

Quorum Quenching: Unexplored Strategy to Control Animal Genital Tract Bacterial Infection

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Abstract: Endometritis was reported as the most serious cause of economic losses in dairy farms. The misuse of antibiotics has contributed to widespread development of antimicrobial resistance among clinically significant bacterial species. Alternative approaches other than those using antibiotics are needed in the fight against infectious diseases. Quorum sensing is an intercellular signaling and gene regulatory mechanism, which is used by a number of opportunistic pathogenic bacteria in determining virulence gene expression. Hence, disruption of bacterial quorum sensing has been proposed as a new anti-infective strategy. A major advantage of that quorum sensing inhibitors is that they are used in concentrations that do not affect bacterial growth. For this reason, it is expected that these compounds would exert less pressure towards the development of resistance. These natural and synthetic quorum sensing inhibitors which are generally regarded as safe may lead to efficient treatment with much lower doses than those antibiotics used at present. Early discovery of disease enables animal breeders to control infection. The discovery of some naturally occurring bacteria, act as reporter strains or biosensors, have the ability of early detection of disease. This clinical perspective should be studied in depth as an early diagnostic tool of inflammation. This review throw light on bacterial quorum sensing, quorum sensing inhibitors, the most recent bacterial reporters and how to make use in disease control in animals breeding farms.

Key words: Endometritis • Antimicrobial resistance • Biosensors • Bacterial quorum sensing • Quorum sensing inhibitors

INTRODUCTION

Inflammation of the uterus in cows causes changes in the endometrium, which disturb fertilization, hamper implantation and may even lead to early miscarriages [1, 2]. It also causes ovarian cycle disorders, including the formation of ovarian cysts, prolongation of the luteal phase and delay of first postpartum estrus [3]. Compared to other types of inflammation of the uterus, subclinical endometritis is diagnosed late, usually when insemination becomes ineffective, due to the lack of noticeable clinical symptoms related to the reproductive system. Despite numerous studies, implementation of new diagnostic methods and the use of different therapeutic methods, endometritis in dairy cows remains a serious economic problem all over the world. This is mainly due to the large economic losses caused by the low rate of artificial insemination intervention and the necessity to cull

animals in the herd [4-6]. Some animals acquire infections of the uterus during late gestation, which may lead to premature parturition, or compromise fetal or calf health.

Approaches such as the use of antibiotics and disinfectants have only had limited success in the prevention or cure of disease. Moreover, their frequent use is leading to the rapid development of resistance [7]. Therefore, there is an urgent need for alternative control techniques for prevention of disease, which is likely to be more cost-effective than treatment. Efforts to disrupt biofilms have enabled the identification of bioactive molecules produced by prokaryotes and eukaryotes. These molecules act primarily by quenching the bacterial intercellular signaling (Quorum quenching). Among the eukaryotes, several Algae and medicinal plants were reported to have quorum quenching effect [8]. Also, some synthetic derivative of the *D. pulchra* halogenated

furanones have quorum quenching effect. Use of these quorum sensing inhibitors would decrease the excessive use of antibiotic for bacterial disease treatment.

This review deals with the alternative strategy for disease control that has not received much attention. This by understanding means of quorum sensing, use of bacterial reporters or biosensors for early detection of bacterial signals to start disease control. Elaboration of means of disruption of bacterial quorum sensing (Quorum quenching) and the possible application to control disease with emphasis on genital tract affection.

What Is Quorum Sensing?: In a process called quorum sensing, group of bacteria communicate with each another to coordinate their behavior and function like a multicellular organism. A diverse array of secreted chemical signal molecules and signal detection apparatuses facilitate highly productive intra and interspecies relationship. Quorum sensing regulates bioluminescence, virulence factor expression, biofilm, sporulation and mating. These behaviors are only achieved when bacteria are at high population (Quorum) while are unproductive when undertaken by the individual bacterium. Bacterial population have to achieve production and release of threshold concentration of signal molecules called autoinducers. Thus the processes are proposed to be a mechanism for census taking. Miller and Bassler [9] elaborated three archetypal quorum sensing systems

