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論　文　名	Effects of piperine, an active ingredient of white pepper, on growth, virulence expression and biofilm formation of *Vibrio cholerae* (白コショウの活性成分であるピペリンがコレラ菌の増殖、病原因子の発現及びバイオフィルム形成に及ぼす影響)
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論文要旨

Introduction

Vibrio cholerae is a Gram-negative aquatic bacterium, a causative agent of disease cholera, which is still prevalent in many developing countries. Cholera toxin (CT, encoded by *ctxAB* genes) is the major virulence factor in *V. cholerae* and mostly responsible for profuse watery diarrhea leading to severe dehydration. Along with CT, by using toxin-co-regulated pilus (TCP, encoded by the *tcpA* gene), a colonizing factor, *V. cholerae* causes pathogenesis to human host. Although over 200 different serogroups of *V. cholerae* have already been documented, only O1 (El Tor and classical biotypes) and O139 are responsible for epidemics of cholera. Recently emerged *V. cholerae* O1 El Tor hybrid strains, which possess some attributes of classical biotype including *ctxB* gene allele, are categorized as El Tor variant produce more CT and cause more severe diarrhea than prototype El Tor. The expression of major virulence factors, CT and TCP are controlled through a regulatory cascade by activation of master regulator ToxT. Furthermore, the ToxT expression is controlled by synergistic coupling interaction of periplasmic ToxR/ToxS with TcpP/TcpH. *V. cholerae* is often associated with biofilm mode of growth, which is known to enhance the transmission and persistence of cholera disease.

With rising antibiotic resistance including tetracycline-, fluoroquinolone-resistant for *V. cholerae* over past decade, much attention has been paid to search for effective antimicrobial agents from natural sources. Spices are cheap and easily available natural compounds, which are known to have antimicrobial properties with limited side effects. However, there is very limited information regarding the effect of spices mainly their active ingredients on virulence expression and biofilm formation of *V. cholerae*. Previous reports from our group have shown the antivirulence activity of methanol extract of some spices such as red chili and sweet fennel along with their active ingredients capsaicin and trans-anethole, respectively. Moreover, preliminary studies with crude methanol extract of another spice, white pepper demonstrated anti-virulence activity at sub-bactericidal concentration and also antimicrobial activity at higher concentrations although the role of its active ingredient was not examined in detail. In this study, the active ingredient of white pepper was determined and its role in inhibiting growth, suppression of virulence and biofilm formation against MDR toxigenic *V. cholerae* including various serogroups and biotypes was explored.

Chapter 1. Piperine, an active ingredient of white pepper, suppresses growth in diverse toxigenic *V. cholerae* strains

White pepper was extracted with various solvents such as methanol, hexane, n-butanol and water and each extract was examined for antimicrobial activity against pathogenic *V. cholerae* O1 El Tor variant strain CRC41 (strain CRC41). At 200 µg mL^{-1} only methanol extract of white pepper showed growth inhibitory effect against strain CRC41. Piperine is known to be the major ingredient of white pepper. Thin-layer chromatography (TLC) analysis confirmed that piperine was indeed the major ingredient in methanol extract of white pepper. Effect of piperine (up to 300 µg mL^{-1}) on the growth of various toxigenic *V. cholerae* strains belonging to different serogroups and biotypes was tested in LB media at 37˚C for 2 h under shaking conditions. It was found that 100 µg mL^{-1} of piperine had no significant growth inhibitory effect against all the tested *V. cholerae* strains. However, nearly 3 log and 5 log reductions in number of *V. cholerae* was observed in the presence of 200 µg mL^{-1} and 300 µg mL^{-1} of piperine, respectively.

To further understand if the growth inhibition caused by piperine was due to killing bacteria or entering viable but non-culturable state, live/dead assay was performed using fluorescent dyes DAPI (4′,6-diamidino-2-phenylindole) and PI (propidium iodide) under a fluorescence microscopy. DAPI can stain both live and dead cells whereas PI can stain only dead cells. When bacterial cells were treated with 100 µg/ml piperine, majority were stained by DAPI but not by PI. However, at ≥ 200 µg mL^{-1} most of the bacteria were stained by PI. Hence, the effect of piperine was

bactericidal against *V. cholerae.* Specificity towards killing of pathogenic *V. cholerae* rather than normal flora is essential for the potential use of piperine for therapeutic applications. *E. coli* C600 and *E. coli* isolated from healthy mouse and human appeared to be more tolerant to piperine at 200 µg mL^{-1} when compared to pathogenic *V. cholerae* and was statistically significant. However, at 300 µg mL^{-1} piperine appears to be bactericidal in both of these groups. Further studies are required to clarify if commensal bacteria are more tolerant to piperine or not.

