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Navigating the Third Frontier of Antimicrobial Therapy to Support Women's Health

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Abstract

This paper explores one of the underappreciated reasons for lack of efficacy in certain cases of antimicrobial therapy, namely the occurrence of a non-genetic resistance to antimicrobial drugs due to a metabolic quiescence of microorganisms. This review has centered on those microorganisms of particular importance in obstetrics and gynecology and accordingly has reviewed the nature and extent of the persister phenotype in relation to infectious agents affecting women's health. We show how the quiescent persister microbial phenotype represents the next significant issue that could compromise successful antibiotic therapy. A brief history of antimicrobial therapy is provided as context for the problem posed by the persister phenotype. This review has been focused on the current literature having relevance for physicians concerned with women's health. The study of this phenotype has led to increasing understanding of the molecular mechanisms for this state which also provides ideas for rational development of drug candidates to interdict these organisms in human disease and explores the possibility of developing specifically targeted molecules to address persisters, research on screening botanicals, existing drugs and chemicals to discover novel approaches to the clinical consequence of microbial persisters. Of interest in this review, is the return to naturally occurring botanical substances, first to be used as anti-infectives, now being considered as possible agents to address persister microorganisms. Overall this paper aims to provide information tailored especially to the obstetrics and gynecology specialists.

Keywords

Gynecologic Infection, Obstetric Infection, Antibiotic Therapy, Antimicrobial Resistance, Quiescent Microorganisms, Mechanisms

1. Introduction

Infectious disease is no respecter of gender, despite the fact that there are exam-

ples of infectious diseases for which attack rates are greater in men than women and other examples in which women have the greater burden of disease. But the special consideration for women is those conditions specifically related to the reproductive apparatus and by extension, the possibility of infectious conditions spreading to or otherwise compromising the fetus with consequences greater in the fetus than in the mother.

The topic addressed here relates to both genders but as therapy is considered, the special case of obstetrical and gynecologic implications will be highlighted.

2. Before Germ Theory

From time out of mind, human history has recognized the concept of contagion, and astute observations led to approaches to deal with communicable diseases that were based on distance or avoidance. Communicable skin diseases were known to the ancients. Leprosy, for example, is believed to have been mentioned in Hippocratic writing (460 BC) and may have related to various skin conditions including what is known today as leprosy or by eponym as Hansen's disease. Quarantine, distancing or banishment was often involved and related, at least in part, to the recognition of communicability. Even though it was not until 1873 that the etiologic bacterium was discovered, the ancients treated leprosy as an infectious disease.

The mechanism of contagion would have to wait until the middle of the 19th century when Louis Pasteur and later Robert Koch were able to convincingly identify microorganisms as the pathogenic principal in specific diseases.

3. The First Frontier: Anti-Infective Substances

3.1. History

Logic would dictate that if microorganisms are able to cause human disease, a method for defeating disease would lie in defeating microbial pathogens. Quinine, found in cinchona bark, had been used to treat malaria by Andean peoples. Cinchona bark began to be imported to Europe in the 17th century, but its use actually predated Alphonse Laveran's recognition in 1880 that a causative parasite was involved in this disease. Certain poisons such as mercury salts were introduced in 1496 for syphilis, even though *Treponema pallidum* would not be identified until 1905. Five years after the discovery of the spirochete, Hata and Ehrlich introduced an arsenical, salvarsan, which was the first effective medication for syphilis.

Paul Ehrlich, a Prussian Doctor working in the latter years of the 19th century found that chemical dyes, particularly methylene blue, had an affinity for microorganisms and reasoned that there could be other dyes or chemical compounds that might be discovered or developed with specificity for microorganisms. His recognition that dyes had selectivity for cellular components led him to the astute conclusion that unless a compound can bind to the etiologic microbe, it cannot exert a damaging effect on it. In particular, he expressed hope that he

would discover "*therapia sterilans magna*" or a general sterilizing agent that may come through the development of a compound that specifically attached to pathogen cells as methylene blue did in the case of *Plasmodium*. Not surprisingly, the early work on chemotherapeutic agents focused on dye molecules, most notably pursued by Bayer in Germany. The concept of Ehrlich became popularized in his term "charmed" or "magic bullet". Combining his knowledge of chemistry and medicine, he was involved in developing the aforementioned arsphenamine as an improved treatment for syphilis. Perhaps as important as the drug itself was the birth of the concept of chemotherapy for infectious disease.

Syphilis, it should be pointed out, has been endemic since about 1500 and has been responsible for unaccountable levels of morbidity and mortality over the centuries. One particular concern continuing up to the present is congenital syphilis, which can result in fetal demise or severely compromised offspring. Despite the availability of penicillin as the most effective therapeutic, in 2017 congenital syphilis reached a 20 year high in the US with more than 900 babies infected [1]. This occurs in the face of effective methods for screening, even in a high-resource nation.

Despite the unfortunate fact that syphilis treatment opportunities are sometimes missed, the historical legacy of the Ehrlichian concept of a magic bullet is modern day chemotherapeutic and antibiotic compounds. These therapies have saved countless lives and have become the mainstay of treatment and for some diseases, and prevention. Chemotherapeutics such as sulfonamides became available around 1935 and were used for streptococcal infections and in relation to women's health were used to treat "childbed fever" which was predominantly the result of Group A streptococcus. Their use was limited by several factors that included the bacteriostatic effect, allergies and limitations due to endogenous folic acid.

The antibiotic concept was expanded when penicillin was discovered by Alexander Fleming in 1928 (though practical use would have to wait until 1942), who showed that an inhibitor of *Streptococcus* was elaborated by another microorganism—a mold. Accordingly, many more naturally-occurring antimicrobials and synthetic and semi-synthetic antibiotic usage ensued. By the middle of the twentieth century it seemed infectious disease was destined to be conquered if only the right drug compounds might be discovered or invented.

3.2. Antimicrobials of Mono-Etiologic Infections in Women

Antibiotics have had an important impact on some of the infectious disease threats to women and infants. As noted above, penicillin has remained the mainstay of syphilis therapy and is guided by detailed protocols in the CDC literature which very specifically addresses syphilis in pregnancy and neonates. It is striking that penicillin remains the required treatment more than 70 years after its discovery.

