoniae is a severe threat to public health and represents one of the greatest justifications for the development of PAS therapies [46–48]. There is strong experimental evidence that mixtures of phages and antibiotics have enhanced kill activity, may act to inhibit biofilm formation, or prevent the emergence of resistant bacterial strains. The role of capsular polysaccharides has become a crucial factor in the PAS of K. Pneumoniae with Many phages against this species capable of producing an enzyme capable of degrading these capsular polysaccharides and hence facilitating phage entry onto the bacterial cell, or increasing the diffusion and penetration of the antibiotic into the cells themselves. This has the potential to increase the sensitivity of bacteria to the treatment.

In this regard Zhao and colleagues have shown that cocktails of phages with antibiotics showed markedly improved killing of pathogenic Klebsiella pneumoniae isolates than was possible with either treatment alone[46]. Moreover, the use of multiple phage strains in combination with the antibiotics reduced the selection for bacterial mutants with altered sensitivities, and the development ofresistance. This offers great potential for treating difficult to treat hypervirulent or carbapenem resistant K. Pneumoniae strains with limited or no other treatment options [46–48].

➢ *Acinetobacter Baumannii*

Acinetobacter baumannii is perhaps the greatest therapeutic challenge among the ESKAPE pathogens given the widespread dissemination of extensively (XDR) and pan-drug resistant strains, and research has shown that phage and antibiotic combinations produce greater reduction in bacteria compared to either single therapy and it has proved beneficial particularly when paired with last resort antibiotics such ascolistin and rifampicin [49–51]. As with P. Aeruginosa, resistant A. Baumannii mutants typically develop mutations that affect outer membrane structure to reduce phage adherence, and that frequently incur a fitness cost resulting in increased permeability to certain drugs or decreased ability to express factors associated with virulence [49,50]. The destruction of biofilm integrity by phages improves penetration of antibiotic into resident cells of the matrix [51]. This suggests PAS could have a significant role to play in combating highly resistant A. Baumannii but clinical validation needs to occur[49–51].

➢ *Methicillin-Resistant Staphylococcus Aureus (MRSA)*

Among the Gram-positive ESKAPE organisms, there is strong evidence for PAS in MRSA. Recent in vitro pharmacodynamic (PK/PD) studies utilising a simulated biofilm reactor have shown that bacteriophage and daptomycin combinations, or a combination of bacteriophage and ceftaroline, can effectively reduce biofilm bacterial populations which are not significantly susceptible to antibiotic alone [52]. Kunz Coyne et al. Investigated combinations of phage and daptomycin and ceftaroline against a biofilm that harboured resistant methicillin resistant Staphlococcus aureus (MRSA) and daptomycin-nonsusceptible vancomycin-intermediate S. Aureus (DNS-VISA) and found a significant reduction in total bacterial numbers achieved with combination treatments versus either treatment alone and limited the generation of both complete phage-resistance and reduced daptomycinresistance.[52] This observed activity of combination therapy against biofilm MRSA may arise from a number of synergystic factors including; the increase in the number of phages present within the biofilms by increased multiplication, a capacity for phages to produce degradative enzymes which in turn degrades structural components within the extracellular matrix thereby improving drug penetration [53,54] or, conversely, increasing the bacteria load in the biofilms, which facilitates further phage amplification, or by reducing the

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

bacterial population enabling greater drug penetration [53,54]. Therefore, these findings support potential applications of PAS for the treatment of chronic infections involving MRSA, particularly those associated with implantable devices and orthopedic infections [52–54].

➢ *Enterococcus Faecium*

Enterococcus faecium, in particular VRE E faecium, is perhaps least studied of the ESKAPE species in relation to PAS and only a few specific enterococcal phages have been identified to date. However, there is suggestive evidence to indicate PAS approaches could combat VRE. In a study involving a simulated endocardial vegetation model in a murine vankcomycinresistant enterococci infectionmodel, mixtures of three phage strains in combination with daptomycin or ampicillin dramatically reduced bacterial numbers in the vegetationcompared with control groups, and largelyprevented thereemergence of phage resistant bacteria [55]. This approach has the potential to maintain the effect of existing antibiotics by reducing bacterial population density and prevent resistance [55]. While more evidence is required, such treatment could potentially be very effective against chronic VRE colonisation of implanted medical device and endovascular biofilm [55,56].