Taken together these data suggested that piperine could be a potential antimicrobial agent against *V. cholerae* and as it might have less inhibitory effect on commensal bacteria, it could be possible to use piperine in preventive and therapeutic applications.

Chapter 2. Piperine inhibit CT production and TcpA expression in toxigenic *V. cholerae* by affecting virulence regulatory cascade

In this chapter, it was examined if white pepper methanol extract and piperine can inhibit virulence expression such as CT and TcpA in toxigenic *V. cholerae* at its sub-bactericidal concentration. Piperine was dissolved and diluted in ≤ 0.5% MeOH as a final concentration, which showed no significant growth inhibition. For optimum CT and TCP production we cultured O1 El Tor and O139 strains in AKI medium and O1 classical or non-O1/non-O139 in LB medium under stationary condition for an initial 4 h, followed by additional 4 h of shaking at 180 rpm at 37ºC. It was found that ≤ 50 µg mL^{-1} of piperine did not have any significant growth inhibition of these strains. Then, we tested the effect of piperine on the production of CT and TCP by culturing these strains in the presence of 50 µg mL^{-1} piperine. CT production in the culture supernatant was measured by bead-ELISA, whereas that of TCP was assessed by Western blotting. It was found that piperine could inhibit CT production in all the tested *V. cholerae* strains irrespective of their serogroups and biotypes. Further, dose dependent effect of piperine on CT and TCP productions was analyzed in the representative MDR and high CT-producing O1 El Tor variant strain CRC41. We found that ≥ 75% CT and ≥ 86% TCP production was suppressed at 50 µg mL^{-1} piperine and these suppressions were observed in a dose dependent manner. These results indicated that the piperine could be useful as an anti-virulence compound against *V. cholerae.*

To better understand how piperine inhibits CT and TCP productions, the transcriptional level of virulence (*ctxA, tcpA*), virulence positive regulatory (*toxS, toxR, tcpP, tcpH*) and negative regulatory (*hns, hapR, crp*) genes was analyzed by qRT-PCR. For optimum virulence expression, strain CRC41 was cultured in the presence or absence of piperine (50 µg mL^{-1}) in AKI medium, and kept under stationary condition for an initial 4 h followed by 2 h of shaking at 180 rpm, at 37ºC. Then, RNA was isolated for cDNA synthesis to check the transcriptional level of target genes. The

relative transcription of each gene was normalized with that of housekeeping gene, *recA*. Transcriptional level of virulence genes in strain CRC41 showed that *ctxA* and *tcpA* were repressed 3 fold ($p<0.01$) and >2 fold ($p<0.01$), respectively. Moreover, transcription of some virulence positive regulatory genes such as *toxT, tcpP* and *tcpH*, was also significantly repressed nearly 2 fold while that of *toxR* and *toxS* was not affected. Interestingly, piperine significantly upregulated the transcription of virulence negative regulatory genes, *hns* (>1.8 fold), *hapR* (>2 fold) and *crp* (>3 fold). Furthermore, promoter activity assay was performed to better understand whether piperine affect transcription of these genes directly or not. *E. coli* strains having plasmid pHRP309 in which promoter region of selected genes (*ctx, tcpPH, hapR, crp, toxT, hns*) was cloned into upstream of β-galactosidase gene without its own promoter was used and β-galactosidase activity was measured in the presence or absence of piperine. The results revealed that piperine upregulated the promoter activity of *hapR* and *crp* and downregulated *tcpPH*. On the other hand, the promoter of *ctx*, *toxT* and *hns* genes was not significantly affected. Taken together, transcriptional analysis indicated that piperine inhibited CT and TCP production through upregulation of virulence negative regulatory genes such as *hapR* and *crp* directly and *hns* indirectly, and suppression of virulence regulatory genes, *tcpPH* transcriptions directly.

