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論　文　名	Occurrence of multidrug-resistant *Escherichia coli* in Egyptian dairy products and effect of subinhibitory concentration of antimicrobials on resistance modulation （エジプトの乳製品における多剤耐性大腸菌の発生状況と抗菌薬の亜抑制濃度が耐性変調に及ぼす影響）
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論文要旨

Introduction

Escherichia coli is a commensal bacterium found in the large intestines of humans and animals. It has a high elasticity to acquire antimicrobial resistance (AMR) genes or even raise intrinsic resistance by genetic mutation under improper antimicrobial treatment. The growing prevalence of AMR *E. coli* represents a significant public health concern which puts it as a priority microorganism for monitoring AMR under the United Nations' sustainable development goals. Furthermore, certain strains acquired virulence genes and were associated with enteric and extraintestinal infections. These potentially virulent *E. coli* strains caused several outbreaks worldwide through the consumption of contaminated raw milk and dairy products, one of the important sources of *E. coli* transmission to humans. In Egypt, the production of milk and dairy products poses a significant risk of *E. coli* contamination since it mainly depends on small farms and small-scale factories operating under improper hygienic conditions. Additionally, antimicrobials are frequently and improperly used in these farms. This results in the presence of antimicrobials at subinhibitory levels, promoting the selection and dissemination of resistant strains and AMR genes. The transfer of these resistance

determinants from animals to humans underscores the urgent need for monitoring the emergence and dissemination of AMR *E. coli* in dairy products in the Egyptian markets and understanding the impact of improper usage of antimicrobials on dairy sectors on hazarding antimicrobials important for humans.

Thus, this study aims to elucidate the role of milk and dairy products in transmitting AMR and potentially pathogenic *E. coli* to humans while investigating the impact of uncontrolled antimicrobial use in dairy farms on resistance development. Chapter 1 describes the screening of raw milk and dairy products from Egyptian markets to detect AMR and pathogenic *E. coli*. Chapter 2 evaluates the effects of subinhibitory concentrations (SICs) of commonly used antimicrobials on the resistance profiles of susceptible *E. coli* strains *in vitro*. Chapter 3 extends this analysis to multidrug-resistant (MDR) *E. coli* strains, exploring the broader implications of such antimicrobial use for human medicine.

Chapter 1. Prevalence and characterization of antimicrobial-resistant and potentially pathogenic *E. coli* in raw milk and dairy products, in Egypt

To examine the contamination of *E. coli* in Egyptian milk and dairy products, 210 samples were collected from small farms, supermarkets, and street vendors in Kafrelsheikh and Algarbia, governorates, Egypt. Samples included raw buffalo milk (n=50), goat milk (n=20), Domiati cheese (n=35), Domiati cheese with pepper (n=25), rayeb (n=40), and yogurt (n=40). The samples were processed and cultured on EMB agar plates. Fifty-five (26%) samples were contaminated by *E. coli* and 196 *E. coli* isolates were obtained. Notably, a higher contamination rate was observed in raw milk (30-68%) compared to fermented dairy products (7.5-13%). The processing steps, probiotic and lactic acid-producing bacteria utilized during the ripening of these products may exert an inhibitory effect on *E. coli*. To avoid selection of clonal isolates for characterization, *E. coli* isolates (n=196) were examined by ERIC-PCR, resulting in 84 non-clonal isolates whose clonal relatedness was also confirmed by PFGE. Antimicrobial susceptibility test of the 84 isolates was conducted using the disc diffusion method. Among 84 isolates, 27% (23/84) were resistant to at least one antimicrobial agent and 3.5% (10/23) of them were classified as MDR (resistant to at least three classes of antimicrobials).

For further profiling of *E. coli* strains (n=84), O-genotyping and phylogenetic grouping were conducted. Forty-eight O-genotypes belonging to four phylogenetic groups (A, B1, B2, and D) were detected, including two strains belonging to OgGp9, phylogenetic group D, like the strain caused a large outbreak after consumption of school milk in Toyama, Japan. To investigate the similarity of Egyptian isolates to the Toyama outbreak strain, H-genotyping, and whole genome sequencing were done for

these isolates. One isolate was determined to be Hg18, contained an intact ETT2 locus (putative virulence factor), and belonged to sequence type 1380 carrying the same genetic characteristics as the Toyama outbreak strain.

