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論　文　名	Effect of natural spices extract on the virulence in *Vibrio cholerae* （スパイス抽出物がコレラ菌の病原因子発現に及ぼす影響）
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論文要旨

General introduction

Vibrio cholerae is a Gram negative aquatic bacterium. Until now more than 200 serogroups of *V. cholerae* are known and among them toxigenic O1 and O139 serogroups are mainly responsible for cholera epidemics. Cholera is a devastating diarrheal disease in many developing countries and claims several thousand lives every year. *V. cholerae* O1 serogroup divided into two biotypes, El Tor and classical. In the recent history of cholera seven distinct pandemics have been traced. The first six pandemics (1817-1923) were presumably caused by O1 classical biotype whereas the current seventh pandemic that started in 1961 is due to O1 El Tor biotype. The O139 serogroup emerged as epidemic strain in 1992. *V. cholerae* strains belonging to other serogroups are collectively known as non-O1/non-O139 and only very few of these strains are toxigenic and can cause sporadic cases of diarrhea. However, all the toxigenic *V. cholerae* strains possess two major virulence factors: cholera toxin

(CT) and toxin-coregulated pilus (TCP).

The CT is composed of one 'A' subunit encoded by *ctxA* gene and five 'B' subunits encoded by the *ctxB* gene. The 'B' subunit of CT of El Tor differs from that of classical in two amino acid residues. Recent cholera outbreaks are caused by O1 El Tor biotype which possesses classical *ctxB* gene and designated as El Tor variants. The TCP helps the bacterium during colonization in human intestine and its major structural subunit is encoded by *tcpA* gene. Expressions of CT and TCP are regulated by ToxT, the master regulator of virulence gene expression. On the other hand, ToxT expression can be activated by coupling of ToxR/ToxS and TcpP/TcpH. In addition, a global prokaryotic gene regulator known as histone-like nucleoid structuring protein (H-NS) encoded by *hns* gene, has been shown to repress the transcription of several virulence genes including *toxT*, *ctxAB* and *tcpA*.

Like many other bacteria, genetic recombination events in *V. cholerae* have aided in the emergence of multi drug resistance (MDR) strains. The use of antimicrobial agent targeting bacterial viability can be expected to impose selective pressure on the development of antimicrobial resistance. On the contrary, targeting virulence regulatory process like interference in quorum sensing (process of bacterial cell-to-cell signaling through chemical molecules known as autoinducer), inhibition of virulence genes expression etc. has advantage by nullifying the chance of developing resistance against antimicrobial agents. Commonly used natural products like spices, herbs, fruits, etc. have many kinds of beneficial effects to our health. Spices and herbs have also been used to treat diarrheal diseases since ancient time. As these kind of natural products have fewer side effects, much attention has been paid for developing antimicrobial drugs from these products. However, the effects of natural compounds have been examined targeting the viability of some pathogens whereas little is known about their specific influence on bacterial virulence regulation.

The objective of this study was to examine the potentiality of spices to inhibit the CT production in *V. cholerae* without affecting the growth and to explore its specific regulatory mechanism at transcriptional level. The research was divided into three parts: 1) checking the effect of spices extracts on the growth and CT

production in *V. cholerae* strains, 2) identification and purification of spice extract fractions which are most active against CT production in *V. cholerae* and 3) determination of possible regulatory mechanism for the inhibition of CT production by using active components of spice.

Chapter 1. Effect of different spice extracts on the cholera toxin production in *Vibrio cholerae*

Six spices were selected such as red chili (*Capsicum annuum* L.), sweet fennel (*Foeniculum vulgare* Miller var. *dulce*), white pepper (*Piper nigrum* L.), cassia bark (*Cinnamomum aromaticum*), red pepper (*Piper nigrum* L.) and star anise (*Illicium verum*). All these spices have medicinal properties and are commonly available in South East Asia, where cholera outbreaks are frequent. The selected spices were extracted with 99.9% methanol (MeOH) and used as crude extract. A total of 52 *V. cholerae* strains including O1 El Tor ($n = 40$), O1 classical ($n = 5$), O139 ($n = 5$) and nonO-1/non-O139 ($n = 2$) were selected. All strains were *ctxA* gene positive. Preliminary results revealed that in many strains (20 out of 52) the growth was not affected with white pepper MeOH extract (100 μg/ml). Among them four high CT producing (determined by bead ELISA) O1 El Tor strains were selected for further study. Effect of all six crude spices extract on the quorum sensing of these strains was examined by measuring autoinducers production [Cholera auotinducer-1 (CAI-1) and autoinducer-2 (AI-2)]. No clear-cut influence of spice extract on autoinducers production was observed. However, all six spices MeOH extract were able to inhibit the CT production in these strains without affecting their growth. Methanol alone was used as a control, did not show any inhibitory effect on the growth as well as CT production. Red chili, sweet fennel and white pepper MeOH extracts showed more than 90% inhibition of CT production in the four *V. cholerae* strains. Among these three spices, red chili MeOH extract was able to inhibit the CT production most strongly with all the tested doses (10, 50, 100 μg/ml) against these strains. Red chilies from various countries (China, Japan, Turkey) were also able to inhibit the CT production indicating that the active

