Introduction

Antibiotics resistance is a growing global health crisis (WHO, 2017; World Health Organization, 2019). A global study by the Institute for Health Metrics and Evaluation (IHME) estimated that bacterial antimicrobial resistance (AMR) associated deaths accounted for most burden after ischaemic heart disease and stroke in 2019 (Antimicrobial Resistance Collaborators, 2022; IHME, 2023). This dire situation is exacerbated by drastically dwindling new antibiotics (Chahine *et al*, 2022; Ventola, 2015). Anti-virulence approach is one of the alternative therapies to combat antibiotic resistance. Virulence factors, for instance, secreted toxins, allow pathogens to invade and infect host cells that cause diseases (Sharmat et al, 2017 cited in Dehbanipour & Ghalavand, 2022). As an anti-thesis to antibiotics, anti-virulence approach disarms pathogens' virulence factors without killing them, thereby minimising selection pressure for resistance development (Dehbanipour & Ghalavand, 2022). Although some evidence has indicated that anti-virulence resistance does surface, it may be limited if the agents act extracellularly or quench "public good" which does not threaten bacteria survival (Lissens *et al*, 2022; Rezzoagli *et al*, 2018). Hence, this review search was limited to anti-virulent agents with **extracellular binding mechanisms of action.** Furthermore, to assess the potential and progress of this field, the search focuses on the well-known ESKAPE pathogens (Table 1), systemic infections, and research that have at least progressed to *in vivo* experiments. Due to the availability of research papers and word limitation, this review will discuss progress and potential two anti-virulence strategies – neutralization of *Staphylococcus aureus* α-toxin and disruption of *Pseudomonas aeruginosa* type III secretion system (T3SS).

Bacterial Species (Gram positive / negative)	**Antibiotics resistance**
Enterococcus sp. (Gram positive)	vancomycin-resistant
Staphylococcus aureus (Gram positive)	methicillin-resistant, vancomycin-intermediate-resistant
Klebsiella pneumoniae (Gram negative)	carbapenem-resistant
Acinetobacter baumannii (Gram negative)	carbapenem-resistant
Pseudomonas aeruginosa (Gram negative)	carbapenem-resistant
Enterobacter sp. (Gram negative)	carbapenem-resistant

Table 1: Summary of ESKAPE pathogens and respective antibiotic resistance (Venkateswaran *et al*, 2023).

S. aureus α-toxin Neutralization

One of the most well-characterised exotoxins as an anti-virulence target is *S. aureus* pore-forming α-toxin due to its driving role in *S. aureus*-related disease manifestations and severity (see α-toxin mechanism of action in Figure 1, structures and host cell effects in figure 2) (Hsieh *et al*, 2023). α-toxin attacks many cell types, leading to cell hemolysis and tissue necrosis (Oliveira *et al*, 2018). This section will review progress and potential in α-toxin neutralisation with monoclonal antibodies, natural molecules and biomimetic nanosomes modalities that utilize extracellular binding mechanisms.