Furthermore, screening of the virulence genes associated with various *E. coli* pathotypes was conducted in all isolates (n=84) by colony hybridization assay using probes for detection of adhesion and toxin-encoding genes. Results revealed that 61% (51/84) of the *E. coli* strains carried at least one of the tested virulence genes. The genes encoding toxins for intestinal pathogenicity such as *astA* and *cdt* were detected in 6.0 and 4.8% of the strains, respectively, and others encoding toxins for extraintestinal infections such as *cnf* and *hlyA* were detected in 4.8% each of the strains. The *cdt* and/or *cnf* gene(s) carrying isolates caused cytotoxicity to CHO cells and *hlyA* gene-positive strains also caused hemolytic activity on sheep blood agar.

These findings indicate that raw milk and dairy products in the Egyptian market have a high risk to cause food poisoning or its outbreaks in Egypt.

Chapter 2. Impact of subinhibitory concentration of antimicrobials used for dairy animals on human antimicrobials MICs in susceptible *E. coli* strains

In Chapter 1, resistance to common antimicrobials used for dairy was detected as well as antimicrobials used for humans. Other studies from Egypt reported the uncontrolled use of antimicrobials as a routine practice for mastitis control in dairy farms. This Chapter aims to evaluate the influence of antimicrobials on minimum inhibitory concentration (MIC) shifts for human-use antimicrobials in susceptible *E. coli*. An *in vitro* experiment was designed to treat two susceptible *E. coli* strains AS109b and AS174a obtained from milk with 0.5 x MIC of antimicrobials commonly used for dairy, specifically AMX, CEX, GEN, and CHL. Treatment with the antimicrobials was done in tryptic soy broth with the continuous passage of culture every 24 h for 21 days. Changes in MIC for various antimicrobial classes were evaluated on days 7, 14, and 21. The treatment with AMX, CEX, and GEN increased MIC within the same class in both strains, including increased MIC to third-generation cephalosporins, which are critical for treating human infections. The most pronounced effect in both strains was for CHL treatment, which induced resistance to not only CHL and but also other classes such as quinolones and β-lactams. This finding is particularly concerning, as CHL is cost-effective and commonly used in small farms lacking adequate veterinary supervision. To exclude the bacteriostatic effect of CHL, CHL-treated strains were cultured without antimicrobials for 5 days, and MIC was measured every other day. MICs of these strains remained elevated towards quinolones and β-lactams. These results suggest that the emerging resistance is not only the transient effects of CHL but might be due to genetic changes. Therefore,

whole genome sequencing was performed to check the presence of silent AMR genes and genetic mutation in common genes related to β-lactam and quinolone resistance or associated efflux systems. No antimicrobial resistance genes were detected in both strains after sequence analysis. Further, no mutations were detected in genes related to β-lactam and quinolone resistance or efflux systems. However, a deletion in the galactose metabolism and colanic acid synthesis genes (*gal* and *wca* operons) was detected in both strains after CHL treatment. The relationship between the deletion of these operons and the cross-resistance to other classes remains unclear.

This data shed light on the CHL-SIC critical effect compared to the other tested antimicrobials and the significant risks associated with using SICs of antimicrobials in dairy.