Likewise, Group B streptococcal infection of infants with early or late onset presentation, with its grave consequences, has been addressed by prophylactic use of penicillin or ampicillin. This is employed in conjunction with testing protocols during pregnancy. The major reason for using vancomycin as an alternative to penicillin or related compounds is penicillin allergies and not the lack of susceptibility of the organism to beta-lactam antibiotics.

Another Gram-positive pathogen of concern in pregnancy is *Listeria monocytogenes*, which causes a 13-fold greater infection rate among pregnant women compared to the general population. Overall cases of infection are still low, in the range of 0.27/100,000, according to the CDC. This foodborne illness is usually prevented through avoidance of some foods and careful preparation and handling of foods. However, when an antibiotic is indicated the drug of choice is ampicillin.

The intrinsic susceptibility of microorganisms to the inhibitory action of antimicrobials defines the spectrum of activity for the antibiotic. While the spectrum of activity can be relatively stable for some microbe-antibiotic combinations as indicated above, the occurrence of antibiotic resistance represents a challenge to therapy as discussed below.

3.3. Antimicrobial Therapy of Polymicrobial Infections in Women

Particular to women's health, are endogenous infections that arise from bacteria that are commensal in the lower genital tract. Some of these organisms are known to be found as polymicrobial communities involved in pelvic inflammatory disease, tubo-ovarian abscesses, post-hysterectomy surgical infections, chorioamnionitis, postpartum endometritis and other related infections. Prior to the advent of molecular methods for characterizing the cervical and vaginal microbiota, culture-based techniques demonstrated that among healthy women, Gram negative facultative organisms as well as Gram negative and positive strictly anaerobic bacteria could be present. These organisms could become the polymicrobial inoculum in infections arising after procedures or parturition. Because cultures of organisms involved in endometritis and post-hysterectomy infections resembled the lower genital tract, empirical therapy was based on obtaining a spectrum of activity to cover most of the common microbial species.

Early work done on postoperative and postpartum cultures in women demonstrated that in addition to bacteria present in the lower genital tract prior to the procedure, within a few days from the operative event a proliferation of Gram negative facultative and anaerobic organisms was observed. This phenomenon informed both concepts of therapy of post-procedure infections, but also suggested how prophylactic antibiotics might be used to interdict this shift in lower tract microbiome composition [2] [3].

Antibiotic treatment regimens that covered Gram negative and positive as well as anaerobic organisms led to empiric use of combinations of antibiotics such as clindamycin-gentamicin, or for monotherapy, ampicillin-sulbactam, or

cephalosporin such as cefoxitin with spectrum extending to Gram negative anaerobic organisms. Thus, the first frontier for therapy involved the discovery or development of a large array of antibiotic compounds, an expansion of the understanding of which organisms are involved in obstetrical and gynecologic infections and the antimicrobial spectrum inherent in the therapeutic drugs.

4. The Second Frontier: Drug Resistance

4.1. Resistance—Antibiotic's Other Property

Some of the greatest challenges in antimicrobial therapy have come with acquired antibiotic resistance, though resistance mechanisms, particularly to beta-lactam antibiotics, have existed before the discovery of penicillin. A more expansive discussion of this topic may be found in the excellent review by Bush, which summarizes the large diversity of beta-lactamases known currently and the parallel development of new drugs [4]. Resistance mechanisms to other classes of antibiotics likewise have challenged physicians as the number of antibiotic resistant strains are continuously emerging.

Several approaches to antimicrobial resistance have occurred in parallel with focus on various topics. While the intent here is not to review each of these, it is useful to realize that the epidemiology of drug resistance has documented the existence and increases seen in clinical settings. One approach to antibiotic resistance has been the development of semi-synthetic analogs of antibiotics and as well as discovery and development of new classes of drugs, perhaps no better illustrated than in the early development of beta-lactamase-resistant penicillins. Because antibiotic use and abuse introduces selective pressure for increased prevalence of antibiotic resistant strains, the whole topic of antibiotic stewardship has developed and ecological considerations, including antibiotic use in agriculture, has played a part in the overall topic of antibiotic resistance. Finally, understanding of the chromosomal and non-chromosomal mechanisms of resistance as well as transferring resistance among strains has been vigorously pursued by molecular biology approaches.

Perhaps one of the most volatile areas of drug resistance pertains to *Neisseria gonorrhoeae*. In a historical review, Hook and Kirkaldy note that, "…development of resistance to antibiotics widely used for treatment appears to be an inexorable process" [5]. Both genders are affected by this sexually transmissible pathogen, but certain aspects of morbidity seem to have special relevance to women, including the tendency toward asymptomatic occurrence, progression to upper tract complications and the possibility of intrapartum infection of vaginally delivered infants. A review by Unemo and Schafer invoked the term "super bug status" as they presented a timeline of the demise of the clinical usefulness of emerging (then declining) therapies including sulfonamides, penicillin, penicillin plus streptomycin, spectinomycin, tetracycline, erythromycin, azithromycin and fluoroquinolones [6]. Current CDC guidelines for uncomplicated gonorrhea specify dual therapy with ceftriaxone and azithromycin but continued modifica-

tions in therapy will undoubtedly be required in the future.

4.2. Resistant Organisms and Women's Health

Antimicrobial resistance is also a concern for *Candida albicans* which is opportunistic, being at times a lower genital tract commensal as well as under alternate circumstances a source of vaginal symptoms. Other less frequently encountered *Candida* species may also have limited susceptibility to antifungal drugs. Multiple mechanisms are in play including efflux pumps, mutations involving cell wall biosynthesis, and physical barriers to antifungal drug penetration in the form of biofilm. While the classes of antimicrobials available to treat fungal infections are limited and amphotericin B, for example, requires cautious use due to toxicity, a number of novel approaches to defeating antifungal drug resistance are being explored as described in great detail in a recent review [7].