V. IN VITRO METHODOLOGIES FOR PAS QUANTIFICATION

In vitro methodologies to quantify PAS reliability in determination of phage-antibiotic synergy (PAS) comes from standardization of methodologies. Bacteriophages possess very specific characteristics that differ them from other kind of antibiotics; they are replicative agents, meaning that their "effective dose" dynamically change during an infection event, being affected by different factors such as multiplicity of infection (MOI), adsorption rate, replication speed, bacterial growth phase, and bacteria resistance against the phages. At the present time two of the most common methods for PAS estimation are checkerboard and time-kill assays [57].

➢ *Checkerboard Assay*

The checkerboard is one of the best known techniques to investigate the activity of two different antimicrobial agents on bacteria, testing different concentration combinations. In a PAS study the use of drug concentration is replaced by the measurement of initial multiplicity of infection as second parameter given the capacity of amplification of the phage during infection, what implies that their effectiveness varies according to their growth curve [57]. The results obtained can be quantitatively analyzed by calculating the so-called Fractional Inhibitory Concentration Index (FICI): *FICI = (MIC of phage in combination / MIC of phage alone) + (MIC of antibiotic in combination / MIC of antibiotic alone)* In general, FICI ≤0.5 implies synergism, 0.5 < FICI < 4 the drugs were indifferent and FICI ≥4 antagonism. However, interpretation should be done cautiously given that the action of phage in these assays may reflect more accurately the interaction based on initial multiplicity than actual phage growth and the consequent effect on the total bacterial population.

Thus, Nicolic et al. Published a novel method, in terms of improved checkerboard design to characterize PAS for interactions between phage JG024 with the antibiotic's ciprofloxacin and ceftriaxone against P.aeruginosa and the interaction between SES43300 phage and ciprofloxacin against S.aureus [57].

Their studies concluded that application sequence of the treatment had a strong impact on synergism; therefore, the application order was considered very important in the treatment of infection as the use of phages first, in a first step caused bacteria disruption, the then increased antibiotic effectiveness [57]. Limitations of checkerboard assays: although are highly effective as an initial tool for assessment of interactions, is important to keep in mind that only provides one, final measure of the outcome of interactions and this is likely to do not perfectly recapitulate the behavior of bacteriophage action such as replica and killing dynamics. That is why it is recommended to complement checkerboard assays with kinetic methods like time-kill analysis [58].

➢ *Time-Kill Assay*

This is one of the more reliable method to confirm a PAS since it indicates the dynamic of bacteria viable cell counts over time of treatment. This methodology represents measurement of viable bacterial cell counts based on their colony Forming Unit (CFU) number over time; these numbers are then obtained for treated, untreated and both treated bacteria cultures to compare how effectively an intervention affects bacteria population [58]. The criteria for defining synergy generally require reduction of >2 log10 CFU/ml from controls of most potent single drug, where bactericidal activity is observed when a decrease greater than 3 log10 CFU/ml is achieved from original bacterial population [58].

The time-kill assay provides key mechanistic data as early lysis from the phage component can be delineated from the slow activity and cell killing of antibiotics, as well as the synergistic enhancement provided by combinations [58].

