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論　文　名	Dynamics of cholera outbreaks in endemic areas of Kenya (ケニアにおけるコレラアウトブレークの発生動態)	
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論文要旨

Introduction

Cholera is an acute secretory diarrhea, which may lead to loss of body fluids and eventually death. The causative agent of cholera is Gram-negative bacterium *Vibrio cholerae*. There are more than 200 'O' serogroups of *V. cholerae* but only O1 and O139 were responsible for cholera epidemics and pandemics. Furthermore, *V. cholerae* O1 is classified into two important biotypes, classical and El Tor. In the history of cholera, so far 7 cholera pandemics were recorded. For first to sixth cholera pandemics *V. cholerae* O1 classical biotype was thought to be responsible while the ongoing seventh pandemic, which was initiated from Indonesia in 1961, was caused by O1 El Tor biotype. The seventh pandemic El Tor strains reached African continent in 1970s. Virulence of *V. cholerae* associated with cholera is depended on the production of cholera toxin and toxin co-regulated pilus proteins encoded by the *ctx* and *tcpA* genes, respectively. The other serogroups referred to as non-O1/O139 normally lack *ctx* and *tcpA* genes but are associated with sporadic cases of diarrhea. Virulence in non-O1/O139 is varying and attributed to type three secretion system (T3SS), heat-stable enterotoxin (also called nag-ST), cholix toxin (*chxA*), etc.

Recently, there has been an emergence of new *V. cholerae* O1 pathogenic variants which posses traits of both classical and El Tor prototypes. These variants can be differentiated by genetic comparative analysis of several genes such as *ctxB, tcpA, rstC, rtxC,* etc. The O1 El Tor variant has been shown to be more virulent than the El Tor prototype and has been reported

from several Asian countries and a few from African. The highest number of cholera outbreaks occurred in countries surrounded by the Bay of Bengal, which was considered the homeland of cholera. However, since the beginning of the millennium, Africa has reported more than 90% of the total global cholera cases and cholera related deaths, suggesting that cholera may have settled in Africa. Kenya is one of the African countries, which experienced the increase of cholera outbreaks. There is inadequate information on the dynamics of cholera in Kenya related to continuous isolation of *V. cholerae*, the role of food handlers and index cases, phenotypic and genotypic changes of isolates from consecutive cholera outbreaks as compared to studies carried out in Asian endemic countries and more recently in Haiti. There is paucity of information on the new pathogenic *V. cholerae* O1 El Tor variants in Africa including Kenya. Antimicrobials are used to reduce transmission of *V. cholerae* and duration of diarrhea. However the increase of multidrug resistant *V. cholerae* strains has threatened the effective use of the recommended antimicrobials such as tetracycline. Lack of systematic research on antimicrobial resistance has created gaps in dealing with cholera outbreaks in Africa including Kenya.

In this study, epidemiological survey of cholera endemic areas of Kenya was carried out prior to the new millennium and between 2007 and 2009. Prevalence of *V. cholerae* was compared with that of *Salmonella* spp. and *Shigella* spp. in patients with diarrhea, food handlers and cholera index cases. By adopting new typing schemes *V. cholerae* isolates were identified to their atypical levels. Prevalence of antimicrobial resistance in *V. cholerae* isolates was determined. Molecular traits, presence of virulence factors and clonal relatedness of *V. cholerae* isolates were assessed. The findings of this study illustrates that cholera in Kenya have not reached endemicity levels like those found in Asian countries, however serotype switching, extended multidrug resistance and replacement of *V. cholerae* O1 El Tor prototype by the *V. cholerae* O1 El Tor variant have been observed.

Chapter 1. Epidemiology of cholera in endemic areas of Kenya

Kenya like other African countries experienced an increase in cholera outbreaks from the year 2000 with surge in 2007 and 2009 and significant case fatality rate (CFR). In 2007, 1,206 cases were reported with CFR of 5.56%, whereas 11,475 cases were reported in 2009 with CFR of 2.31%. The highest CFR of 11% was reported in a cholera outbreak in 2008. Previous studies focused on characterization of *V. cholerae* O1 isolates obtained from various outbreaks in Kenya or risk factors involved in transmission. A holistic study to gauge the dynamics of cholera has not been carried out in Kenya. This study was conducted to investigate systematically the dynamics of cholera in endemic areas of Kenya in consecutive outbreaks.