Chapter 3. The effect of subinhibitory concentration of antimicrobials used for dairy animals on MICs of human antimicrobials in multidrug-resistant *E. coli* strains

In Chapter 2, the resistance modulation after antimicrobials SIC treatment was observed. In the context of infections, MDR strains may also colonize udder tissues. Therefore, this Chapter aims to investigate the effects of antimicrobial SICs on MDR strains. *E. coli* D72 (MDR), was treated with 0.5 x MIC of selected antimicrobials for 21 days as described above. Results indicated that treatment with AMX and GEN led to increased MIC values within the same class as susceptible strains. Notably, CHL treatment also demonstrated a pronounced effect on MDR strain, the MIC of aminoglycosides was increased by fourfold. This finding contrasts with the results observed in susceptible strains. To further investigate this effect, another *E. coli* strain KC90 with a similar AMR profile was treated with 0.5 x MIC of CHL. This strain also exhibited an increase in aminoglycoside MIC, corroborating the initial findings. The CHL resistance gene (*cmlA*) was flanked by aminoglycoside resistance genes (*aadA1, aadA2, aac(3)-II*) and downstream to integron 1 in both strains. Therefore, it was hypothesized that the induction of *cmlA* gene by CHL might induce the expression of the flanking aminoglycoside resistance genes. So, the expression of these genes was measured in both MDR-treated strains using qRT-PCR. The results indicated significant upregulation of the tested genes which might explain the increase in the aminoglycoside MIC. Previous studies have reported that the *cmlA* gene product affects the inner membrane structure, which is crucial for the proton motive force (PMF) that aminoglycosides rely on for cellular entry. Therefore, an additional mechanism was hypothesized to contribute to the increased aminoglycoside MIC in *cmlA*-carrying strains. The PMF activity in CHL-treated strains was measured using DiOC2 dye. A significant reduction in PMF activity was detected in both strains after

CHL treatment. Furthermore, qRT-PCR was performed to analyze the expression of PMF-related genes, which showed significant downregulation in both strains, confirming the reduction of the PMF activity at the transcriptional level. Since MDR strains possess multiple factors that might influence PMF activity, *cmlA* gene was cloned into pUC18 and transformed into a *E. coli* strain JM109. After treatment of the *E. coli* JM109+pUC18::*cmlA* transformant by CHL, a fourfold increase in aminoglycoside MIC and CHL resistance was observed. Additionally, this transformant exhibited reduced PMF activity and downregulation of PMF genes. To further explore the relationship between increased aminoglycoside MIC and its internalization, gentamicin was conjugated with Texas Red dye (GTTR) and used to treat both *cmlA* transformant and an empty vector control strain at a concentration of 0.5 x MIC of GEN for 1 h. The fluorescence intensity was measured by flow cytometry. Results revealed a significant reduction of GTTR internalization in the *cmlA* transformant. Furthermore, to ensure the assembly of CmlA protein in the membrane, membrane fractionation was done for the transformant strains and CmlA protein was detected in the membrane fraction by western blotting. This finding suggests that the elevated aminoglycoside MIC in MDR strains after CHL treatment may be linked to increased expression of AMR genes, including *cmlA* and its flanking aminoglycoside resistance genes. In addition, the effect of *cmlA* gene product on the inner membrane and PMF dynamics appeared to synchronize with the observed reduction in aminoglycoside internalization.

Conclusions

1. Milk and dairy product samples from Egyptian retail markets might be a source of gastrointestinal infection or its outbreaks due to their contamination by antimicrobial-resistant and potentially pathogenic *E. coli*.–
2. SICs of antimicrobials used in dairy animals might lead to the emergence of resistance against human-related antimicrobials.
3. The CHL effect was pronounced against other antimicrobial classes in both susceptible and MDR strains.
4. *cmlA* gene product might be involved in increasing the MIC of aminoglycoside by decreasing the PMF activity and internalization of aminoglycoside.

審査結果の要旨

大腸菌は人や動物の大腸に生息する正常細菌叢を構成する細菌の一種である。また、大腸菌は薬剤耐性遺伝子や病原遺伝子の水平伝播により薬剤耐性能や病原性を獲得することも知られている。生（未殺菌）乳は病原性大腸菌の人への伝播に関わることが知られている。事実、ドイツ、英国、フランスにおいて生乳が食中毒の原因とることが報告されている。我が国においても 2021 年富山で学校給食を介した牛乳が原因となる OgGp9:H18 という非定型な下痢原性大腸菌による集団食中毒事例があった。エジプトでは様々な乳製品が生乳で作られているが生乳の生産は多くが小規模農場に依存している。そのため、非衛生的な環境で製造されるため、大腸菌汚染や抗菌薬の不適切使用に基づく薬剤耐性菌も問題となっている。本研究では、エジプトで市販されている生乳および生乳から製造された乳製品がどの程度大腸菌で汚染されているか、どの程度薬剤耐性化しているか、どのような病原遺伝子を保有しているかについて調べること、さらに、亜抑制濃度の抗菌薬が大腸菌の薬剤耐性化にどのような影響を及ぼすか調べることを目的とした。