Gordillo Altamirano and Barr points out the value that this methodology has to reveal the evolutionary benefits that result from combinatorial therapies as for instance selection of phage resistance conferring phage mutated with normal or restore antibiotic resistance in order to ensure an increased rate of phage propagation after initial reduction of bacteria population with the antibiotic, which is not revealed with static assays [58]. It is necessary to mentioned that there is no a clear consensus for bacteriophages' dose calculation, use of multiplicity of infection (MOI) selection, potency calculations (pfu per mL, MOI, bacterial-phage ratio) or incubation times and protocols, and the interpretation of the statistical significance of results and data. Therefore, the present data will not be directly transferable to an international standard and to be implemented clinically in a global perspective until the community agrees on universal criteria to assess PAS [59]. In regard to regulations, European Pharmacopoeia Commission initiated work a few years ago to include a new chapter regarding determination of bacteriophage potency [59].

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

Table 2 Summary of Representative Phage-Antibiotic Synergy Studies against ESKAPE Pathogens (2021–2025).

Pathogen	Phage(s)	Antibiotic(s)	Interaction / Outcome
P. aeruginosa MDR	PAW33 (Bruynoghevirus)	Ciprofloxacin, levofloxacin	Synergistic eradication of all tested strains
P. aeruginosa PAO1	Locally isolated lytic phages	Multiple classes	Drastic biofilm reduction vs. monotherapy (orthopedic model)
K. pneumoniae MDR	Phage cocktails	Multiple antibiotics	Synergistic clearance; reduced phage resistance emergence
K. pneumoniae XDR	Targeted phage	Inactive antibiotic (resistant strain)	Successful eradication of recurrent UTI; resensitization
A. baumannii MDR	vB_AbaSi_W9	Rifampicin	100% mouse survival (vs. reduced in monotherapy arms)
A. baumannii XDR (clinical)	Nebulized phage	Polymyxin B, amikacin, fosfomycin	Sputum clearance + pulmonary resolution in 8 days (ICU case)
MRSA DNS-VISA (biofilm)	Intesti13 + Sb-1 + Romulus	Daptomycin + ceftaroline	Bactericidal in 168-h biofilm PK/PD model; no resistance emergence
E. faecium VRE	Phage cocktail	Daptomycin + ampicillin	Eradication of biofilm-embedded MDR E. faecium; preserved phage susceptibility

DNS = Daptomycin-Nonsusceptible; VISA = Vancomycin-Intermediate S. Aureus; XDR = Extensively Drug-Resistant; UTI = Urinary Tract Infection; PK/PD = Pharmacokinetic/Pharmacodynamic.

VI. CLINICAL EVIDENCE AND TRANSLATIONAL EXPERIENCE

➢ *Compassionate Use Case Series*

Present clinical evidence of phage-antibiotic synergy (PAS) relies primarily on case reports, retrospective case series, and compassionate-use programs. Largest multicentre analyses include the report by Pirnay et al. Who presented the analysis of 100 consecutive personalized phage therapy cases country-wide [60]. In their cohort, phages were nearly always given concomitantly with antibiotic(s) because these agents are commonly used as an adjunct to current antibiotic therapy [61].

Several case studies illustrate that clinical outcomes depend on the etiology of infection, the target pathogen and bacterial resistance profile, and phage specificity selection [61].

Case reports provide support for clinically useful outcomes in ESKAPE pathogens including A. Baumannii, P. Aeruginosa and K. Pneumoniae, but highlight the need for proactive phage susceptibility testing and patient-specific treatments for optimal response [61]. Some groups have successfully employed personalized phage selection integrated with antibiotics on a case basis. These are cases reported through specialized TAILR (Tailored Antibacterials and Innovative Laboratories for Phage Research) program which customized therapeutic phage options, demonstrated that bacteriophage selection and the resulting combination strategies can deliver a positive clinical outcome against multidrug-resistant bacterial infections [62]. Another targeted study evaluating the phage PASA16 in severely infected P. Aeruginosa cases emphasized that a substantial number of patients who received this specific phage in addition to antibiotics also benefit from the phage, demonstrating feasible use of these targeted agents within complex management strategies although with no control arm to infer definitive value of PAS [63].