The prevalence of enteropathogens in particular *V. cholerae, Salmonella* spp. and *Shigella* spp. was studied in patients with gastroenteritis, food handlers, cholera index cases and cholera patients. The study areas were coastal regions of Malindi and Kwale, and an inland lake region, Nyanza. Follow up studies in Nyanza were discontinued because of security problems following a disputed presidential election. Among the 862 patients with diarrhea from the Malindi region between 1991 and 1993, *V. cholerae* was not isolated although *Salmonella* spp. and *Shigella* spp. were isolated at a rate of 7.3% and 6.5%, respectively. A total of 3,006 specimens from Kwale were analyzed for enteropathogens between 2007 and 2009. The rate of isolation of enteropathogens from the patients was *Shigella* spp. (11.0%), *Salmonella* spp. (6.9%), *V. cholerae* O1 (2.8%), *V. cholerae* non-O1/O139 (0.9%) and *Aeromonas* spp. (0.1%), while no pathogen was detected in 1,257 specimens (78.3%). Unlike *Salmonella* spp. and *Shigella* spp., *V. cholerae* were isolated only during the cholera outbreak periods in 2007 and

2009. *V. cholerae* O1 Ogawa and non-O1/O139 were isolated in 2007, whereas only *V. cholerae* O1 Inaba was isolated in 2009. A total of 1,402 stool specimens from the food handlers analyzed yielded *Salmonella* spp. (1.6%) and *Shigella* spp. (0.7%). whereas 1,369 (97.7%) were negative. *V. cholerae* was not isolated from the food handlers. Eighty-one *V. cholerae* isolates were obtained from Nyanza in 2008 cholera outbreak. Nine cholera index cases were investigated for *V. cholerae.* Six were positive for *V. cholerae* O1, two yielded *Aeromonas* spp. and *Salmonella* spp. was isolated from one case.

Although *Salmonella* spp. and *Shigella* spp. were isolated throughout the study period, *V. cholerae* was only isolated during outbreak periods. Misdiagnosis among index cases was common. Food handlers may not play an important role in transmission of cholera. This study reports for the first time the serotype switch in *V. cholerae* from Ogawa to Inaba during two consecutive cholera outbreaks not only in Kenya but also in the rest of Africa. It is important to consider serotype switch when adopting new cholera vaccines. Cholera cases may have increased in Africa but more studies are required to confirm whether *V. cholerae* is established in this region. The dynamics of cholera outbreaks in Kenya are different from that observed in Asian endemic countries.

Chapter 2. Prevalence of antimicrobial resistance in *Vibrio cholerae* isolates

Prevalence of multidrug resistant *V. cholerae* O1 has been increasingly recognized. The guideline for antimicrobial use has been adopted mainly from studies carried out in endemic Asian countries. Yet, one of the earliest reports on tetracycline resistant *V. cholerae* O1 emanated from Africa. With the number of cholera outbreaks increasing in Africa then there is a need to systematically monitor antimicrobial resistance among *V. cholerae*. This study was designed to map prevalence of antimicrobial resistance among circulating strains from the different endemic regions of Kenya and monitor development of resistance in consecutive cholera outbreaks. A total of 130 *V. cholerae* isolates were analyzed for antimicrobial susceptibity to 12 antimicrobial agents including trimethoprim sulfamethoxazole (SXT), fosfomycin (FOF), ampicillin (AMP), imipenem (IPM), chloramphenicol (CHL), tetracycline (TET), norfloxacin (NOR), nalidixic acid (NAL), streptomycin (STR), kanamycin (KAN), cefotaxime (CTX) and ciprofloxacin (CIP). The *V. cholerae* O1 isolated in Nyanza, 2008 and in Kwale, 2007 were more susceptible to the tested antimicrobials. With the exception of a few Nyanza isolates all these were susceptible to FOF, AMP, IPM, CHL, TET, NOR, KAN, CTX and CIP. Most of the Nyanza isolates (88%) were resistant to CHL.

Interestingly, *V. cholerae* O1 isolated in Kwale, 2009 exhibited the highest level of multidrug resistance. The strain was resistant to 9 out of the 12 antimicrobials tested, with the exception of TET, NOR and CIP. This level of antimicrobial resistance in *V. cholerae* has not been reported previously from Kenya. Similarly the study presents first cholera outbreak in Kenya caused by a toxigenic *V. cholerae* O1 showing resistance to third generation cephalosporin. *V. cholerae* non-O1/O139 isolated in Nyanza, 2008 was susceptible to all antimicrobials except AMP. On the other hand *V. cholerae* non-O1/O139 isolated in Kwale, 2007 were susceptible to all the antimicrobials tested except for SXT and NAL. Multidrug resistance pattern to the antimicrobials varied within the different endemic regions as well as in the year of isolation of *V. cholerae*. It is important to carry out antimicrobial susceptibility test in the course of each cholera outbreak and within each region in Kenya. The use of cephalosporins is not recommended as an alternative to tetracycline. A larger national systematic study is required to develop local policy on antimicrobial use for cholera management.