The first type of quorum sensing is typical Gram negative bacterial quorum sensing circuit in which the autoinducer is an acylated homoserin lactone (AHL) The I protein is the AHL synthase enzyme (luxI). The AHL molecules diffuse freely through the plasma membrane. As population density increases, the AHL concentration increases as well and once a critical concentration has been reached, AHL binds to the R protein (luxR), a response regulator. The AHL-R protein complex activates or inactivates transcription of the target genes. (Fig 1)

The second paradigm quorum sensing circuit takes place in Gram positive bacteria where The autoinducers (Pheromones) are short Peptide-mediated quorum sensing. A peptide signal (PS) precursor protein is cleaved, releasing the actual signal molecule. The peptide signal is transported out of the cell by an ATP binding cassette (ABC) transporter. Once a critical extracellular peptide signal concentration is reached, a sensor kinase (SK) protein is activated to phosphorylate the response regulator (RR). The phosphorylated response regulator activates transcription of the target genes (Fig 2).

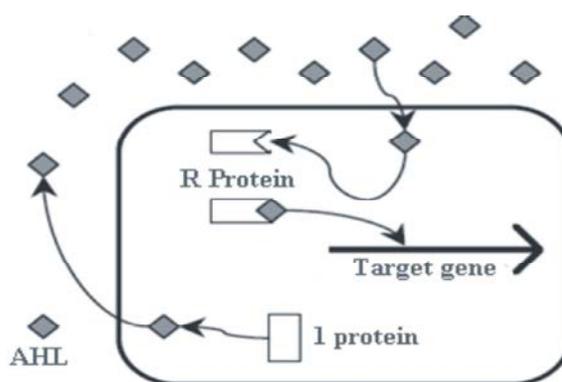


Fig. 1: AHL mediated quorum sensing [10]

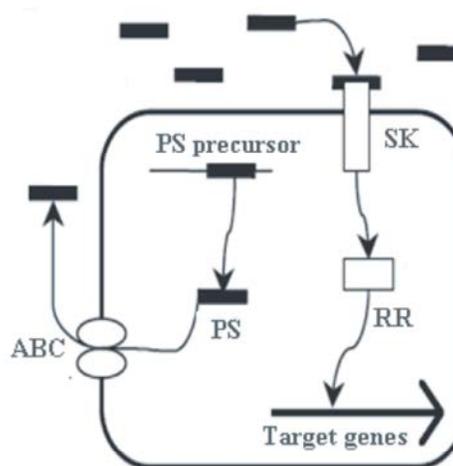


Fig. 2: Peptid mediated quorum sensing in Gram positive bacteria [10]

The third model system of quorum sensing is the hybrid quorum sensing in Gram-negative bacterium *Vibrio harveyi*. This quorum sensing circuit controls bioluminescence. In this system, there are two types of signal molecules (Fig 3).

AI-1 is an AHL and its biosynthesis is catalysed by the luxLM enzyme. AI-2 is a furanosyl borate diester; its biosynthesis is catalysed by the LuxS enzyme. AI-1 and AI-2 are detected at the cell surface by the LuxN and LuxP–LuxQ receptor proteins, respectively. At low cell density, LuxN and LuxQ autophosphorylate and transfer phosphate to LuxO via LuxU. The phosphorylated LuxO is an active repressor for the target genes. At high cell density, LuxN and LuxQ interact with their autoinducers and change from kinases to phosphatases that drain phosphate away from LuxO via LuxU. The dephosphorylated LuxO is inactive. Subsequently, transcription of the target genes is activated by LuxR.