components in this spice are innate. Hence red chili was selected for further purification to obtain the active ingredients responsible for inhibition of CT production.

Chapter 2. The effect of purified fractions and compounds from red chili and other spices on the cholera toxin production in *Vibrio cholerae*

Red chili was extracted with MeOH and filtrate was evaporated under reduced pressure to obtain MeOH extract. The MeOH extract was partitioned into H_2O/EtAcO mixture and EtAcO soluble portion was concentrated and further partitioned into n-hexane/90% aq MeOH mixture. H_2O soluble portion was further partitioned with n-butanol. Each soluble portion was evaporated under reduced pressure to give n-hexane, 90% aq MeOH, n-butanol and H_2O extracts, respectively and was applied to examine the effect on the CT production in a *V. cholerae* O1 El Tor, CRC41 strain. The CT production in this strain was inhibited more than 90% with n-hexane and 90% aq MeOH extracts, respectively whereas the aqueous and n-butanol extracts were able to inhibit the CT production in lesser extent, respectively. These results indicated that the active components of red chili against CT production are hydrophobic in nature. Next the n-hexane extract was separated by SiO_2 column (n-hexane/EtAoc solvent system) to furnish two fractions, namely Fr. A and Fr. B. Fr. A and Fr. B were confirmed to mainly include fatty acids mixture and capsaicin, respectively, by TLC analysis. 90 % aq MeOH extract was also separated by SiO_2 column (n-hexane/EtAcO solvent system) to furnish four fractions (Fr. 1 to Fr. 4). Fr. 1 was confirmed to be fatty acids mixture and Fr. 3 was confirmed to be capsaicin by TLC analysis. All these fractions were also examined against the CT production. Fr. A and Fr. B of n-hexane and Fr.1 of 90% aq MeOH extract inhibited the CT production exceedingly in all tested doses (10, 50, 100 μg/ml). From mixture of fatty acids, one saturated fatty acid (palmitic acid) and two unsaturated fatty acids (linolenic and arachidonic acids) were selected and also examined against the CT production in CRC41 strain and linolenic acid was observed to inhibit the CT production most efficiently.

Since Fr. 3 contained capsaicin, which is one of the most abundant ingredients in red chili, the effect of capsaicin on the CT production was examined in detail using 23 *V. cholerae* strains of different serogroups including El Tor variants (classical CT producer, $n = 12$), El Tor ($n = 5$), classical ($n = 2$), O139 ($n = 2$) and non-O1/non-139 ($n = 2$) strains. Capsaicin (100 μg/ml) was able to drastically inhibit the CT production in all tested El Tor, O139 and non-O1/non-O139 strains without affecting their growth. Whereas, in case of two classical strains the CT production was inhibited with 50 μg/ml of capsaicin (at higher concentration growth inhibition was observed) without affecting their growth. Up to our knowledge this might be the first report showing the inhibition of CT production in *V. cholerae* strains by capsaicin. The major components of sweet fennel such as *trans*-Anethole and 4-Allylanisole were also able to inhibit the CT production in sub-bacteriocidal concentration whereas piperine from white pepper was relatively less effective against the CT production in CRC41 strain. All these results indicate that the active components from red chili, sweet fennel and white pepper are potential natural sources for inhibiting CT production in *V. cholerae*.

Furthermore, to see if the inhibitory activity of red chili extract on CT production is observed *in vivo*, rabbit ileal loop assay was performed with *V. cholerae* O1 El Tor CRC41 strain (10^7 cfu/loop) in the presence (5 mg/loop) or absence of red chili 90% aq MeOH extract. Fluid accumulation was reduced with red chili 90% aq MeOH extract after 6 h in comparison to control. It was also observed that in accumulated fluid the CT production was 71% inhibited with red chili 90% aq MeOH extract in comparison to control. This data suggested that the red chili 90% aq MeOH extract could inhibit the CT production not only *in vitro* but also *in vivo*.