Fortunately, Toxoplasmosis is an uncommon threat to pregnancy in the US but remains highly prevalent elsewhere in the world and is a known threat for congenital infection. According to recent information there are known treatment failures and demonstrated resistance to pyrimethamine and sulfadiazine and clinical consequences remain under scrutiny [8]. Another parasite that is a threat in pregnancy is *Plasmodium*, especially in *falciparum* malaria. Current concerns about drug resistance are related to the presence of strains with *P. falciparum* chloroquine resistance transporter (PfCRT), which has been reported to alter malaria treatment in some geographical areas [9].

While the emphasis thus far has been on the refractoriness of individual microbial species to antibiotic or antifungal drugs, the lower female genital tract is known to have a diverse microbiome and dysbiotic conditions are associated with symptoms as in the case of bacterial vaginosis. The first descriptions of bacterial vaginosis (BV) were accompanied by the discovery of an organism now known as *Gardnerella vaginalis* and there was enthusiasm at that time that this organism was the etiologic agent of what was then called "non-specific vaginitis". Over many years and with both debate and fundamental research, it became accepted that BV represents a state of microbial diversity with a diminution of lactobacilli and an abundance of anaerobic organisms. In the context of this paper, it should be noted that the antibiotic treatment of this condition is oral metronidazole or topical clindamycin, but treatment is not directed toward just one microbial species. In contrast to more classically understood infections, this is a polymicrobial condition and the goal of therapy is not necessarily directed at *Gardnerella vaginalis* alone, but at a microbial community.

BV is also characterized by the presence of a biofilm which is also polymicrobial and the biofilm is considered to be an important part of the condition and likewise an important part of maintaining the microbial community. In addition, because of the refractoriness of biofilms to antibiotic therapy, the biofilm is considered to be a significant element in recrudescent or recurrent BV. BV is common among reproductive aged women and is a risk factor for other conditions such as pelvic inflammatory disease, preterm birth and postpartum infection and is a continuing

concern for the obstetrician-gynecologist. The biofilm that complicates antibiotic therapy is part of a larger matter which is considered in the next section.

5. The Third Frontier: The Persister Phenotype

Genetics plays a very large role in the overall phenomenon of microbial resistance to therapeutic antibiotics through a broad variety of mechanisms. Chromosomal or extrachromosomal mutations affecting the target of an antibiotic may be ways of genetically acquiring resistance. The results of these genetic changes lead to a strain that propagates its resistance through future rounds of replication. Efflux pumps are also involved in some cases of resistance to antimicrobial drugs and these may have a genetic upregulation of an existing gene. But in this section, emphasis is given to a mechanism of refractoriness to antimicrobial therapy that is epigenetic and related to phenotype rather than genotype.

An excellent working definition of the persister is provided by Barrett and co-workers: "A subpopulation of slow-growing or growth-arrested cells that have a decreased susceptibility to killing by normally effective cytotoxic agents. Persisters survive treatment with drugs because of their altered metabolism during a temporary state of quiescence and are genetically identical to other, drug-susceptible cells in the population. Persisters may arise stochastically, or in responses to environmental cues such as nutrient starvation" [10].

5.1. Elaborating the Persister Phenotype

One of the first general concepts taught in microbiology is the kinetics of microbial (bacterial) growth, which takes the form of the growth curve that consists of the familiar lag phase, logarithmic phase, stationary phase, and the death phase. The classical growth curve is readily demonstrated in a closed system, such as a broth culture containing a finite amount of nutrient and with no means of eliminating bacterial end products. Natural environments—including infected living hosts—are open systems and would not be expected to have such a precise population dynamic. But the discovery of quorum sensing is somewhat analogous to the stationary phase in closed cultures, and its characteristics have shaped the understanding of many infectious processes.

Microbial ecologists have long known that in natural environments, microorganisms undergo attachment to surfaces, growth in situ, quorum sensing and formation of biofilm. Biofilms are characterized by physical organization that allows them to resist removal through shear forces. The organisms within the biofilm matrix also show a high level of refractoriness to antibiotics and disinfectants. The differences between planktonic growth and biofilm have genetic determinants that guide organisms' transition to this highly resistant state. The three dimensional structure of biofilms and the extracellular matrix that both fixes the organism to a substrate and provides a barrier has often been cited as the reason why biofilm organisms are refractory to disinfectants and antibiotics.

However, the abundance of persisters within the biofilm matrix are also in play. The process of biofilm formation has elements related to microbial stress responses which provide for microbial survival during adverse conditions and, as will be noted later, processes in biofilm formation and persister formation have overlapping elements.

Biofilm has profound implications for medicine, first as an organized mass of microorganisms that form on abiotic surfaces of medical devices such as catheters or other implanted materials. The organisms that are part of the biofilm are difficult to eradicate, but also provide a potential source of systemic infection when viable organisms break away. In addition, biofilm can form on biotic structures. The heart valve vegetation long known to be associated with alpha hemolytic streptococci represents the first recognized medical problem associated with biofilm. Other normally colonized sites such as alimentary tract and genital tract can host biofilms as well, and these have been associated with difficulty in providing therapy to the patients colonized in this manner. In contrast, biofilms may be a mechanism through which beneficial indigenous organisms can stably associate with a host, which indicates that biofilms are not always threats to health.

Related to the altered state of microorganisms in biofilm is a concept that had its roots in the earliest history of antibiotic use. Early observations with penicillin showed that cultures exposed to this bactericidal treatment were accompanied by killing of microorganisms, but the process was not complete. This same phenomenon was observed with other physical and chemical sterilization methods. As currently understood, microorganisms possess a pathway that leads to some members of a microbial community to enter a state of dormancy and instead of multiplying as rapidly as the environment will support, some stochastically-determined percentage of planktonic cells exist in this indolent state, which renders them insusceptible or tolerant to antibiotics and other antimicrobial agents.

5.2. Semantic Cautions

As mentioned above, survivor populations in antibiotic-treated *Streptococcus* cultures were recognized decades ago. Describing these cells as persisters rather than survivors has become a more recent nomenclature. Beyond the strictly academic interest in persistership, there is obviously a clinical consideration as well. The clinical implication of this particular microbial phenotype is the possibility that recurrent or recrudescent symptomatic disease may arise from the presence of persisters re-growing after apparently effective therapy.