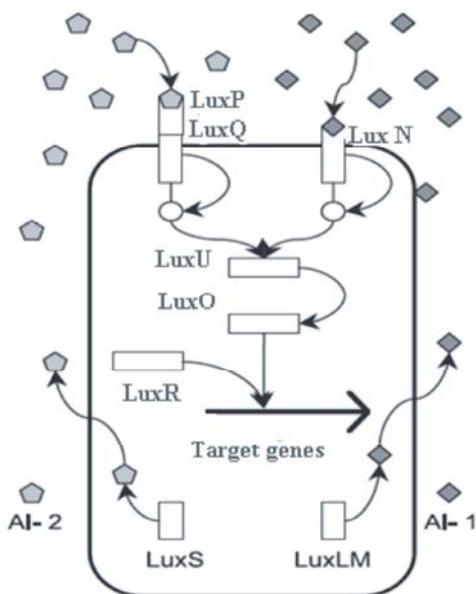


Fig. 3: Quorum sensing in *V. harveyi* [10]

In natural habitats, bacteria can exist in highly ordered communities composed of multiple species. These communities are constructed such that each species carries out a specific subset of functions that, collectively, allow the conglomerate to thrive. Successful associations of this type require effective intra- and interspecies cell-cell communication. AHLs and peptide autoinducers are highly specific and are used for intraspecies cell-cell communication. AI-2 and its synthase LuxS, on the other hand, exist in over 40 species of gram-negative and gram-positive bacteria and AI-2 is proposed to act as a more universal interspecies chemical language.

Interspecies cell-cell communication allows bacteria to exploit the diverse metabolic functions that exist in a mixed-species consortium. AI-2 has been shown to control specific target gene expression in *V. harveyi*, *Vibrio cholerae*, *Escherichia coli*, *Salmonella typhimurium*, *Porphyromonas gingivalis*, *Shigella flexneri*, *Streptococcus pyogenes*, *Neisseria meningitidis*, *Actinobacillus actinomycetemcomitans*, *Borrelia burgdorferi* and *Clostridium perfringens*, suggesting that this molecule plays a critical cell-cell communication role in and possibly among a diverse range of bacteria. Because AI-2 allows communication among species, bacteria that consume AI-2 could be actively interfering with the signaling process, and therefore, directly tied to cell growth. As such, AI-2 harbors information regarding how well a bacterial population is doing. Elimination of AI-2 from the extracellular environment could be a form of

“censorship” that allows one species of bacteria to avoid alerting other species to its presence. Consumption of AI-2 could provide a competitive edge to a bacterial species by rendering other species of bacteria that rely on AI-2 as information at a disadvantage. This could be critical for disrupting the delicate balance that exists between mixed populations competing for colonization of a particular niche [11].

Disruption of Bacterial Quorum Sensing: Some of disease outbreaks caused by pathogenic bacteria are considered one of the largest constraints to development of animal’s wealth sectors. So far, antibiotics and disinfectants have only limited success in the prevention of disease. Moreover, the frequent use of biocides, especially in subtherapeutic doses, is leading to the rapid development of resistance. Therefore, there is an urgent need to develop alternative ways to control infections caused by bacterial pathogens. This can be achieved by controlling virulence factor expression controlled by cell-to-cell communication system. Hence, disruption of bacterial quorum sensing has been proposed as a new anti-infective strategy and several techniques that could be used to disrupt quorum sensing have been investigated. These techniques comprise the inhibition of signal molecule biosynthesis, the application of quorum sensing antagonists (Including natural occurring as well as synthetic halogenated furanones, antagonistic quorum sensing molecules and undefined exudates of higher plants and algae), the chemical inactivation of quorum sensing signals by oxidized halogen antimicrobials and finally by signal molecule biodegradation by bacterial lactonases and by bacterial and eukaryotic acylases.

Phenotypes that are controlled by a quorum sensing system include luminescence, conjugation, nodulation, swarming, sporulation, biocorrosion, antibiotic production and most importantly biofilm formation and the expression of virulence factors such as lytic enzymes, toxins, siderophores and adhesion molecules [12, 13].