While many case reports are encouraging, they often lack strict control groups, have the confounding effect of antibiotic therapy in the background, suffer from heterogeneity of patients, pathogens and treatment approaches and, consequently, do not prove but provide some valuable insights and experience regarding clinical application of phage therapy in combination with antibiotics.

➢ *Randomized Controlled Trials*

The available evidence on phage therapy from randomized controlled trials (RCTs) is limited and, critically, does not currently contain studies aimed at proving the effectiveness of PAS. The first human randomized double-blind controlled phase 1/2 clinical trial comparing a cocktail of phages to the control group for treatment of Pseudomonas aeruginosa burn wound infections (PhagoBurn study) was recently published [64]. Safety and tolerability was demonstrated, but it failed to show superiority over the best available treatment in this trial due to possibly a lower dose of phage administration to the patients that arose as a result of phage production challenges [64].

The lack of proper controls to test PAS as a definitive modality is reflected in other case studies which could offer evidence but fail to establish definitive PAS clinical benefit due to multitude of uncontrolled factors including background antibiotics and patient heterogeneity.

Phage therapy must be studied using adequate protocols and with carefully planned, controlled studies to truly determine its potential to expand treatment options with particular attention to a synergistic approach with antibiotic therapies. Future studies should consider controlled randomization and stratification for pathogen type, its phage susceptible pattern, nature of infection, presence of biofilm, and combination of phages with antibiotics of specific types.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

VII. CLINICAL TRANSLATION CHALLENGES

➢ *Phage Resistance Development*

Although phage-antibiotic synergy (PAS) can be enabled by evolutionary trade-offs where phage resistance leads to an increased sensitivity to antibiotics, the emergence of resistance against phages poses a threat to the clinical use of phage therapy. Phage resistance can arise via numerous different mechanisms, including mutations to phage receptors, alterations in restriction-modification systems, the presence of CRISPR-Cas defense systems, or abortive infection systems [65]. These resistance traits do not necessarily exhibit the same fitness consequences across species or affect resistance to multiple agents in a comparable manner; rather they can impose very different evolutionary costs or benefits that influence their ability to spread in a bacterial population in conjunction with or independent of changes in susceptibility to antibiotic agents.

In cases of biofilm-associated infections, the development of resistance is further complicated, since the spatial structure of the bacterial communities may preclude phage penetration and thus permit surviving bacterial clones, with the potential to grow as an antibiotic-resistant population.

Biofilm matrices themselves can restrict phage mobility [66], and bacteria residing within them often survive the phage challenge through metabolic inactivation or due to decreased susceptibility to antibiotic antibiotics due to increased tolerance [66]. These conditions facilitate a potential co-existence between resistant and sensitive bacterial clones or a shift toward the more resistant variants. Phage cocktail-treatment in which different phages act by targeting different receptor proteins of target bacteria offers a way to increase the number of genetic changes required for the bacterial cell to be resistant to both agents and this increases the barrier to the evolutionary acquisition of resistance compared to a mono-phage treatment approach [65]. Nonetheless, cocktail design is limited by the available host spectrum of particular phage strains and must take into account possible interactions that could reduce potency (antagonism between phages). Moreover, and very significantly, phage resistance does not necessarily correspond to the restored antibiotic susceptibility.

Receptor mutations may be associated with a fitness burden that restores susceptibility of the bacteria to antibiotic drugs, yet resistance towards phages and antibiotic agents can also co-occur (and even cooperate) via entirely different bacterial defense strategies. Biofilm development itself may provide a means to tolerance that provides simultaneous resistance towards phages and antibiotic antibiotics [66,67]; similarly changes in bacterial membrane fluidity and in host cellular stress response can contribute to global increases in bacterial tolerances [66].