In a semantically pure sense, persistent disease is to be distinguished from the term persister referring to the microbial phenotype, despite the fact that persistent disease may be the result of persister microorganisms. However, it is also possible that persistent or recurrent disease may arise from pharmacologic considerations such as early termination of a bacteriostatic drug or from genetically

resistant organisms representing that second frontier of treatment. The latter situation could be confirmed by re-isolation of the causative microorganism coupled with evidence that the organism is stably-resistant to the therapeutic drug.

Perhaps no better source for clarity surrounding the distinction between persister organisms and persistent infection and the relevance and relationship between them will be found in the treatment of the topic by Zhang [11]. Here is also described two types of persisters, non-growing and slow growing and the relationship to other phenomena such as bacterial L-forms and biofilm bacteria. The purpose of this paper will be best served by considering the persister phenotype generally and unless otherwise noted, will not focus on different persister types. In addition, it should be noted that additional terms (in addition to survivor) have been applied to persisters, including dormant cells, quiescent cells or inert cells.

5.3. Breadth of the Persister Phenomenon

Research on persisters shows that these organisms are associated with a vast array of bacterial species and also extend to fungal pathogens as well. Zhang describes it as a phenomenon common to all bacteria. It would follow that even though the persister phenotype has not been demonstrated in a particular microbial species, it may nevertheless occur and could link to prolongation of clinical diseases [11]. Demonstration of the persister phenotype among bacteria requires experiments conducted on organisms that are capable of laboratory cultivation, and some of the organisms of interest relative to women's health may be difficult or impossible to cultivate. As with organisms only identified through molecular taxonomy, it may be possible to predict the ability of organisms to elaborate persisters through pathways identified by gene sequencing, even among organisms that cannot be cultivated.

Because persister organisms have a relationship to persistent disease, it becomes a collateral interest to know why some diseases do not seem to be persistent (for example streptococcal pneumonia) versus others that are persistent (like tuberculosis). This worthy topic will not be explored in this paper, but probably at a minimum, involves differences in the manner in which the innate and adaptive immune systems handle different microbial species *in vivo*.

In addition to the assertion that persister generation applies to all bacteria, it also applies to eukaryotic organisms as well. Among the organisms with relevance to women's health, *Candida albicans* and other *Candida* species are of significant importance and have been extensively studied for their pathways to persister generation [12]. As noted in the next section, eukaryotic and prokaryotic mechanisms of persister generation differ, but may have similar implications for chronicity of infectious disease. Protozoan persisters have been described and even in the case of cancer cells refractory to chemotherapy, persister cancer stem cells may play a role in prolongation of disease [13] [14].

Some viral diseases such as those caused by the Human Herpes Virus, have mechanisms for prolonging the life of their host cells [15]. Cytomegalovirus, a concern for pregnant women who may transmit the virus to their offspring transplacentally, is able to induce a specific phenotype of macrophage that is long-lived and resists apoptosis [16]. Other examples that have unique molecular mechanisms exist, but will not be elaborated here. To focus more directly on pathogenic microorganisms that have a specific persister phenotype it will be useful to consider the epigenetic and metabolic properties of bacterial and fungal persister cells.

5.4. Metabolic Mechanisms of Persistence

Bacterial persister phenotypes have a "high tolerance" to antibiotics and are able to survive various doses of bactericidal or bacteriostatic treatments. Once the antibiotics are removed from the environment, the bacteria will begin dividing again and resume the metabolic processes of normally functioning cells. These persisters can also survive other environmental stressors, including nutrient depletion. The persister phenotype is a dormant state that enables the bacteria to survive the environmental stress and antibiotics without reliance on any antibiotic-resistance genes. The general consensus developing around the persister phenotype in bacteria relates to a model known as the Toxin-Antitoxin (T-AT) model. In this system, the Toxin downregulates cellular activities such as replication, biosynthesis and growth. The toxin suppressed by the antitoxin under desirable conditions such as logarithmic growth, in the presence of nutrient repletion. However, under stringent conditions, the Antitoxin is degraded. This allows the Toxin to apply brakes to the cellular systems and prompt the cell into dormancy [17]. Klimina *et al.* found that numerous strains of *Lactobacillus rhamnosus* had T-AT systems, therefore driving these strains to express persister phenotypes to evade and survive environmental stressors [18]. From the standpoint of gynecology, probiotic lactobacillus may be spared under conditions of environmental stress. Conversely, the T-AT system may promote the survival of pathogenic organisms under the stress of therapeutic drugs.

Yeast species have also been found to contain persister phenotypes, but fungal persistership is mechanistically different from that of bacteria. Yeast persisters can survive antimicrobial agents too, and resume their normal cellular activities and growth once the agents are removed. Eukaryotic persisters, like prokaryotic persister phenotype, are not genetically transmitted. The metabolic machinery that allows for persistence is genetically programmed, but the persister state itself is not inherited. It should be mentioned that some mutations have been found in yeast to promote a propensity to produce persisters (high persister strains) and the propensity is heritable [19] [20].

In yeast species, the target of rapamycin complex 1 (TORC1) pathway alters cell growth by various mechanisms: cell cycle initiation and translation of ribosomal proteins. TORC1 itself is in turn regulated by rapamycin and cell nutri-

ents. Rapamycin treatment induces a stressful environment for the yeast and increases expression of the persister phenotype. Bojsen *et al.* did a study with *Saccharomyces cerevisiae* mutants, which had inhibited TORC pathways and found that they had an increased persistence after amphotericin B treatment. This result made it appear that amphotericin B induces persister phenotypes itself by applying environmental stress. *Candida albicans* and *Candida glabrata* have been found to have a higher persister phenotype when exposed to rapamycin. When yeast cells are subjected to a stressor, the TORC1 pathway is inhibited or downregulated, allowing the cell to enter a dormant phase and take on a persister phenotype [21].

5.5. Identification of Persister Organisms

The percentage of a population growing in culture which are persister cells seems to be smallest in logarithmic planktonic growth. As a culture reaches its stationary phase, when replication and death rates equalize, more persisters are to be found. Finally, when microorganisms form biofilm, the biofilm is likely to harbor the highest percentage of persisters.