1. Inhibition of Signal Molecule Biosynthesis: A first quorum sensing disrupting technique aims at inhibiting signal molecule biosynthesis (Fig. 4A). In many cases, homologues LuxI protein catalyse in an attempt to block AHL biosynthesis. Parsek *et al.* [14] found that analogues of S-adenosylmethionine (Such as S-adenosylcysteine) could inhibit activity of the *Pseudomonas aeruginosa* LuxI homologue RhlI by up to 97%. Database research revealed that no AHL synthase sequence motifs were present in other enzymes with S-adenosylmethionine

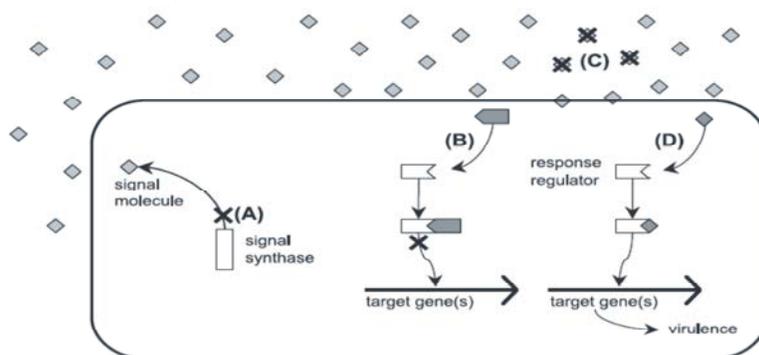


Fig. 4: Schematic overview of different strategies that have been developed to disrupt bacterial quorum sensing. (A) Inhibition of signal molecule biosynthesis by the application of substrate analogues. (B) Blocking signal transduction by the application of quorum sensing antagonists. (C) Chemical inactivation and enzyme biodegradation of signal molecules. (D) Application of quorum sensing agonists to evoke virulence factor expression at low population density [10]

binding sites. Therefore, it might be possible to use the S-adenosylmethionine analogues as specific quorum sensing inhibitors, without affecting other vital processes in prokaryotic and eukaryotic organisms [12].

2. Application of Quorum Sensing Antagonists:

2.1. Antagonists for AHL-Mediated Quorum Sensing:

2.1.1. Halogenated furanones: In such mechanisms the production of quorum sensing antagonist molecules can bind to quorum sensing response regulators, but fail to activate them (Fig.4B). The red marine alga *D. pulchra* has developed such a defense mechanism to protect itself from extensive bacterial colonization [15]. The alga produces halogenated furanones as antagonists for AHL mediated quorum sensing. Because of their structural similarity with AHLs [10]. Halogenated furanones most probably bind to LuxR type proteins without activating them [16]. This furanone could also suppress the expression of bioluminescence genes, located on a reporter plasmid in *S. liquefaciens*, without affecting the growth rate of the bacterium.

2.1.2. Natural Antagonistic AHL Molecules: Some naturally occurring AHL molecules could stimulate quorum sensing-regulated pigment production in the Gram-negative bacterium *Chromobacterium violaceum*, while other AHLs completely inhibited this phenotype [17]. The stimulatory or inhibitory effect was linked to the structure of the acyl side chain of the molecules.

AHLs with an acyl side chain containing up to eight carbons were stimulatory, acting as quorum sensing agonists. While, AHLs with an acyl chain containing 10 carbons or more, were inhibitory and acted as quorum sensing antagonists.

2.1.3. Synthetic AHL-Mediated Quorum Sensing

Antagonists: As far as we know, a synthetic derivative of the *D. pulchra* halogenated furanones, (5Z)-4-bromo-5-(Bromomethylene)-2(5H)-furanone, is the most active AHL antagonist mentioned in literature thus far. This furanone, dosed in a concentration of 10 μ M, could almost completely reduce virulence factor expression in pure cultures of *P. aeruginosa* PAO1 [18]. Interestingly, the furanone was equally active on biofilm bacteria compared to planktonic cells, making them susceptible to sodium dodecyl sulphate and antibiotics. In the absence of the furanone, on the contrary, 100- to 1000-fold higher doses of antibiotics are required to eradicate biofilm bacteria compared to their planktonic counterparts [19].

2.1.4. Antagonists Produced by Higher Plants and

Micro-Algae: Reverse phase high-performance liquid chromatography revealed that there are several different AHL mimicking substances present in extracts. Flavanoids have been the focus of research for their roles as antioxidant, anti-inflammatory and anticancer agents. Keeping in view these health benefits, flavanoids such as naringenin, kaempferol, quercetin and apigenein were evaluated for their quorum sensing inhibitory activities. All these flavanoids inhibited HAI-1 or AI-2 mediated bioluminescence.