Chapter 3. Molecular characterization of *Vibrio cholerae* strains associated with cholera outbreak

New pathogenic variant of *V. cholerae* O1 have spread in not only Asia but also Africa. However, only few African countries have reported emergency of the new variants. In Kenya the prevalence of O1 El Tor variant has not been determined. This study proposed that one of the contributing factors to the severity of cholera outbreaks in Kenya was due to the more toxigenic *V. cholerae* O1 El Tor variant. As such molecular characterization of *V. cholerae* O1 isolated from outbreaks was carried out by employing several specific PCR, multiplex PCR and mismatch amplification (MAMA) PCR targeting *rfb, ctxB, rstR, rstC, tcpA* and *toxR* genes for *V. cholerae* O1 and colony hybridization targeting heat stable toxin (*nag-ST*), T3SS related (*vcsC*$_2$, *vcsN*$_2$ and *vopF*) and *chxA* genes for *V. cholerae* non-O1/O139. All the *V. cholerae* O1 possessed the El Tor *rstR* and *tcpA* genes, *rstC* gene and all harbored the classical type *ctxB* allele. This study suggests that the O1 El tor variant has replaced the O1 El tor prototype in Kenya. The *V. cholerae* O1 isolates were negative for *nag-ST*, T3SS related and *chxA* genes except the only *V. cholerae* non-O1/O139 from Nyanza/2007 isolate which was positive for *chxA* gene. Analysis of *Not*I restriction enzyme digested PFGE profile of *V. cholerae* O1 strains isolated from Nyanza province revealed that they were most likely of clonal origin. The PFGE patterns of Kwale *V. cholerae* O1 isolates showed subtle differences with an insertion of a single band (~90kb). Interestingly, the *V. cholerae*O1 from Kwale/2009 seems to be more closely related to the *V. cholerae* O1 strains isolated from Nyanza/2007.

Conclusions

V. cholerae O1 El tor variant has replaced the *V. cholerae* O1 El tor prototype in Kenya and may be associated with severity of the disease. We are reporting for the first time in Kenya, serotype switch and replacement of less resistant strains by multidrug resistant strains in the same area of Kenya during consecutive outbreaks. Unlike endemic areas in South Asia *V. cholerae* is isolated only during outbreak periods and food handler may not play a key role in transmission of *V. cholerae*. Cholera outbreaks in different endemic areas in Kenya can be caused by *V. cholerae* O1, which share close clonal relatedness. Cholera outbreak has increased in Africa but it has not reached to endemicity levels found in Asia.

審査結果の要旨

コレラは現在においても、南アジアやアフリカで猛威を振るっており、小児を中心に多くの命が失われている重要な感染症である。現在コレラ菌には 200 種類以上の O 血清型が知られているが、コレラの原因となるのは O1 と O139 コレラ菌のみである。一方、O1 と O139 以外のコ

レラ菌を一括して non-O1/non-O139 コレラ菌と呼び、重症下痢症患者から分離されるが、いわゆるコレラの原因とはならない。O1 コレラ菌は生物学的性状の違いから 2 種類に分類される。1 つは第 5 次と 6 次の世界流行に関わり致命率が高い古典型、もう 1 つは現在も続く第7次の世界流行に関わり致命率は高くないが環境中での生存率が高いエルトール型である。O1 及び O139 コレラ菌の病原因子として、コレラ毒素（Cholera toxin: CT）や Toxin co-regulated pilus (TCP) がある。通常、non-O1/non-O139 コレラ菌は、*ctx* や *tcpA* 遺伝子を持たず、3型分泌装置（T3SS）、耐熱性溶血毒（NAG-ST）、コリックス毒素（ChxA）等が病原因子として知られている。さらに近年、エルトール型と古典型の両方の性質を持ち合わせ、より病原性の強いハイブリッド型の O1 コレラ菌エルトールバリアントが出現し大きな問題となっている。今世紀に入る前はほとんどのコレラがベンガル湾周辺国で発生していたが、21 世紀に入り世界で発生するコレラの 90%以上はアフリカで起こっている。ケニアはアフリカの中でも最もコレラの発生が多い国の1つである。しかしながら、ケニアでのコレラの疫学情報や薬剤感受性を含む分離菌の細菌学的性状に関する情報はほとんどない。