第一章ではエジプトの生乳と乳製品がとの程度大腸菌で汚染されているかを調べるためにエジプトの小規模農場、スーパーマーケット、露店で販売されている生の水牛乳や山羊乳、チーズや発酵乳などの汚染状況を調査した。その結果、生の水牛乳（68%）の汚染割合が最も高く、次いで山羊の生乳（30%）、生乳で作ったドミアテチーズ（14%）、ヨーグルト（13%）、胡椒入りのドミアテチーズ（8%）、ライブ（7.5%）であった。分離菌のうち、クローナルと確認された 84 株の大腸菌について 12 種類の薬剤を用いて感受性を調べた結果、23 株が薬剤耐性と同定され、そのうちの 10 株が多剤耐性であった。また、84 株中、51 株が少なくとも 1 つの病原遺伝子を保有していた。系統解析と O 遺伝子型別、全ゲノム解析を行ったところ、2 株で富山の集団食中毒に関わった株と同じく系統は D 型、O 遺伝子型は OgGp9:Hg18、大腸菌の 3 型分泌装置 2 を有していた。以上より、エジプトの生乳や生乳から製造された乳製品は人への病原性がある大腸菌に汚染されていることが明らかとなった。

第二章では、エジプトでは乳房炎の予防に抗菌薬が日常的に使用されている背景から、亜抑制濃度の抗菌薬が人に使用されている抗菌薬に対する最小発育阻止濃度 (MIC) に与える影響について調べた。水牛の生乳から分離された薬剤感受性の大腸菌 2 株について 1/2MIC の AMX、CEX、GEN、CHL 存在下で 1 日置きに 3 週間継代培養し、各種抗菌薬に対する MIC の変化を調べた。AMX、CEX、GEN で処理した場合同種の抗菌薬には耐性化したが、CHL で処理した場合 CHL に加え NAL、CEX、CAZ、CRO あるいは AMX など異種の抗菌薬にも耐性化した。これら 2 株の全塩基配列の解析から、薬剤耐性に直接関わる遺伝子は検出されず、薬剤耐性に関係のない *gal* 遺伝子オペロンあるいは *wca* 遺伝子オペロンに欠失が

認められた。以上の結果より亜抑制濃度の抗菌薬使用、特に CHL にはリスクがあることが明らかとなった。

第三章では、亜抑制濃度の抗菌薬使用が多剤耐性菌の薬剤耐性パターンにどのような影響を与えるかを、2 種類の異なる多剤耐性大腸菌について AMX、CEX、GEN、CHL を用いて調べた。両株とも CHL のみアミノグリコシドなど異種の薬剤に対しても耐性能を付与した。耐性化機構を調べたところ、本菌が有するアミノグリコシド耐性遺伝子の転写の促進に加え、内膜に存在し CHL の薬剤排出ポンプとして働く CmlA が、同じく内膜に存在しアミノグリコシドの取り込みにも関わるプロトン駆動関連タンパクの機能を抑制していることを明らかとした。以上の結果は、亜抑制濃度の抗菌薬は薬剤耐性菌の耐性化に寄与すること、CHL とプロトン駆動関連タンパクを介しアミノグリコシドに耐性化することを明らかとした。

以上の結果は、家畜に使用される抗菌薬が乳製品に汚染している大腸菌の多剤耐性化に寄与していること、亜抑制濃度の抗菌薬使用が同種のみならず異種の薬剤耐性化に寄与する新規の機構を明らかとした。本研究成果は獣医学の分野のみならず、医学の分野において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。