In *V. harveyi* BB886 and MM32. Quercetin and naringenin were found to inhibit biofilm formation by *V. harveyi* BB120 and *E. coli* O157:H7 [20]. Extracts from different plant parts like leaves, flowers, fruit and bark of *Combretum albiflorum*, *Laurus nobilis* and *Sonchus oleraceus* were also found to possess anti quorum sensing activities [21, 22].

Flavan-3-ol catechin, one of the flavonoids from the bark of *Combretum albiflorum* reduces the production of quorum sensing mediated virulence factors- pyocyanin, elastase and biofilm formation by *P. aeruginosa* PAO1 [23]. grapefruit could inhibit AI-1 and AI-2 activities of *V. harveyi* reporter strains BB886 and BB170 and also inhibited biofilm formation by pathogens such as *E. coli* O157:H7, *Salmonella typhimurium* and *P. aeruginosa*. Purified furocoumarins dihydroxybergamottin and berggamottin caused AI inhibitions in the range of 94.6–97.7%. Interestingly, similar results were obtained for micro-algae [24]. The algae *Chlamydomonas reinhardtii*, *Chlamydomonas mutablis*, *Chlorella vulgaris* and *Chlorella fusca* all stimulated quorum sensing-regulated luminescence in wildtype *V. harveyi*. In contrast, colonies of *C. reinhardtii* inhibited AHL-mediated luminescence in several different *Escherichia coli* AHL reporter strains.

2.2. Antagonists for AI-2-Mediated Quorum Sensing:

It has been found that the halogenated *D. pulchra* furanone Compound 2, previously described as an AHL antagonistic analogue, could completely inhibit AI-2-regulated swarming of *E. coli*. Moreover, the furanone decreased thickness of *E. coli* biofilms by 55% and the percentage of live cells in the biofilms by 87% [25]. The furanone also inhibited AHL-mediated as well as AI-2-mediated luminescence in *V. harveyi*. Foods such as turkey patties, chicken breast, home made cheeses, beef steak and beef patties showed 84.4–99.8% inhibition of AI-2 activity [26].

3. Chemical Inactivation of Quorum Sensing Molecules:

It has been previously established long time ago that AHLs are chemically inactivated via alkaline hydrolysis, yielding the cognate acyl-homoserine [27] then, the oxidizing halogen, such as hypobromous or hypochlorous acid, is used as antimicrobials (Fig. 4C). using concentration of approximately 0.14 mM of these halogens, were found to decrease the concentration of 3-oxo-substituted AHLs to about one-fourth after 1 min incubation, but had no effect on unsubstituted ones [28]. Moreover, the inactivation of 3-oxo AHLs was shown to proceed in the presence of polysaccharide biofilm compounds despite the much higher concentration of the latter compared to the AHL concentration.

4. Enzymatic Biodegradation of Quorum Sensing Molecules: Some bacteria might block the quorum sensing systems of their bacterial competitors to obtain a selective advantage over them. This could be the case,

for those microbes that regulate the production of antibiotics via quorum sensing [29]. The actual inactivation of the signal compound (Fig 4C) can be mediated by two types of enzymes: AHL lactonases and AHL acylases [30]. AHL degradation by the *Bacillus* sp. Strain offered a protection as effective as or better than antibiotic production by a *Pseudomonas chlororaphis* biocontrol strain. Moreover, degradation of AHLs had not only a preventive, but also a curative biocontrol activity. Recently, similar results were obtained with *Bacillus thuringiensis* [31].

5. Application of Quorum Sensing Agonistic Analogues:

All techniques discussed so far aim to inactivate quorum sensing-regulated virulence factor expression. However, some authors tested an opposite strategy where they activated quorum sensing-regulated virulence factor expression by using quorum sensing agonists. This was applied by adding the signal molecule of a pathogen that lead to activated virulence factor expression at low population density (Fig. 4D). Subsequently, the virulence factors could trigger the activation of the host's defense system allowing resistance to develop [32].