Chapter 3. Possible mechanisms for the reduction of virulence genes transcription with red chili fractions and capsaicin in *Vibrio cholerae*

Possible mechanisms behind the inhibition of CT production with different fractions of red chili (100 μg/ml) were examined at transcriptional level in a *V.*

cholerae El Tor CRC41 strain. Initially, *ctxA* gene transcription was analyzed through real-time quantitative reverse transcription-PCR (qRT-PCR) using TaqMan probe method. Furthermore, transcription of another virulence gene *tcpA* as well as other regulatory genes such as *toxT, toxR, toxS, tcpP, tcpH*, and *hns* were also analyzed. For qRT-PCR specific primers and probes were designed by Primer express 3.0, software. The housekeeping gene *recA*, was used as an internal control. Total RNA was purified from the CRC41 strain cultured up to late log phase in the absence and presence of crude red chili methanol extract, red chili 90% aq MeOH extract, Fr. 1 (obtained through SiO_2 chromatography), linolenic acid and capsaicin, and subjected to the qRT-PCR. The transcription of *ctxA* and *tcpA* genes was drastically repressed with above mentioned red chili fractions. The *toxT* gene transcription was also repressed by all these fractions. In contrast, the *hns* gene transcription was enhanced by red chili fractions. Whereas other regulatory genes such as *toxR, toxS, tcpP, tcpH* was apparently less affected with red chili fractions. These data suggested that the enhancement of *hns* gene transcription by all red chili fractions might be responsible for the repression of *ctxA*, *tcpA* and *toxT* genes in O1 El Tor CRC41 strain.

Conclusions

1. Red chili, sweet fennel and white pepper MeOH extracts could strongly inhibit the CT production in sub-bacteriocidal concentration in *V. cholerae* O1 El Tor strains.

2. The n-hexane and 90% aq MeOH extracts of red chili were able to inhibit the CT production in a *V. cholerae* O1 El Tor strain *in vitro*. Besides, the red chili 90% aq MeOH extract was also able to reduce the fluid accumulation as well as CT production *in vivo*.

3. Capsaicin and mixture of fatty acids, such as linolenic acid, arachidonic acid and palmitic acid were able to drastically inhibit the CT production in *V. cholerae* strains belonging to various serogroups and biotypes including O1 El Tor, O1

classical, O139, non-O1/non-O139 and newly emerged O1 El Tor variant strains. The major components from sweet fennel such as *trans*-Anethole, and 4-Allylanisole were also able to inhibit the CT production in sub-bacteriocidal concentration. To the best of our knowledge this is the first report demonstrating that red chili, sweet fennel and white pepper can inhibit the CT production in *V. cholerae* without affecting their growth.

4. The *ctxA, tcpA* and *toxT* genes transcription was drastically repressed by red chili 90% aq MeOH extract, Fr. 1 (obtained through SiO_2 chromatography), linolenic acid and capsaicin. In contrast, *hns* gene transcription was enhanced with these red chili fractions indicating that inhibition of the CT production might be due to the repression of *ctxA*, *tcpA* and *toxT* genes by H-NS.

Thus, routine intake of natural spices may be an alternative approach to prevent cholera.

審査結果の要旨

コレラ菌は、汽水域に生息するグラム陰性桿菌である。現在までに 200 種類以上の O 群血清型が報告されているが、コレラの原因となるのはコレラ毒素（CT）産生性の O1 と O139 の O 群血清型のみであると考えられてきた。O1 コレラ菌は生物学的性状の違いによりさらに古典型とエルトール型に分けられる。現在までにコレラの世界流行は 7 回報告されている。第 1 次から第 6 次は古典型 O1 コレラ菌（古典型 O1）が、1961 年から始まり今日まで続いている第 7 次はエルトール型 O1 コレラ菌（エルトール型 O1）が、原因菌として知られている。1992 年に突如として新型コレラ菌 O139（O139）によるコレラが流行し、第 8 次の世界流行の始まりかと危惧された。一方、O1 と O139 以外のコレラ菌は non-O1、non-O139 コレラ菌（non-O1/O139）と呼ばれ、散発性下痢症の原因菌とし問題となっている。