As with studies of the bacterial growth curve, persistership may best be demonstrated in vitro and perhaps cannot truly and irrefutably be demonstrated *in vivo*. Demonstrating persisters is closely related to the definition of persister, which is a cell that remains viable under conditions that ordinarily render cells in the same culture non-viable. In other words, if an antibiotic is added to a growing culture of bacteria and the culture is allowed to incubate until killing should be complete, subculture shows that a number of cells will be able to recover in fresh media, though their recovery will be characterized by a very slow growth rate. This release from the persister state also seems to vary within the same culture, with some individual cells rebounding faster than others. Persister resuscitation results in small colonies described as "deep persisters" and larger colonies described as "shallow persisters" while viable non-persisters produce the largest colonies.

This phenomenon can be demonstrated on agar plates on which a presumably sterilized culture is plated. Over a period of time small colonies will begin to form, much slower than normal colonies would. These colonies, though small, will be of varying sizes which implies that persisters recovering from their persister state have differing levels of indolence, even in a pure culture of the same species. This variation among persisters has led to the concept of deep and shallow persisters, somewhat analogous to deep or shallow sleep.

5.6. Persisters in Obstetric and Gynecologic Infections

5.6.1. Bacteria

Many bacterial species are involved in infections or related conditions that impinge on the wellbeing of female patients. In the paragraphs that follow, we briefly catalog significant species that have been documented to produce persisters. If persisters have not been identified, we will discuss the evidence that

persisters are likely because the persister phenotype is considered a common feature of bacteria and the absence of persisters would generally be considered anomalous. This discussion has been organized generally in the order of prevalence of these organisms.

Bacterial vaginosis (BV) is one of the most commonly encountered conditions in women. While it is considered to be a dysbiotic condition of the vaginal microbiome, virtually all cases have an abundance of *Gardnerella vaginalis* in concert with a diversity of Gram negative bacteria and reduced abundance of *Lactobacillus* species. Although we have not identified specific reports of persisters among *Gardnerella*, several clinical features are consistent with a role for persisters. Despite its frequency, recurrent episodes are common, even after treatment with metronidazole or clindamycin. One reason for recurrent BV may be polymicrobial biofilm that includes *Gardnerella vaginalis*. Biofilms in general are considered to have a significant level of persisters as will be subsequently addressed further. This polymicrobial biofilm is characteristic of BV. Such a biofilm promotes recurrent infection in over 50% of patients within 6 - 12 months after initial treatment [22]. A 2010 experiment testing the response of the polymicrobial biofilm to the antibiotic moxifloxacin found that within 5 days post-treatment, a surge in *Gardnerella vaginalis* cell numbers due to persisting biofilm was identified [23]. Subsequent studies suggested inhibition of biofilm production using quorum-inhibiting agents or disruption of existing biofilm using boric acid could, in combination with bacteriostatic or bactericidal medications, provide improved efficacy in preventing recurrence [24] [25].

Urinary tract infection, frequently due to Gram negative enteric bacteria, may also be a recurrent clinical problem and persister organisms with mucosal biofilms are implicated. UTIs commonly occur during pregnancy with further complications such as pyelonephritis and increased risk of maternal sepsis, preterm labor, and premature delivery in pregnant women [26]. *Escherichia coli* is the most common bacterial pathogen of UTI in women and persister populations have been identified in a number of studies [27] [28]. Literature suggests that overexpression of chromosomal Type II TA toxin molecules may be related to development of persistership by inhibiting cell growth after exposure to environmental stressors [27] [29]. Although this is a well-studied phenomenon, it is unclear as to whether the TA system is the main mechanism by which persistership arises and the exact role of (p)ppGpp signaling (see later discussion) drives the persister generation [27] [30]. This persister-development model has been applied to different types of bacteria to explain how persisters arise and is currently being further studied.

Historically some of the earliest evidence that antibiotic survival was due to the persisters in a population was discovered among Streptococci. Group A Streptococcus (GAS) was scourge in obstetrics in the mid 1800s as "childbed fever", stemming from GAS infection, contributed to significant maternal mortality. Today GAS infections in developed countries are infrequent, but still cause about 40% of septic deaths among patients with postpartum endometritis, ne-

crotizing fasciitis, and along with *Staphylococcus aureus*, toxic shock syndrome [31]. Biofilm formation in GAS has been documented and hypothesized to be part of the cause for failed antibiotic treatment of GAS infections. The antimicrobial resistance of GAS biofilms can be partly attributed to the fact that cells within the biofilm have reduced metabolic activity, which typifies the persister phenotype [32]. *Streptococcus mutans* and *Streptococcus suis* persister phenotypes have been identified which furthers the notion that GAS organisms likely have persister phenotypes as well [33] [34].

Group B Streptococcus (GBS) is found within the gastrointestinal tract and genitourinary tract as a commensal organism in as frequently as 30% of women. GBS has declined as a cause of perinatal and neonatal disease due to aggressive screening and prophylaxis programs, but presence and continued colonization of women remains a cause for vigilance. Korir *et al.* state GBS is the leading cause of neonatal sepsis and meningitis, adding to the importance of treating and eradicating these infections in pregnant patients, which unfortunately may recolonize the vaginal flora even after antibiotic treatment. Different GBS strains can survive inside macrophages, evade the host defense and lead to disseminated infection. GBS surviving antibiotic treatment can also be partly attributed to its ability to form biofilms, which as previously mentioned hints at the fact that GBS can undertake persister phenotypes [35]. The variety of streptococcal species found to have persister phenotypes suggests a commonality that should encompass GBS.