Limitations to Disruption of Bacterial Quorum Sensing:

A first limitation to the use of quorum sensing disrupting techniques as a new anti infective therapy might be resistance development where bacteria could simply circumvent quorum sensing blockade by over expressing quorum sensing genes [33]. Also, the lack of specificity could also confine the use of quorum sensing disrupting techniques to control pathogens. Most AHL degrading bacteria, for instance, inactivate a wide range of AHL molecules. However, not all bacteria found to contain a quorum sensing system are pathogens.

And this may disrupt growth promoting and biocontrol activities of unknown favourable quorum sensing-regulated processes. It is clear that much more knowledge about the occurrence and function of quorum sensing will be necessary in order to be able to develop a new anti-infective strategy [10].

Criteria for Selecting Quorum Sensing Inhibitors:

For selecting an effective quorum sensing inhibitors, it has been proposed that it should meet at least the following few criteria. First it should be a small molecule with ability to efficient reduction of the quorum sensing regulated gene expression, Second, it should be highly specific for a given quorum sensing regulator with no adverse effect on the bacteria or the host, third, it should be chemically stable and resistant to degradation

by various host metabolic systems and lastly it preferably should be longer than the native AHL. As a consequence of these characteristics of a quorum sensing inhibitors, the bacteria are not likely to become resistant to such drugs, which generally exert selection pressure during treatment of infections and these compounds are not likely to affect the population of beneficial bacteria present in the communities harbouring the host [34]. Finally, these Quorum sensing inhibitors, which do not show antigenicity due to their low molecular weights, are expected to expedite drug discovery against infectious [18, 35, 36].

Bacterial Reporters to Detect Quorum Sensing Signals:

One of the most important prerequisites for circumventing the pathogenicity issues related to quorum sensing is its detection. Bacteria can thus be detected during early stages of infection and are likely to be more susceptible to antibiotic treatment [37].

The need for biosensors was realized quite early primarily because of the increasing variation in the quorum sensing signals produced by diverse organisms. In order to establish the link between cause and effects, AHL and AI-2 reporters were developed over a period of time by different researchers. Although these reporter strains have a functional regulator protein, they lack the AHL synthase enzyme. The promoter activity gets induced by exogenous quorum sensing signals. Here, the receptor gets activated by the presence of AHLs, which binds to its cognate LuxI promoter and initiates the expression of certain genes. The expression of the relevant genes is proportional to the concentration of the signal molecules. In brief, it mimics the natural quorum sensing system with certain easily identifiable phenotypes [38].

Chromobacterium violaceum has high sensitivity for quorum sensing signal compounds with 4–6 carbon acyl side chains, *E. coli* harbouring pSB410 is effective for 6–8 carbon side chains and pSB1075 is sensitive for detecting AHLs with 10–14 carbon side chains lengths [39, 40].

The class of reporter strain needed for identifying quorum sensing inhibitors which may target long-chain AHLs — C16–C20 is represented by *S. meliloti* Rm41 sinI::lacZ (pJNSinR) (Llamas et al., 2004). A more recent addition to reporters for detecting long-chain AHLs is *C. violaceum* VIR24, which was derived from *C. violaceum* type strain ATCC12472 [38]. *In vitro* methods including high throughput genetic tools have proven effective in screening of non-toxic quorum sensing inhibitors from natural sources and for elucidating their effects [34, 39].