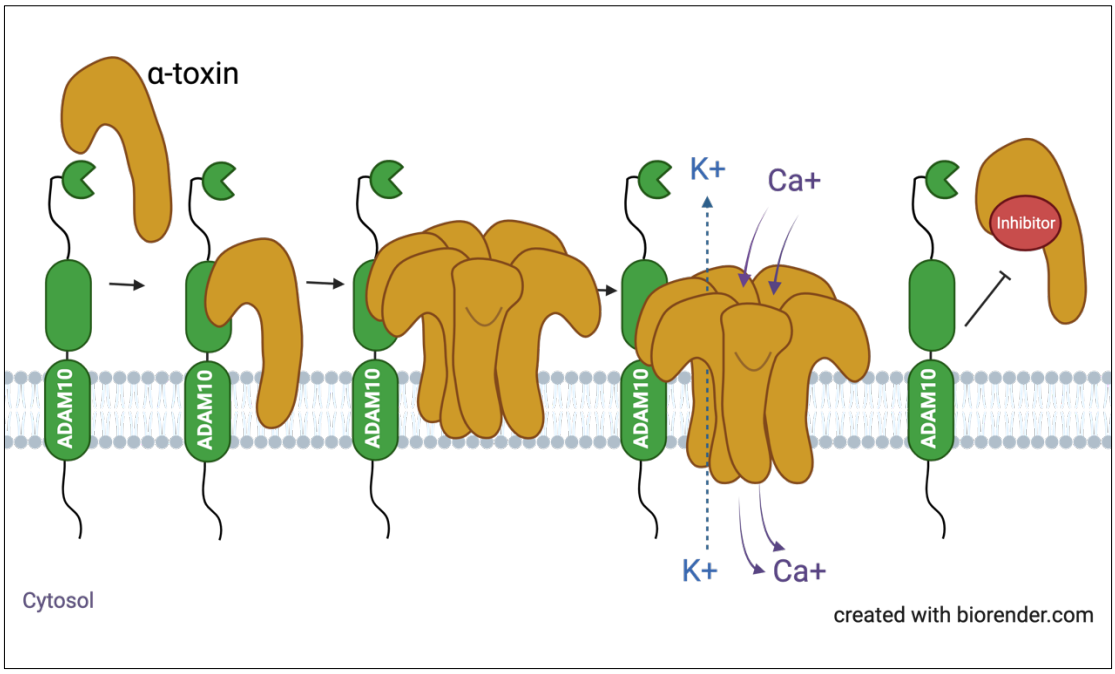

Figure 1. α-toxin pore-formation mechanism of action adapted from (Oliveira *et al.*, 2018). α-toxin is a 33kDa water-soluble polypeptide monomer. First, an α-toxin monomer binds to Adam10, a metalloprotease found ubiquitously on the membrane of many human cell types. Then, other six monomers join and oligomerize into a pre-pore β-barrel structure, which is then extruded through the membrane to form a hydrophilic transmembrane channel. This causes the rapid release of intracellular ATP and K^+ out of the cytoplasm, followed by an influx of Ca^{2+}. Subsequent signalling cascade leads to activation of pro-inflammatory pathways and cell lysis.