Chlamydia trachomatis is another high-prevalence infection with significant consequences for the female patient. In addition to its prevalence as primary sexually transmitted infection, it is associated with the subsequent complications of salpingitis, tubal scarring, ectopic pregnancy, tubo-ovarian abscess and peritonitis. This Gram negative obligate intracellular bacterium has been shown to have an interesting persister form. *Chlamydia* is generally characterized as having two different forms, an infectious elementary body (EB) that is extracellular, and a replicative reticulate body (RB) that is intracellular. When exposed to stressors like iron deprivation, Beta-lactam antibiotics or Fosmidomycin, a third form for the bacteria, the aberrant body (AB), arises. When the stress is removed, the AB then converts back to its normal cell type and resumes cell growth and division. The AB is then considered to be a persister phenotype of *Chlamydia trachomatis* [36]. The persister phenotype allows *C. trachomatis* to evade antibiotic treatment and reestablish an infection once the drug treatment is finished. This knowledge of *Chlamydia* persistership needs to be utilized to create antibiotics that will directly target the persister cells and prevent recurrent infections [37].

Neisseria gonorrhoeae is less prevalent than *Chlamydia trachomatis*, but can also engender complications of pelvic inflammatory disease, tubal infertility, ectopic pregnancy and perinatal infections [38] [39]. A literature search did not show any studies directly identifying persisters, but because the history of antibiotic therapy for *Neisseria gonorrhoeae* focused mainly on genetic resistance, a

search for persister phenotypes deserves further exploration [38].

Described in the early 1970's, Lyme disease caused by the Spirochete of the genus *Borrelia*, is characterized by a chronic and recurrent set of symptoms and can be extremely challenging to treat even with appropriate antibiotic regimens. In a recent review, a detailed discussion of the persister state of *Borrelia burgdorferi* described morphologic and metabolic aspects of this organism as a response to antibiotic exposure suggesting that dealing with the persister state in these organisms may be exceedingly important in overcoming the chronicity of infection [40]. The authors also drew parallels to other chronic infections including leprosy, syphilis and tuberculosis.

With regard to women's health, Lyme disease is of concern, but certainly less studied than Syphilis, another spirochetal disease. A systematic review by Waddell *et al.* found 45 relevant reports in the world literature and while the numbers of cases were small, some adverse pregnancy outcomes related to Lyme disease were noted and some indication of transplacental infection was reported [41]. Syphilis, on the other hand, has a larger literature describing both maternal and fetal consequences. Because it cannot be cultivated, there is no demonstration of the persister phenotype in the literature, although there are references to bacterial persistence that seem to relate to persistent infection as opposed to the specific phenotype.

5.6.2. Fungi

Candida albicans is typically found in the vaginal canal as a commensal organism. However, overgrowth and the establishment of biofilm increases the risk of persistent infection [42]. The development of persister cells in infectious *Candida albicans* is well documented and is believed to make up 0.1% - 2% of the total number of cells in a biotic biofilm at any one time [43]. Recurrent vulvovaginal candidiasis (RVVC) refers to 3 - 4 consecutive *Candida* infections within a 12-month period and remains a clinically challenging entity. A 2019 study identified RAFT-derived polymethacrylates as a more effective treatment for RVVC than popular antifungals like clotrimazole and nystatin. Polymethacrylates have been found to successfully disrupt biotic biofilm formation on vaginal epithelium and therefore inhibit persistership [44]. This has significant implications for women who may suffer from undiagnosed or diagnosed HIV/AIDS, which increases the probability of contracting RVVC and recurrent oral thrush. Because of its generally significant role among immunocompromised hosts, the literature on this organism is voluminous and research efforts to study the persister phenomenon have fostered research but as yet no specific therapy for quiescent forms of the organism.

5.6.3. Protozoa

Earlier in this paper parasitic infections that are of concern in pregnancy were noted and specific parasites of interest were *Toxoplasma gondii* and the agents of malaria, especially *P. falciparum.* The existence of persisters and their refrac-

toriness to therapeutic drugs is considered consequential for these organisms and in a comprehensive treatment of the topic, a side-by-side comparison with characteristics of bacterial persisters is presented [10]. Bacterial persisters are genetically identical to non-persisters and for the majority of parasites reviewed, the dormant forms are genetically identical to the replicating form.

Hypnozoites are well-known stages that occur in *P. vivax* and apparently in the liver some stochastically generated forms have long periods of dormancy while others revert to schizogony readily. For *P. falciparum*, the ring form of the parasite is refractory to dihydroartemisinin. For *Toxoplasma gondii*, it is the bradyzoite that is the cognate of bacterial persisters.

5.6.4. Virus

Although viruses are unable to become "persisters" on the basis of metabolism in the same sense that bacteria and fungi can, viruses such as Herpes Simplex Virus-1 & -2 and Human Papilloma Virus (HPV) can persist in human hosts. One study states that approximately 90% of HPV-infected women will become HPV-negative after 2 years, although certain strains of HPV (such as HPV-16 & -18) are more likely to lead to persistent latent infection. This persistent infection can increase risk of cervical and anogenital cancers 20 years down the line [45] [46]. In order to maintain persistent infection in host's daughter cells, HPV E2 proteins tether extrachromosomal viral episomes to the host cell's mitotic chromosomes. This results in maintenance replication of viral genomes, which ensures that the virus remains in the host's nucleus without immune system detection and clearance [46]. Prophylactic vaccination with Gaurdasil has proven to be an effective measure in preventing infection by HPV-6, 11, 16, and 18.

Herpes Genitalis is caused by Herpes Simplex Virus (HSV)-1 and -2 and is transmitted through direct contact with infected people who have clinically manifested herpes genitalis or asymptomatic viral shedding. Primary infection of HSV is often asymptomatic and is followed by life-long persistent latent infection in the local sensory neural ganglions of the region infected. Latent HSV infection can be reactivated by psychological and environmental factors and often manifests as skin lesions at mucocutaneous sites. In the case of obstetric patients, both primary and recurrent HSV infection of pregnant women can lead to intrauterine viral transmission. This can lead to congenital HSV infection, abortion of pregnancy, or stillbirth [47].