Significance of Quorum Sensing Inhibitors for Animal Breeding:

Uterine microbial disease affects half of all dairy cattle after parturition, causing infertility by disrupting uterine and ovarian function. *Trueperella pyogenes* (43.5%), *Escherichia coli* (21.5%), *Bacillus* spp. (21.0%) and *Streptococcus uberis* (18.5%) were the most frequently isolated microbes. Analyses of different sampling time points revealed that the presence of *S. uberis* on day 3 increased the risk of subsequent *T. pyogenes* infection on day 9 [41]. The effect of pathogen-associated molecules on uterine cells is not limited to inflammation, but also affects endocrine function. The principal hormones secreted by the endometrium are PGF2 α and PGE2, respectively and the secretion of these hormones is modulated by *E. coli* or LPS [42,43]. The uterine disease would extend the luteal phase. *In vitro*, LPS stimulates progesterone secretion from mixed populations of luteal cells (including steroidogenic, endothelial and immune cell types) to a level similar to that seen with luteinising hormone (LH), but at higher concentrations LPS kills the cells [1]. It has been proposed that biofilms play an important role in chronic bacterial endometritis [44]. It has been repeatedly observed that bacteria within biofilm are around 1000 times more resistant to antibiotics than their planktonic counterparts [45]. Bacterial behaviour within biofilms is regulated by the phenomenon of quorum sensing, where bacteria release chemical signals and express virulence genes in a cell density dependent manner [46,47]. Efforts to disrupt biofilms have enabled the identification of molecules produced by prokaryotes and eukaryotes with abilities to quench the quorum sensing system, termed as quorum quenching [48–49]. In addition, synthetic compounds have also been found to be effective in regulating quorum sensing [50]. These quorum sensing inhibitors can competitively inhibit quorum sensing signaling system, providing an opportunity to develop new drugs against these targets to combat pathogens.

Among prokaryotes, the most promising organisms for producing quorum quenching enzymes under a wide range of conditions and expression systems are strains belonging to diverse *Bacillus* spp. This privileged “Kingdom” is because of the unique properties of *Bacillus*, which undergoes sporulation to withstand extremes of environmental stresses. It is an industrial work-horse with abilities to produce a wide range of enzymes. As far as quorum quenching is concerned, it produces AHL-lactonase, which in principle is sufficient to inactivate the quorum sensing signals and disrupt biofilms produced by infectious pathogenic bacteria.

However, it may be more desirable to look for organisms which may be more versatile in terms of their range of quorum quenching enzymes. So far *R. erythropolis* is the only organism which has been reported to produce AHL-acylase and -lactonase.

Among eukaryotes, some plant extracts have been found to act as quorum sensing inhibitors because of similarity in their chemical structure to those of quorum sensing signals (AHL) and also because of their ability to degrade signal receptors (LuxR/LasR) [51, 36]. GABA (γ -aminobutyric acid) produced by plant acts as promoter for the degradation of AHL signal OHC8HSL by lactonase (AttM) of *A. tumefaciens*, attenuating the quorum sensing dependent infection process [52, 53]. Also, pyrogallol extracted from medicinal plants such as *Emblica officinalis* and its analogues exhibit antagonism against AI-2 [54].

The use of garlic as a quorum sensing inhibitor against *P. aeruginosa*, which is intrinsically resistant to many antibiotics and also causes chronic infections, has been shown through inhibition of biofilms produced by them [34]. This treatment made the biofilm susceptible to antibiotics such as tobramycin and the phagocytosis by neutrophils [43]. It has been envisaged that disruption of biofilms is likely to make bacteria more susceptible to even low doses of antibiotics [55]. In fact, in this case, down regulation of virulence is complemented by the activation of the innate immune system of the host [43].

CONCLUSION

Endometritis causes great economic losses to animal breeders specially in dairy farms. Bacterial infection represent about 64% of infectious agents. It has been proposed that bacteria within biofilm are around 1000 times more resistant to antibiotics than their planktonic counterparts. Bacterial behaviour within biofilms is regulated by the phenomenon of quorum sensing, where bacteria release chemical signals and express virulence genes in a cell density dependent manner. Efforts to disrupt biofilms have enabled the identification of molecules produced by prokaryotes and eukaryotes with abilities to quench the quorum sensing system, termed as quorum quenching. This treatment made the biofilm susceptible to low doses of antibiotics and the phagocytosis by neutrophils. Early diagnosis of disease is available using bacterial reporters the thing enables breeders to early control disease. Use of quorum quenching agents should be studied in depth to control genital tract infections in farm animals to achieve profit and reduce the extensive use of antibiotics.

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