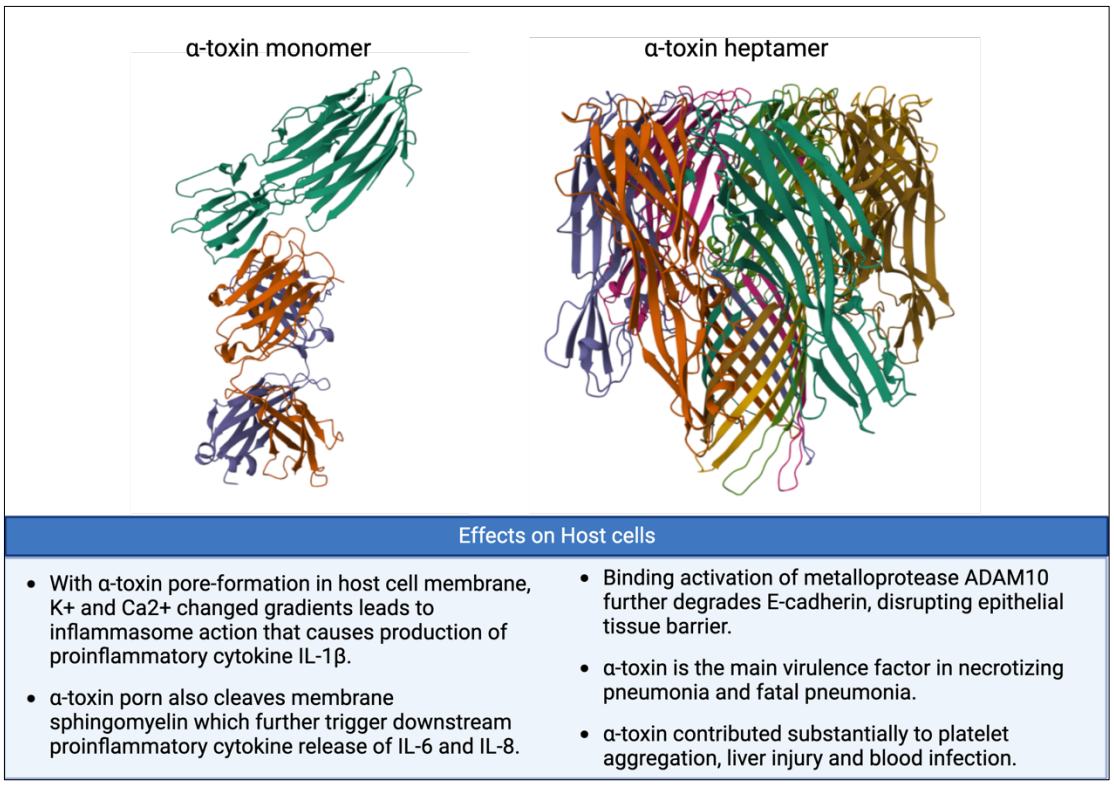

Figure 2. The X-ray structure of α-toxin monomer (PDB 41DJ) and heptamer (PDB 7AHL) structures from Protein (Foletti *et al*, 2013; Song *et al*, 1996), and α-toxin effects on host cells adapted from (Hsieh *et al.*, 2023).

Monoclonal Antibodies (mAb)

MEDI4893, a humanised mAb that neutralizes α-toxin, showed a significant relative risk reduction (RRR) of 47.4% (p=0.075) in the incidence of ventilator-associated pneumonia vs. placebo in *S. aureus* colonisation of lower respiratory tract in Phase II clinical trials amongst patients aged 65 years or younger, with no difference in adverse events between treated and placebo-treated groups (François *et al*, 2021). Despite MEDI4893 showing a non-statistically significant RRR between the overall treated vs placebo-treated group, Phase III clinical trial (NCT05331885) is underway (François *et al.*, 2021). Earlier preclinical structure characterisation study showed MEDI4893 bound to a highly conserved rim region of α-toxin to sterically block its binding to ADAM-10 receptor and also prevented its oligomerization (Oganesyan *et al*, 2014). Previously, in a pneumonia-mouse model, Hua *et al* (2014) also showed that MEDI4893 delivered significantly improved survival and lung function in both prophylaxis and therapeutic regimes, and MEDI4893 displayed synergistic results with vancomycin (systemic) or linezolid (subcutaneous) relative to monotherapy (Hua *et al.*, 2014).

Similarly, another human mAb AR-301 targeting α-toxin is currently in Phase III clinical trial (NCT03816956) as an adjunctive treatment for ventilator-associated pneumonia after being given FDA's priority review. However, no pre-clinical data was published (Aridis Pharmaceuticals, 2023; Deswal, 2023).

To date, α-toxin neutralization with monoclonal antibodies has progressed to clinical trials and showed clinical translation potential. Notably, four other monoclonal antibodies for toxin neutralization of other pathogens had also received FDA approval, beckoning hope for future development (Lau *et al*, 2023).

Biomimetic Nanosomes

Biomimetic toxin nanosponges (hRBC-NS), erythroliposomes and nanovesicles (ANVs) with sonosensitizer are nanotechnological innovations developed to combat α-toxin.