5.7. The Broader Picture: Stress, Quorum Sensing, Biofilm, Persistership

5.7.1. Growth and Quorum Sensing

All of the life processes of living organisms are highly integrated and as noted previously, the persister phenotype is connected to metabolic activities that are highly regulated and metabolic processes connected to persisters, deserve mention as part of the overall place of persisters in the life and death of microorganisms. It is theorized that there are two general ways in which persisters arise,

stochastically within an active culture or those that are induced through environmental stressors. The spontaneous persisters are enigmatic, as they are challenging to research and characterize [48]. On the other hand, stress-induced persisters are receiving research attention because of the connection to therapy of human infections and refractoriness to antibiotics [48]. There are a multitude of stressors that promote persister generation including nutritional stress, oxidative stress, antibiotic treatment, heat shock and DNA damage, all resulting in organisms that survive adverse environments through quiescence [48] [49]. Stress, in turn, is connected to other processes including natural progression of organisms from exponential growth to the well-known stationary phase of the growth curve.

5.7.2. Biofilm

The stationary phase of the growth curve occurring in a closed environment is characterized by a depletion of nutrients, accumulation of end products of metabolism and the presence of large numbers of organisms. Quorum sensing is a mechanism used by organisms to determine the cell density of their population via autoinducers [50]. The autoinducers are "small diffusible signal molecules" that mediate cell-cell communication [51]. When the concentration of the autoinducers reaches and then surpasses a certain threshold, specific gene expression stimulates quorum-sensing-regulated processes. This means that once the population is large enough, biofilm formation and virulence processes are regulated via quorum sensing [50]. Bacteria present in biofilms are different from free-living bacteria in the sense that they have more tolerance to antibiotics and host immune response. It appears that these altered characteristics are because of the cells' coordinated efforts and activities that are controlled by quorum sensing [51]. The quorum of microorganisms can also sense the presence of a surface to allow the generation of biofilm.

As mentioned before, the T-AT system is involved in the bacterial stress response and can alter bacterial metabolism. It has been found that both biofilm and quorum sensing can also be influenced by various T-AT systems to help promote their communication and formation when the host is exposed to stress [52]. Persisters develop from planktonic bacteria collectively forming bacterial colonies within three-dimensional biofilms. Because persisters are found inside the biofilm's bacterial community, the host immune response to eliminate persister organisms initially rests on its ability to degrade biofilm itself. The composition of biofilm is that of a thick, aqueous mass of polysaccharides, extracellular DNA, proteins and lipids. Biofilm serves as a physical barrier that prevents host immune cells from penetrating and phagocytosing biofilm bacteria, thereby leaving non-persister and persister bacteria protected. Thus, dealing with biofilm in a clinical setting is a major challenge for the future [53] [54].

As a matter of clinical concern, the presence of antibiotics represents a stressor that may promote some viable organisms to enter the persister state. Investigators have conducted numerous detailed examinations of the mediators, path-

ways and results of generalized stress and these findings are informative in the context of antimicrobial therapy.

5.7.3. Generalized Stress

In the case of stress-induced persisters, the rise of antibiotic persistence is believed to be mainly due to nutritional or oxidative stress. In the case of nutritional stress, there is evidence that the deprivation of metabolic substrates causes an inhibition of normal metabolic processes and therefore suspends growth. Conversely, there is also maintenance of high-enough levels of ATP to sustain non-growth-related cellular processes in persister cells. This response to nutritional stress is referred to as the "Stringent Response" in the literature. In relation to oxidative stress, damage to bacterial DNA can elicit the "SOS Response", which can also promote persistership [48].

In bacteria the stringent response is connected to (p)ppGpp that serves as an alarmone in what is believed to be a second messenger response system activated by nutrient depletion. As observed in *E. coli*, the starvation-induced stringent response involves release of (p)ppGpp, which inhibits translation and replication while promoting DNA repair [50]. Studies have shown that across multiple organisms, mutants with an inability to produce (p)ppGpp also have reduced ability to form persisters. Release of (p)ppGpp has been observed to be stimulated by the formation of biofilm itself and is generally accepted to be involved with persistership development in general [55].

5.7.4. Mediators T-AT

As previously discussed, bacterial T-AT has been shown to be related to persistership in stressful environments and is regulated by the stringent and SOS responses [56]. Increased levels of (p)ppGpp activate Type II antitoxin degradation and therefore increases unregulated "self-intoxication" by toxin [55]. High levels of toxin ultimately inhibit bacterial growth and promote cellular dormancy under the right conditions, such as nutrient and oxidative stress. However, studies have shown that certain environmental stresses such as phosphate starvation, acid stress, and osmotic stress result in upregulation of Type II TA with no increase in persister cells [56].

5.7.5. The SOS Response

The SOS response to DNA damage caused by oxidative stress or antibiotic treatment serves as a complementary pathway for stress signaling, and triggers Type I and II Toxin [56]. The SOS response also promotes DNA repair, which is important for persister resuscitation once the stressor is removed. In (p)ppGpp-deficient mutants, studies have shown that SOS-deficient mutant cells show a decreased number of persisters, particularly when treated with DNA-damaging agents [55]. This is related to studies that have observed that the introduction of antibiotics as treatment may also be implicated in the development of persistership and supports the idea that generalized bacterial stress responses are

related to increased persistership [48].

6. Looking Ahead

It may be concluded from the foregoing discussion that microbial quiescence or persister phenotype is broadly encountered among microorganisms of concern to women's health. But the follow up question centers on defeating the organisms that persist after other therapeutic measures have been used. Several potential approaches might address this issue, but few proven methods have entered clinical practice. Intact host defenses may be considered as a potential mechanism for managing persisters, but as indicated in an earlier treatment of the topic, knowledge of the host-microbe interaction with persister organisms has not been studied extensively. It would be important to investigate whether microbial virulence is diminished in quiescent organisms, leading to down-regulated immunity or if the quiescent organisms are refractory to host defense mechanisms as they are to antibiotics.

Based on past successes, it may be hoped that chemical antimicrobial and disinfecting molecules might emerge that can disable persister cells and inactivate them before the re-emerge from the dormant state. At present, the best example of a drug that targets persisters is pyrazinamide which interdicts persister survival through multiple mechanisms including reduction of CoA needed for survival and disruption of membrane energetics [57].