コレラ菌の主要な病原因子に、CT と定着線毛（TCP）がある。古典型とエルトール型の CT の間で 2 アミノ酸残基が異なり抗原性に影響を与えている。近年、コレラの流行時にエルトール型と古典型の両方の性質を併せ持つハイブリッド型の O1 コレラ菌が相次いで分離された。分離菌を詳細に調べた結果エルトール型 O1 であるが古典型の CT を

産生する菌(エルトールバリアント)が高頻度に分離されていることが明らかとなった。また、コレラの治療の第一選択薬として用いられるテトラサイクリンやニューキノロンなどに対する耐性菌の出現が深刻な問題となっている。それゆえ、新たな抗菌物質の探索研究が精力的に行われておりその対象として天然物が注目を集めている。しかしながら、新たな抗菌薬が開発されたとしてもやがては耐性菌が出現してくることが懸念される。本研究では、コレラの流行地域で日常的によく用いられているスパイスに注目し、コレラ菌の病原因子特に CT の産生を抑制する物質がスパイス中に含まれているか否か、また、含まれている場合はその抑制に関わる物質の性状並びに抑制機構について解析した。

第１章では、熱帯・亜熱帯のコレラ流行地で比較的よく使用されている 6 種類のスパイス、すなわち白胡椒、赤胡椒、赤唐辛子、桂皮、八角、ウイキョウのメタノール抽出物のCT産生に及ぼす影響について調べた。まず、白胡椒のメタノール抽出物のエルトール型01　40 株、古典型01　 5 株、0139　5 株、non-01/0139　2 株の計 52 株の増殖に及ぼす影響を調べた。その結果、増殖に影響を受けなかった 20 株から、CT産生性の高い 4 株を選び、さらにCT産生性及びクオーラムセンシングに対する影響を 6 種類のスパイスのメタノール抽出物について調べた。その結果、クオーラムセンシングとは無関係にCTの産生性が抑制されること、特に赤唐辛子、白胡椒およびウイキョウが強い抑制活性を示すことを明らかとした。

第２章では、CT産生に対する抑制活性が最も高い唐辛子のメタノール抽出物に含まれる抑制因子を明らかにすることを目的に、活性物質の精製・分画を試みた。メタノール抽出物を極性の異なる様々な溶媒で分画したところ、１つの画分のみならず複数の画分でCT抑制活性が認められた。中でもn-Hexaneや 90%メタノール抽出画分で強いCT産生抑制活性が認められた。さらに、n-Hexaneと 90%メタノール抽出物をシリカゲルクロマトグラフィーで分画したところそれぞれで分画されたフラクションに脂肪酸類とカプサイシンが含まれることがTLCによって確認された。また両フラクションにCT抑制活性が確認でき、さらに市販のカプサイシン、パルミチン酸、リノレン酸及びアラキドン酸を用いてCT抑制活性を調べたところ、カプサイシンとリノレン酸に強いCT産生抑制活性があることを見いだした。

第３章では、カプサイシンのCT産生抑制活性をさらに調べることを目的に、エルトール型01　5 株、古典型01　5 株、エルトールバリアント 12 株、0139　2 株、non-01/0139 2 株を用いて調べた。その結果、調べたコレラ菌全てにおいてカプサイシンによるCT産生抑制活性が認められた。さらに、90%メタノール抽出物は、コレラ菌による液体貯留を*in vivo*でも抑制することがウサギ腸管ループ試験によりわかった。カプサイシンのCT産生抑制機構を明らかにすることを目的に、CT産生を制御すると報告されている遺伝子の転写レベルをリアルタイムRT-PCRで調べた。その結果、*hns*遺伝子の転写促進に基づく*toxT*、*tcpA*、*ctx*遺伝子の転写抑制である可能性を示した。

以上の結果は、赤唐辛子の中にCTの産生を抑制する物質が複数含まれること、特に赤唐辛子の主要な成分の１つであるカプサイシンに非常に強いCT産生抑制活性を有する

ことを明らかにした。さらにその作用がCTの発現調節に関わる遺伝子群の転写レベルで起こっていることを明らかにした。これらの成果は、赤唐辛子の中に含まれる物質が特定のコレラ菌の増殖に影響を与えず病原因子の転写を抑制すること、即ち新たな細菌感染症の治療薬となる可能性を提供するものであり、獣医学の分野のみならず医学領域においても多大な貢献をすると考えられる。従って、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。