Chapter 3. Effect of piperine on the biofilm formation in *V. cholera*

Upon oral ingestion of *V. cholerae* its biofilm enhances infectivity, protects the bacteria during passage through stomach and helps in colonization in small intestine. Therefore, inhibiting the biofilm formation in *V. cholerae* is crucial for preventing disease transmission in host. As described in chapter 2, it was found that piperine upregulated *hapR* transcription, which is also a master regulator for controlling biofilm formation in *V. cholerae*. So, it was speculated that piperine might have some role in inhibition of biofilm formation. To evaluate it, the biofilm forming *V. cholerae* strains were screened initially by culturing the bacteria in 24 well plates under stationary condition in LB broth at 37°C for 24 h. After 24 h, biofilm was stained with 0.1% crystal violet and further quantification by recording the absorbance at 540 nm. Among the tested strong biofilm forming *V. cholerae* strains RC1239 (O1 classical), VC29 (O1 El Tor), Vc129 (non-O1/O139) and VC5 (O1 El Tor variant), it was found that piperine (up to 50 μg mL^{-1}) inhibited more than 70% of biofilm formation without significantly inhibiting bacterial growth. The major autoinducers, CAI-1 binds to CqsS receptor while AI-2 binds to LuxPQ receptor and plays a crucial role in controlling the biofilm formation by affecting the *hapR* transcription. To check the role of piperine on interfering with autoinducer production, autoinducer reporter assay was performed. *V. cholerae* MM920 [Δ*cqsA* Δ*luxQ* pBB1 (*luxCDABE* from *V. harveyi*)] was used as reporter strain for CAI-1 production while *V. harveyi* BB170 ($AI\text{-}1^{-}$, $AI\text{-}2^{+}$) was used as

reporter strain to check AI-2 production. Cell-free culture supernatant (CFS) from piperine treated/untreated *V. cholerae* strains were incubated with each reporter strain at 30°C under shaking for 4 h and bioluminescence was recorded. It was found that piperine inhibited nearly 60-80% CAI-1 production however there was no change in the AI-2 production level. These results indicated that piperine affected CAI-1 production levels to inhibit the biofilm formation in *V. cholerae.* Structural similarities between autoinducer CAI-1 and piperine indicates that piperine might utilize CqsS receptor to inhibit biofilm formation which was confirmed when a mutant of CqsS receptor, Δ*cqsS* VC29 failed to inhibit biofilm formation even in the presence of piperine which support our hypothesis that piperine might affect CqsS/CqsA quorum sensing system by interfering with CqsS receptor, although further investigations are required.

Conclusions

Piperine showed potential antimicrobial and anti-virulence activity against *V. cholerae* strains irrespective of their serogroups and biotypes. Piperine suppressed CT and TCP productions through upregulation of virulence negative regulatory genes *hapR* and *crp* directly and *hns* indirectly, and suppression of virulence positive regulatory genes *tcpPH* directly. Piperine inhibited the *V. cholerae* biofilm formation by affecting CAI-1 production, which is one of the key molecules in quorum sensing. White pepper, in particular, piperine could be used for preventive and therapeutic applications against cholera.

審査結果の要旨

コレラ菌は O 抗原の違いに基づき現在 200 種以上の 0 群血清型に分類されている。この中で、いわゆるコレラの原因となるのはコレラ毒素（CT）と定着線毛（TCP）産生性の O1 と O139 の血清型である。さらに O1 コレラ菌は生物学的性状の違いに基づき、古典型とエルトール型の 2 種類がある。しかし、近年、エルトール型であるが古典型の CT を産生する O1 エルトールバリアントと呼ばれる流行株が出現し問題となっている。また、CT や TCP の発現は ToxT、ToxR/ToxS、TcpP/TcpH など様々な因子によって調節を受けている。

一方、テトラサイクリン耐性等様々な薬剤耐性コレラ菌が出現し大きな問題となっている。スパイスには抗菌活性を有するものがあり、比較的安価で利用し易いという特徴がある。しかしながら、スパイスにコレラ菌の病原因子の発現やバイオフィルム形成を抑制する活性があるか、また、それはどのような物質がそのような活性を示す