Chen *et al* (2018) developed biomimetic toxin nanosponges (hRBC-NS) consisting of polymeric nanoparticles coated with human red blood cell membrane to circumvent host immune response and mimic red blood cells (RBCs) as decoys for bacterial toxin binding and adsorption. hRBC-NS (CTI-005) has been approved by the FDA for Phase 1b/2a clinical trial (Cellics Therapeutics, 2022). hRBC-NS demonstrated *in vitro* hemolytic inhibition ability across four structurally distinctive pore-forming toxins – melittin, α-toxin (*S. aureus*), streptolysin O (Group A *Streptococcus*) and listeriolysin O (*Listeria monocytogenes*). Furthermore, in a systemic-infected-mouse model, the survival rates of the mice were very significantly better in both prophylaxis and therapeutic regimes compared to non-treated groups where survival was 0%. As further reinforcement, Chen *et al* (2019) demonstrated the neutralization ability of hRBC-NS *in vitro* and *in vivo* using whole-secreted proteins (wSP) of methicillin-resistant *S. aureus* (MRSA) with similar good results.

Another biomimetic nanosponge is erythroliposomes. In contrast to polymeric nanoparticles, erythroliposomes contain artificial PEGylated lipid membrane infused with red blood cell (RBC), with the promise of greater stabilisation by PEGylated lipid membrane, easier modification and scale-up production through an extrusion

process (He *et al*, 2019). The group demonstrated superior *in vitro* neutralisation ability of erythroliposomes on α-toxin compared to PLS (PEGylated lipid vesicles) and RMVs (RBC membrane vesicles) in hemolysis assay. Interestingly, He *et al* compared the neutralization capability of erythroliposomes to be 139-fold more than Chen *et al*'s nanosponges (hRBC-NS) made from one RCB. Subsequently, in the systemic lethally-infected-mouse model, erythroliposomes-treated mice showed survival of 80% and 40% in prophylaxis and therapeutic regimes respectively, compared to no survival for untreated, RMVs or PLS.

Pang *et al* (2019) developed biomimetic nanovesicles (ANVs) with sonosensitizer meso-tetrakis (4-sulfonatophenyl) porphyrin (TPPS) encapsulated together with fractions of cell membrane displaying mAb MEDI4893 that target α-toxin actively. Moreover, the TPPS generates reactive oxygen species (ROS) for bactericide upon ultrasound exposure. ANVs showed significant in vitro hemolysis reduction compared to NVs (cell membrane fractions infused with sonosensitizer nanovesicles) and RBC NVs (RBC fractions infused with sonosensitizer nanovesicles) without impacting on *S. aureus* survival, and yet with ultrasound, ANVs exhibited very significant bactericidal action. Furthermore, this technique allows real-time monitoring using magnetic resonance imaging (MRI). In a myositis-mouse model, ANVs showed high specificity in targeting infection sites and discriminated infection from sterile inflammation compared to free TPPS or NVs (sonosensitizer nanovesicles). Furthermore, ANVs significantly repressed infection progress without ultrasound and eradicated thigh muscular abscesses with ultrasound.

Nanotechnology encapsulation has brought impetus to both specific and broad-spectrum toxin neutralization for future clinical applications.

Natural molecules

Natural products as anti-virulent agents against α-toxin have made some research progress with aloe-emodin, myricetin and ligustchuane B.

Jiang *et al* (2019) demonstrated aloe-emodin as a potential anti-virulent α-toxin inhibitor. Aloe-emodin was first demonstrated to have no bactericidal effect on methicillin-resistant *S. aureus* (MRSA) within the tested concentration. Aloe-emodin

also showed significant RBC hemolysis inhibition (>90%) and cell-protecting ability *in vitro*. Molecular binding characterisation showed aloe-emodin molecular bound to α-toxin "stem" region that disrupted its oligomerization. Using an MRSA-infected pneumonia-mouse model, aloe-emodin treatment further showed significantly improved mice survival of 70% compared to placebo-treated at 20%, with significantly less bacteria load and pulmonary histopathology.

Flavonoid myricetin also exhibited some potential as an anti-virulent agent against α-toxin (Wang *et al*, 2020). Like aloe-emodin, myricetin did not affect bacteria viability within the tested concentration and demonstrated strong hemolysis inhibition *in vitro*. However, myricetin also down-regulated α-toxin production. Myricetin was shown to inhibit α-toxin oligomerization with binding but binding characterisation remained unclear. While *Wang et al. (2020)* further showed that myricetin down-regulation of phosphorylated proteins in MARK and NF-kB pathways, it was unclear if such mitigation was due to reduced α-toxin or myricetin. In the pneumonia-mouse model, Myricetin visibly reduced histopathology, but mortality nor bacteria load were not assessed.