➢ *Host Immune Interactions*

Host immune responses towards the phages administered have a crucial role in their fate in the body and thus in therapeutic success or failure. Following intravenous or systemic administration of phages to the bloodstream, they can be quickly eliminated by immune-mediated clearance pathways, including complement-mediated opsonization, phagocytosis by macrophages, and sequestration in the reticuloendothelial system [68]. Host-generated antibodies that neutralize the phages can therefore reduce their effective concentration and limit the success of subsequent or multiple therapeutic doses of phage, especially if administered in rapid succession. For phages administered intravenously, pre-formed as well as treatment-induced anti-phage antibodies targeting various components of the phage structural proteins, have demonstrated decreased phage presence in the bloodstream and may compromise therapy depending on phage type and administration route [68].

However, it should not be overlooked that phages can also facilitate interactions between bacteria and the human immune system in a potentially beneficial way. Because of bacteriophage-induced lysis, components of the bacteria, such as endotoxin or lipopolysaccharide (LPS), are liberated and can act as stimulus for activation of the human innate immune system, contributing to the removal of the bacteria [69]. Thus an optimally effective combined treatment with phage and antibiotics would imply the simultaneous and combined effect of direct antibiotic killing, phage killing and phagocytosis. Whether a dose reduction of antibiotic antibiotics is therefore justifiable to account for immune-mediated clearance of phages from blood has remained undetermined [68].

➢ *Regulatory Framework Immaturity*

Given the unique biological characteristics of phages-their replication, genetic material and host specificity, as well as the requirement to use a personalized and tailored formulation-the regulatory frameworks, both on national and international level, are still immature for PTMPs. As with other biologics (but unlike conventional drugs) their manufacturing needs stringent GMP (Good Manufacturing Practices) standards that are significantly different from those pertaining to the pharmaceutical industry.

While on one side access can be gained through compassionate use-based regulatory procedures in most of the countries that apply compassionate access use procedures and the hospitals own responsibility and regulations (so called magistral preparation, use in a local context of a hospital pharmacy in which only preparations intended for direct and personalized use of an individual patient under physician responsibility are allowed by law in many jurisdictions)), access based on regulatory approved medicinal products (MPTMP) is only starting up and remains more limited[70].

There have been important steps towards regulation of therapeutic phages. In Europe, the European Medicines Agency (EMA) recently issued guidance for applicants on the scientific aspects of good quality practices when manufacturing clinical trial on bacteriophages therapeutic. EMA emphasizes a need for specific phage characteristics such as identity, genetic characterisation of each phage strains used, genomic purity of each phage strain, standardisation of

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

the method of determining potencies for each phage, and consistent production (quality assurance and control) for PTM [71].

Nonetheless, determining the therapeutic potency and optimal treatment dosage for a given phage remains a critical challenge. Standardized phagogram (plaque assay performed with various bacteria to identify susceptible strains) using appropriate control phages remains important in standardizing relative phage activities, however, differences in bacterial strains, growth conditions, as well as plaque reading may limit the comparison across different labs [72].

- *Standardization of PAS Testing*

An issue that can strongly delay the clinical uptake of PTM is the lack of standardised tests to measure PAS [73]. Many studies report significant synergistic activity, however, the experimental conditions are frequently quite heterogeneous with respect to the types of bacteria tested, their growth phase and inoculum, MOI and concentrations of phages and antibiotics used, type of test conducted (e.g., tube or plate assays), and definitions for synergy.

This diversity limits the comparative evaluation of results between various studies, and also hinder identification of the predictors of clinical outcomes based on in vitro parameters. Pre-therapeutic testing of the patient's infecting isolates for their susceptibility to bacteriophages is necessary and is of the utmost importance. Phagogram identification and optimal concentration and dosage of the selected phage to be prescribed to a patient would enable precise personalization and efficacy of the phage-drug combination therapy [73]. Daubie et al. Points out that the development of sensitive, specific, reproducible and easily achievable bacterial susceptibility tests is required for phage therapy being integrated into conventional antimicrobial therapy, to guide the choice of appropriate phage drugs and phage combination(s) to ensure maximum clinical success [74].