かについての報告は限られている。過去に赤唐辛子やウイキョウのメタノール抽出物中にコレラ菌の病原因子の発現を抑制する活性を見出され、その活性はカプサイシンやトランスアネトールであることが明らかとなっている。白胡椒のメタノール抽出物中にもコレラ菌の増殖や病原因子の発現を抑制する可能性が見出されているがその詳細については解析されていない。そこで、本研究では、1) 白胡椒に含まれる抗菌活性を示す物質を同定し、その物質がコレラ菌の増殖に及ぼす影響、2) その物質がどのようなメカニズムで病原因子の発現を抑制するか、3) さらにその物質がコレラ菌のバイオフィルムの形成を抑制するかについて調べた。

第 1 章では白胡椒のメタノール抽出物を様々な溶媒で抽出し、コレラ菌に対する抗菌活性について調べた。その結果、90%メタノール抽出物で最も強い抗菌活性を示し、TLC によって抗菌活性を示す物質をピペリンと同定した。200 μg/mL 以上のピペリンは多剤耐性のコレラ菌、緑膿菌、EHEC O104 に対して抗菌活性を示した。本抗菌活性は、異なる血清型や生物型のコレラ菌に対しても認められ、またいわゆる「viable but non-culturable」状態と誘導するのでなく菌を死滅させていることを確認した。以上より、白胡椒に含まれる抗菌物質はピペリンであり、コレラ菌及び多剤耐性菌に抗菌活性を有することが明らかとなった。

第 2 章ではピペリンが抗菌活性を示さない低濃度でコレラ菌の CT 及び TCP の発現に影響を与えるかについて調べた。その結果、抗菌活性を示さない 50 μg/mL の濃度でピペリンが CT や TCP の発現を抑制することを免疫学的手法を用い確認した。さらに CT や TCP の発現調節に関わる遺伝子を qRT-PCR によって転写レベルで解析し、ピペリンがどのようなメカニズムで病原因子の発現を抑制しているか調べた。その結果、正の調節因子である *toxT*、*tcpP*、*tcpH* の転写が抑制され、負の調節因子である *hns*、*hapR*、*crp* の転写が亢進されていた。これらの調節因子は互いに影響し合うネットワークを構成しているため、実際どの遺伝子が直接ピペリンの影響を受けたかを明らかにするため、それぞれのプロモーター領域を *lacZ* の上流にクローニングしプロモータアッセイを行なった。その結果、*hapR* と *crp* のプロモーター活性が上昇し *tcpP* と *tcpH* のプロモーター活性は減少した。以上の結果より、ピペリンは正の調節系である *tcpP* と *tcpH* の転写を抑制し、負の調節系である *hapR* と *crp* の転写を促進しコレラ菌の病原因子の発現を抑制している可能性が示された。

第 3 章ではピペリンが第 2 章において、バイオフィルム形成に関わるマスターレギュレーターである *hapR* の転写を直接上昇させたことから、ピペリンがコレラ菌のバイオフィルム形成に何らかの影響を及ぼす可能性を考え、その可能性について調べた。最もバイオフィルム形成能の高いコレラ菌を用いて調べたところ、ピペリンはコレラ菌のバイオフィルム形成能を 70%以上阻害した。バイオフィルム形成にクォーラムセンシングが関わっていることから、CAI-I と AI-2 の 2 種類のオートインデューサーについて調べたところ、CAI-I の産生を抑制していること、CAI-I のレセプターである *cqsS* 欠損株ではピペリンによってバイオフィルム形成が抑制されなかった。以上より、ピペリンは、*hapR* の転写を促進させ、CAI-I の発現を亢進させることで、バイオフィル

ム形成を抑制する可能性が考えられた。

以上の結果は、白胡椒に含まれるピペリンは抗菌活性のみならずコレラ菌の病原因子の発現抑制活性やバイオフィルム形成阻害活性があることを明らかとし、さらにそのメカニズムについても明らかとした。本研究成果は獣医学のみならず医学の分野においても多大な貢献をすると考えられる。従って、本論文の審査ならびに最終試験の結果と併せて博士（獣医学）の学位を授与することを適当と認める。