Wan *et al* (2022) demonstrated bioactive ligustchuane B extracted from Chinese medicine Lingusticum chuanxiong could be a potential α-toxin inhibitor. First, the group showed that ligustchuane B has an in vitro inhibitory effect on methicillin-resistant *S. aureus* (MRSA) α-toxin haemolysis. Through molecular docking study, the group postulated that Ligustchuane B bound directly to α-toxin. Finally, the group further demonstrated this effect using an intraperitoneal-infected mice model where treated mice have significantly less bacteria load in the visceral organs and less pulmonary damage than untreated mice. However, the survival of mice was not presented. In addition, ligustchuane B effect on *S. aureus* survival was not presented.

It can be seen that aloe-emodin is the only potential anti-virulent α-toxin inhibitor while the other two candidates need further investigation.

P. Aeruginosa Type III Toxin Secretion systems (T3SS) Deactivation

T3SS is a needle-syringe apparatus commonly found in gram-negative bacteria (Fasciano *et al*, 2019). T3SS is the only secretion system for P. aeruginosa to inject cytotoxic effector toxins (ExoS, ExoT, ExoU and ExoY) into host cytoplasm, leading to cell death, and its functionality is closely related to the severity of disease manifestations (Figure 2) (Liao *et al*, 2022). As such, it is one of the most well-characterized virulence factors. This section will discuss the modalities of monoclonal antibody MEDI3902 and drug molecule fluorothiazinon targeting *P. aeruginosa* T3SS deactivation.