VIII. FUTURE PERSPECTIVES

- *Future Outlook*

In the era of the growing knowledge of PAS phenomena the concept of therapeutic application has already moved from discovery on empirical base towards mechanistic consideration and rational approach. The growth in bacteria genome sequencing, phage biology knowledge, as well as computational methods of modeling are anticipated to enable a better rational choosing of the combinations of phage-antibiotics for treatment of multidrug-resistant infections [75].

Among the current prospective trends, the most obvious direction consists in association between the testing results on bacteriophage susceptibility to isolates of pathogenic bacteria (so called “phagogram”) and sequence of the whole genome of these microorganisms [75,76]. By means of bacteria genome sequence analyses it is possible to predict genetic mechanisms of resistance, type of capsules and type of receptors on surface, which participate in adherence to bacteriophages. Such analysis may considerably promote effective choice of phage preparations and of therapeutic concentration range of bacteriophages for synergistic effects [75,76]. By integrating machine learning techniques and AI algorithms, the process of prediction the most effective phage-host interaction as well as selection of phage-antibiotic combination may be drastically accelerated [76].

Other exciting direction related to enhancement of phage preparations application lies in modification of bacteriophage. Genetic manipulation methods used in synthetic biology are being used to change the bacteriophage’s genome, including their receptors-binding molecules such as tailspikes and tail fibers. These alterations lead to the increase of host tropism, as well as adsorption properties against resistant bacterial strains [77]. It is also possible to engineer phages that would target the elements of bacterial cells structure directly linked with antibiotic resistance mechanisms, promoting a trade-off that strengthens interactions between phages and antibiotics and contributes to the restoration of sensitivity [77,78].

To move to further levels of research development it is needed the further modeling and analysis of PK/PD characteristics of bacteriophages. Contrary to classical antibiotics they are selfreplicativebiological agents whose local concentration at the infection site will depend not on their introduction rate but on their multiplication rate, and local concentrations of target bacterial cells and on immune system of host [79]. The detailed investigation of interactions between multiplication rate of bacterial cells, their sensitivity towards antibiotic action, growth rate and number of immune cells will enable a more precise modeling and forecasting of phage replication process at infection site which will improve selection of treatment schemes [79,80].

Last but not least, there is still great deal of work related to the establishment of standard guidelines and regulatory requirements for phage applications. A large-scale randomized clinical trial, standardization of bacteriophage suscertibility testing protocols and harmonious international regulatory rules for phage products would enable wide use of laboratory findings in clinical practice [80,81].

IX. CONCLUSIONS

Phage-antibiotic synergy represents a mechanistically rich and therapeutically compelling strategy for addressing infections caused by ESKAPE pathogens that resist conventional antibiotics. The molecular mechanisms underpinning PAS—efflux pump exploitation, SOS-mediated filamentation, outer membrane permeabilization, biofilm matrix disruption, and evolutionary fitness cost imposition—are increasingly well characterized, and the pathogen-specific experimental and clinical evidence base has grown substantially in the period 2021–2025. The conceptual unification of these mechanisms under the evolutionary steering paradigm provides a rational framework for selecting phage-antibiotic pairings that are not merely additive but that convert bacterial resistance adaptations into therapeutic vulnerabilities.

ISSN No:-2456-2165 https://doi.org/10.38124/ijisrt/26jun1484

Clinical translation remains constrained by a series of interlocking challenges: the near-absence of randomized controlled trial data; the lack of standardized in vitro PAS assay protocols; regulatory frameworks only now developing the specificity needed for phage products; and the biological complexity of phage resistance evolution and host immune interactions. Progress on each of these fronts is occurring, with the 2024 European Pharmacopoeia harmonization, the EMA's 2025 draft guideline, and the growing network of specialized phage therapy centers collectively constituting a foundation from which more rigorous clinical investigation can proceed. The prospective integration of AI-guided phage selection with rational PAS-based combination regimen design may ultimately provide the mechanistic precision and clinical reproducibility needed to establish phage-antibiotic therapy as a standard-of-care modality for MDR ESKAPE infections.

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