As previously mentioned, another intracellular pathogen of interest and concern is *Chlamydia trachomatis* (as well as other chlamydial species) and in an excellent review by Schoborg the factors which promote the persister state which consists of AB, distinct from the well-known EB and RB are driven by stressors that include beta-lactam drugs and interferon gamma [58]. Interferon gamma serves to down-regulate indolamine 2 - 3 dioxygenase to create a dearth of tyrosine. In a recent study, the tryptophan operon of chlamydial isolates from a patient with recurrent disease showed a consistent mutation in the tryptophan biosynthesis pathway that resulted in development of AB's making a connection between persister phenotype and persistent infection [36]. Detailed dissection of the pathways involved in generation of persisters will suggest new targets for persisters in the future. Other stressors may be involved in AB development including iron starvation or other nutrient deprivation which seems to be a common theme for many types of persister organisms [59].

Targeting pathways may depend in part on detailed identification of common and unique elements in various microbial strains. Drug design may be based on such detailed information, but other approaches are possible. The work of Feng *et al.* [60] explored the stationary phase of *Borrelia butdorferi*, which is enriched with persister organisms. They found dapsone or daptomycin could be employed in combination with other drugs to reduce persisters. If reliable methods can be developed for isolating persisters, chemical, drug or biological reagents could be screened for the ability to inactivate persisters while in their quiescent

states. With an appropriate screening method, large chemical or drug libraries may be screened for new therapeutic candidates.

The addition of a drug or compound that has the ability to damage the microorganism even while it is quiescent presents an attractive concept for defeating persister organisms. With respect to *Candida* persisters, de Oliveria Santos and co-workers have presented an extensive exploration of novel compounds to approach drug resistant strains, although their primary concern did not focus on the persister phenotype [61]. Their paper describes several substances that are associated with human host defense which have promise in this context. These include lysozyme, lactoferrin, and small peptides such as beta defensins. Defensin peptides (or other synthetic peptides) could alter membrane integrity even while the organism is in the quiescent state, and enzymes or other substances could damage structures such as bacterial cell wall, which may limit the ability of organisms to exit the persister state when conditions become conducive to growth. The ability of compounds of plant origin to inhibit drug resistant organisms was also raised in the de Oliveria Santos paper.

Persisters may be indirectly targeted as the persister phenotype is intricately involved with microbial stress responses that push populations toward persister generation and biofilm formation. As a result of these relationships, the search for clinical interventions to prevent biofilm may also prevent the accumulation of persisters associated with the biofilm. In their extensive presentation on biofilms of several *Candida* species, Cavalheiro and Teixeira discussed many of the current efforts directed at preventing biofilm formation [62]. They describe many of the treatments that address biofilms on abiotic surfaces, but also mention some of the compounds with potential effect against biofilms forming on mucosa. Notably, such plant compounds as phenylpropanoids and terpenoids, synthetic peptides and repurposed drugs discovered through screening compound libraries are possible candidates for biofilm interdiction. The parallels with compounds mentioned in the previous paragraph are interesting. The role of transition to hyphal growth is also significant in biofilm formation and compounds that alter this process, such as aspirin, have intriguing implications [63].

A recent study has also indicated lysozyme may be useful in preventing *Gardnerella* biofilms and can accordingly serve as an adjunct to antibiotics [64]. Several other natural and synthetic compounds have been directed against *Gardnerella* biofilm including retrocyclin, DNase, chitosan, subtilosin, poly-L-lysine and lauramide ethyl ester [65] [66] [67]. Whether these compounds affect persisters directly, indirectly, or not at all remains to be established.

As previously discussed, quorum sensing is also an element in biofilm and persister generation and as a consequence, modulation of quorum sensing may be another mechanism to indirectly limit persistership. The discovery of multiple quorum sensing and quorum inhibiting mediators in *Candida albicans* suggests the possibility of rational approaches to recurrent and recrudescent vaginitis due to this organism. The breadth of these autocrine mediators is more fully described elsewhere but suggests the possibility of several potential targets to ex-

plore in future work [68].

There are three main quorum sensing mediators in bacteria specific for Gram negative bacteria (acyl homoserine lactone), Gram positive bacteria (autoinducer peptide), or for both Gram negative and Gram positives (autoinducer-2) [69]. Targeting these is predicted to have beneficial effects on microbial virulence and biofilm production. Interestingly, some of the relevant discoveries in this area include phytochemicals with activity against *Gardnerella vaginalis* biofilm and the previously mentioned subtilosin which inhibits autoinducer-2 in *Gardnerella vaginalis* [70] [71]. Additional botanicals in the form of essential oil have also been studied as inhibitors of biofilm using various bacterial species as test organisms and activity against *Candida* biofilms and hyphae development was reported for plant essential oils as well [72] [73].

The future for discovering direct and indirect means for addressing the problem of persisters and in turn, the problem of stubborn and recurrent infections seems bright. Some answers will come from better understanding of the molecular processes of persister generation and targeting specific pathways, while other progress may be made through high throughput screening of drug and chemical libraries. Interest has also turned to natural products from plants and marine organisms.

While in this paper we have presented the matter of microbial persisters as the third frontier of therapeutic challenge, we will not identify it as the last. As with therapeutics before, resistance is certain to develop and has precedence even as PZA is being used against Mycobacterial persisters, evidence for occasional refractoriness to that drug is being reported [57].

7. Summary

In this paper we have contextualized the history of antimicrobial therapy in the form of three frontiers, the discovery and development of antibiotics, the threat to antibiotics posed by drug resistance and its genetic spread, and the problem posed by the persister phenotype which is related to an altered metabolic state of quiescence. This phenomenon is widely distributed through the diversity of microorganisms that affect women's health. The study of this phenotype has led to increasing understanding of the molecular mechanisms for this state which also provides ideas for rational development of drug candidates to interdict these organisms in human disease. Despite the possibility of developing specifically targeted molecules to address persisters, work continues on screening botanicals, existing drugs and chemicals to discover novel approaches to the clinical consequence of microbial persisters. The origin of antimicrobials includes naturally occurring botanical substances, like cinchona bark. This may seem unsophisticated by current standards, but we are currently turning again to botanicals and other natural products for potential answers to the persister problem.

Declaration

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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