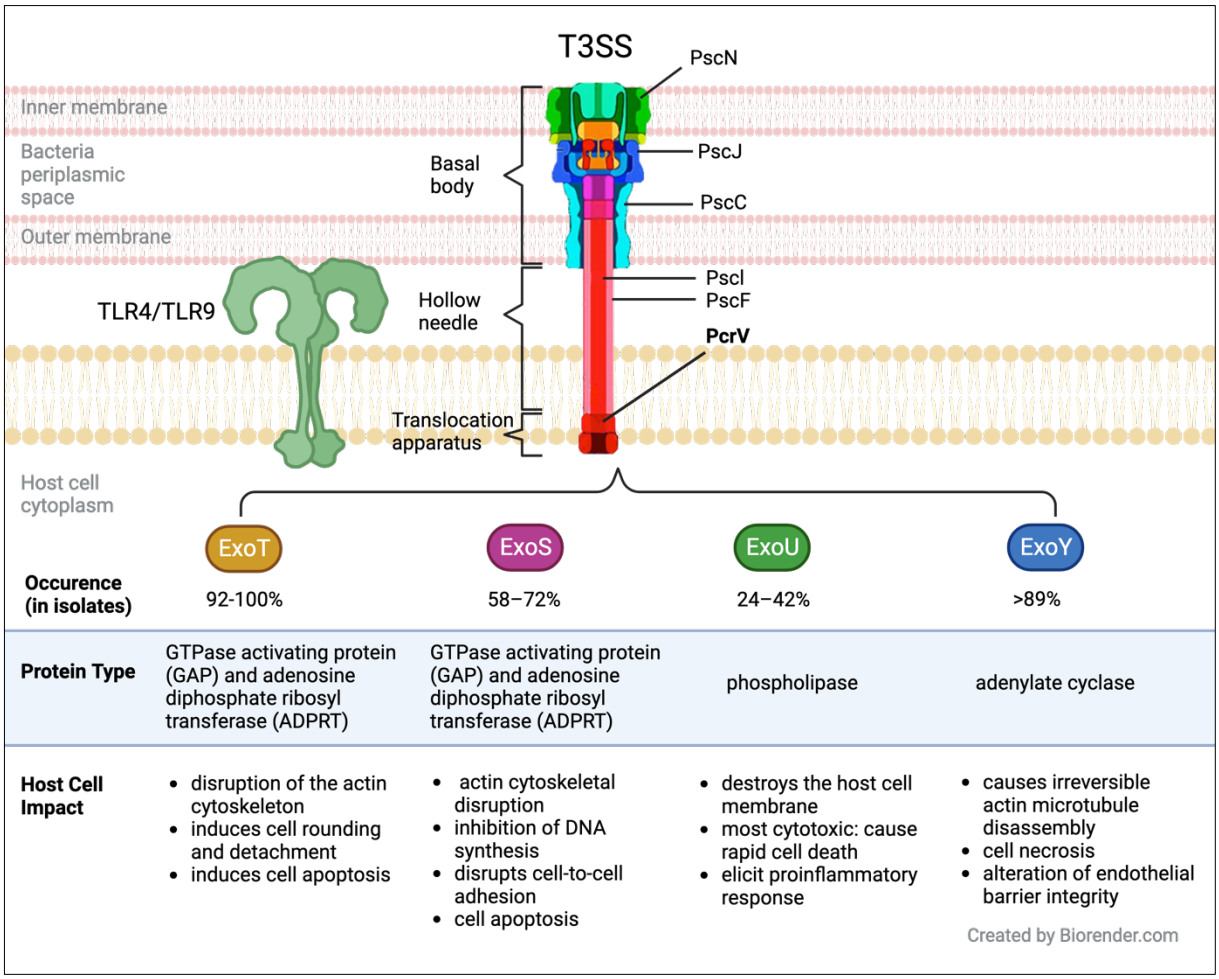

Figure 3. Type 3 Secretion System (T3SS) and host cell impact from injected cytotoxic effector proteins of *P. aeruginosa*. TLR 4/9 recognises LPS on *P. aeruginosa* and then activates T3SS. T3SS structure adapted from (Qin *et al*, 2022). Chart created from (Jurado-Martín *et al*, 2021; Qin *et al.*, 2022).

Monoclonal antibody (mAb)

MEDI3902 is a bispecific monoclonal antibody that combines the target of T3SS protein PcrV to inhibit T3SS injection into host cells and the exopolysaccharide Psl adhesion to epithelial cells (Le *et al*, 2018). Unfortunately, AstraZeneca halted MEDI3902 development as there was no significant reduction of nosocomial pneumonia prevention in mechanically ventilated patients vs placebo after Phase II (Chastre *et al*, 2020).

Small molecule

Another candidate that has reached Phase II clinical trial is fluorothiazinon, an adjuvant to Cefepime (NCT03638830). Sheremet *et al* (2018) demonstrated fluorothiazinon to be a promising anti-virulent T3SS inhibitor of *P. aeruginosa*. Fluorothiazinon showed an *in vitro* inhibitory effect on T3SS secretion of ExoU and ExoS and cell internalization of *P. aeruginosa.* Furthermore, in an airway-infection mouse model in both therapeutic and prophylaxis regimes, fluorothiazinon significantly improved survival rate. Likewise, the bacteria load in the lungs and spleen were significantly reduced vs untreated mice, and blood infection was completely cleared compared to untreated mice. Correspondingly, pulmonary damages were more pronounced for untreated mice. Moreover, in two previous studies on chlamydia and salmonella, fluorothiazinon showed suppression ability of T3SS for *in vitro* and *in vivo* experiments yet had no impact on bacteria growth, promising a wider scope of therapeutic application. Fluorothiazinon was elucidated in earlier research to block the secretion of T3SS effector proteins (Zigangirova *et al*, 2012).

Despite the disappointment of MEDI3902, fluorothiazinon is a promising anti-virulent agent against T3SS.