申請者は、ケニアにおけるコレラの発生動態及び患者から分離されたコレラ菌の細菌学的性状を明らかにすること目的に、1) 1990 年前半と 2007 から 2009 年のケニアにおけるコレラ様下痢症の疫学的調査を行い、2) 分離されたコレラ菌の薬剤感受性、3)コレラ菌の病原因子プロファイルを含む分子疫学的解析を行った。

第1章では、コレラ様の下痢症が流行しているインド洋に面したマリンディとクワレ、ビクトリア湖に面したニャンザで疫学調査を行った。マリンディでの 1991 から 1993 年の調査では、下痢症患者からサルモネラ (7.3%) や赤痢菌 (6.5%) は分離されたがコレラ菌は分離されなかった。2000 年代に入ると、下痢症の致命率が一気に上昇し、2007 年には 5.6%、2009 年は 2.3%、2008 年には 11%となった。2007 から 2009 年にクワレで行った調査では、赤痢菌 (11%)、サルモネラ (6.9%)、O1 コレラ菌 (2.8%) が主要な下痢原因菌として分離された。サルモネラや赤痢菌と異なり O1 コレラ菌はアウトブレーク時以外には分離されなかった。また、2007 年は稲葉型が流行し、2009 年は小川型が流行した。コレラ菌の血清型変換はケニアのみならずアフリカで初めての報告である。調理従事者からは O1 コレラ菌は分離されず、ケニアでは南アジアと異なり調理従事者がコレラの感染源となっていないことを明らかとした。

第2章では、コレラ様アウトブレーク時に分離された O1 コレラ菌の薬剤感受性について調べた。コレラの治療の第一選択薬として一般的にテトラサイクリンが挙げられる。しかし、アフリカにおいてテトラサイクリン耐性の O1 コレラ菌が原因のコレラアウトブレーク時にテトラサイクリンを使用したことで、多くの犠牲者を出した苦い経験がある。そこで、適切な抗菌薬の選択を行えるよう、分離した O1 コレラ菌 139 株について 12 種類の抗菌薬に対する薬剤感受性を調べた。その結果、2007 年にクワレ、2008 年にニャンザで分離した O1 コレラ菌は調べたほとんど全ての抗菌薬に感受性であったが、2009 年にクワレで分離した O1 コレラ菌はテトラサイクリン

とニューキノロン系の 3 種類を除く 9 種類で耐性を示す多剤耐性菌であった。また、薬剤耐性パターンは分離された年や都市で異なっていることを明らかとした。

第3章では、コレラ様アウトブレーク時に分離されたコレラ菌の病原因子プロファイルについて解析した。その結果、全ての O1 コレラ菌が *ctx* 遺伝子を保持し、*ctx* 遺伝子はエルトール型でなく古典型であった。その他は、エルトール型の性状を示す病原因子及び病原因子関連遺伝子を保持していた。よって、2007 年以降ケニアで発生したコレラ様アウトブレークは、いわゆる典型的なエルトール型 O1 コレラ菌によるものでなく、O1 コレラ菌エルトールバリアントによるものであることを明らかとした。一方、コレラ様アウトブレーク時に分離された non-O1/non-O139 コレラ菌の 1 株で、近年新たな病原因子として見いだされた *chxA* II 遺伝子が陽性であった。それぞれのアウトブレークで分離された O1 コレラ菌の PFGE パターンの解析から、それぞれのアウトブレーク株はクローナルであること、また 2009 年のクワレ株が 2007 年のニャンザ株と類似していることを明らかとした。

本研究において、ケニアでのコレラアウトブレークはエルトール型 O1 コレラ菌でなく、南アジアで流行し重症化率の高い O1 コレラ菌エルトールバリアントが原因であることを初めて明らかにした。また、ケニアのコレラアウトブレークで稲葉型から小川型への血清型変換が起こったことや薬剤感受性菌が多剤耐性菌に置き換わったことも初めて示した。コレラの流行時以外にコレラ菌は分離されず、調理従事者がコレラの感染に関わっていないことやアフリカにおけるコレラ流行様式は南アジアとは異なることを明らかにした。本研究で行なったコレラの疫学研究は、アフリカにおける致命率の高いコレラの原因の一端を明らかとするものであり、獣医学領域のみならず医学領域にも大きく貢献するものと評価できる。本論文の審査および学力確認の結果をあわせて博士（獣医学）の学位を授与することを適当と認める。