Advantages and Disadvantages of anti-virulence approach

Table 2 lists both the advantages and disadvantages of anti-virulence approach in the light of comparison with antibiotics or other therapies that might elicit bacterial resistance development. The core principle of the anti-virulence approach is a double-edged sword where the benefits of non-annihilation of bacteria became a burden in its struggle to reach conventional clinical success. Considering that

pharmaceutical companies already have dwindled interest in antibiotics, anti-virulent agents would be of an even lower priority for their pipeline development. Furthermore, the disadvantages of limited scientific knowledge and complexity are not limited to anti-virulence approach and exist in any untrodden therapies. Consequently, while anti-virulence research undoubtedly needs to establish clear success criteria and standardized methodologies of evaluation and validation, the commercial burden must be prioritized by AMR government agencies and regulatory approval agencies such as the FDA. This can be in the form of research funds, alternative clinical trial endpoints and specialised "fast-lane" approvals. In the face of a myriad of virulence factors of an infection, a single bullet anti-virulent agent would be hard-pressed, hence, alternative experimental testing needs to be developed for deployment of multiple anti-virulent agents which could subsequently be translated into clinical trial testing.

Table 2: Advantages and Disadvantages of Anti-virulence Approach

Advantages	**Disadvantages**
i. Anti-virulence resistance can be limited in spread possibly if the inhibition is mechanistic binding inhibition (Rezzoagli *et al.*, 2018).	i. No anti-virulent agent is employed in clinical settings and very few in clinical trials (Lau *et al.*, 2023). Compared to diseases with limited alternatives e.g. cancer, standard-of-care antibiotics largely remain effective which muted the urgency.
ii. Targeting specific non-survival-dependent or public good virulence factors could impede horizontal gene transfer of resistance gene, thereby reducing resistance spread (Ross-Gillespie *et al*, 2014).	ii. Identification and characterisation of key virulence targets across pathogens are still lagging and may not be well-understood (Theuretzbacher & Piddock, 2019), which may hinder therapy development.

iii. Anti-virulence modalities can range from narrow-spectrum, high affinity e.g. mAb (Aridis Pharmaceuticals, 2023; François *et al.*, 2021) to broad-spectrum yet effective, e.g. nanosponges (Chen *et al.*, 2018).	iii. Limited knowledge of the interplay between bacteria microbiological systems, their virulence factors, bacteria inter-species interactions and human immune response (Theuretzbacher & Piddock, 2019). This complicates the elucidation of potency, side effects and anti-virulence resistance.
iv. Antibiotics dosage is reduced with synergistic anti-virulent agent adjuvant, thereby further reducing the risk of antibiotics-resistance development (Parra-Millán *et al*, 2018).	iv. Anti-virulence therapies cannot eradicate pathogens, thereby making it more difficult to establish superior endpoints in clinical trials (Theuretzbacher & Piddock, 2019). It also niches itself invariably as an adjuvant or as prophylaxis.
v. Extended antibiotics usage annihilates beneficial gut microbiota (Ramirez *et al*, 2020). Anti-virulent agents are harmless, and synergistic usage also attenuates such annihilation.	vi. Similar to antibiotics (Plackett, 2020), anti-virulence agents have a short duration of treatment that translates to lower profit per patient compared to other diseases e.g. cancer treatment. Consequently, being a less-profitable enterprise limits funding sources or buyovers by pharmaceutical companies that have the financial might to shoulder the substantial follow-up clinical trial costs.

Conclusion

Even though very few therapies have advanced into clinical trials, nevertheless there has been increasing research published on anti-virulence strategies in the last 20 years (Totsika, 2016). Endotoxin neutralisation and T3SS inhibition have a few promising candidates that can potentially translate into clinical therapies and some promising research studies with *in vivo* proof of concept. Nanotechnology also brought new innovations into the field, e.g., ultrasound-induced nanosomes. With expanding knowledge on bacteria microsystems and interplay with virulence factors in the advent of bioinformatics, the anti-virulence approach in future may take on a multi-prong approach of combining several synergistic anti-virulent agents to elicit better patient outcomes.

(1789 words)

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