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Mini-Review

Host-microbe-drug triad: Role of chloroquine/hydroxychloroquine in Covid-19 treatment-focus on inflammatory cytokine inhibition

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The threat caused by the recent coronavirus disease 2019 (COVID-19) virus pandemic has thrown everyone into a panic mode including scientists, medical practitioners and pharmaceutical firms trying to discover a drug for its treatment. This has seen many clinical studies registered within the past few months. This has called for repositioning of some drugs in order to manage the crisis with hydroxychloroquine and chloroquine being in the front line. The two have been with us over 50 years and have been demonstrated to have strong antiviral activities. Studies have shown that Covid-19 induces an inflammatory response while chloroquine and hydroxychloroquine induce an anti-inflammatory response in the body. Here, we review available information on the interaction between Covid-19 and the innate immune systems of the hosts, the type of inflammatory responses induced by Covid-19 and the anti-inflammatory response conferred by the CQ and HCQ in a bid to understand if there is a justifiable link between the two to support the latter being used as a treatment.

Key words: Covid-19, inflammation, cytokines, chloroquine, hydroxychloroquine, treatment.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) is a new global pandemic caused by the novel Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) belonging to the same family of coronaviruses such as SARS (SARS-Cov) and MERS (MERS-Cov) (Chen et al., 2020; Prompetchara et al., 2020). The virus has spread to over 213 countries and territories infecting over 50 million people and leaving over 1.2 million deaths as at 9th November 2020 and over 30 million recoveries. Due to its rapid spread and severe symptoms to some patients, the virus has caused strain in health facilities and caused panic globally. There is currently no recommended treatment for Covid-19 and researchers are working round the clock to find a cure. As the cure takes longer and the disease goes on spreading; it would also call for drug repositioning to test what has worked before as we wait for the new cure. It is in this search and the thought for repositioning the old anti-malarial drugs; chloroquine (CQ) and hydroxychloroquine (HCQ) has come into limelight.

Persons infected with Covid-19 have shown varying symptoms ranging from some being asymptomatic (Rothe et al., 2020), to others having hyper-inflammatory cytokines (Cao, 2020; Shi et al., 2020). The pathogenicity

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of covid-19 is proposed to be through production of inflammatory cytokines such as interleukin-6 (IL-6), IL-1β, as well as IL-2, IL-8, IL-17, G-CSF, GM-CSF, IP10, MCP1, MIP1α and tumor necrosis factor alpha (TNF-α) among others (Cao, 2020; Chen et al., 2020; Huang et al., 2020) especially at the severe stage. Hence any drug that suppresses the production of these cytokines would be a good candidate for the management of the virus. Currently there is no drug approved for the treatment of Covid-19 (Cao, 2020) and mostly the drugs being used are all on trial or being recommended based on studies targeting other SARS-CoV viruses.

Chloroquine (CQ) and Hydroxychloroquine (HCQ) have been in the market as malaria drugs for the longest time. While they are primarily anti-malaria, the drugs have demonstrated to have anti-inflammatory effects (Vincent et al., 2005), a property that brought about the hypothesis that they could be viable option for the treatment of Covid-19 infections. Hydroxychloroquine and Chloroquine have been demonstrated to be able to reduce the production of anti-inflammatory cytokines IL-1, IL-6, TNF and IFNγ by mononuclear cells through various signaling mechanisms (Al-Bari, 2015; Schrezenmeier and Doerner, 2020). It is against this background that we thought of reviewing available information to back this triad, where the body infected by Covid-19 produces pro-inflammatory cytokines and administration of CQ and HCQ suppresses the same cytokines restoring the body to normal. This review aims at consolidating the existing knowledge on the host-microbe-drug interactions and fills the gaps that could shed some light in support of CQ and HCQ in the treatment of Covid-19.

LITERATURE REVIEW

A literature review was performed in PubMed, Google Scholar, EMBASE, other trial Registries for studies on the use of chloroquine in patients with COVID-19. The search words included Host-microbe interaction, Microbes and Cytokines, Covid-19, SARS-Cov-2, Pro-inflammatory Cytokines, MERSCoV, Chloroquine and Hydroxychloroquine and Covid-19 treatment. The search focused on clinical trials, review articles and case studies and this resulted in four hundred and twenty relevant articles. The article was written based on full paper and abstract reviews and 44 relevant articles were selected, independently reviewed and referenced. Only articles that focused on modulation of the immune response were considered in this review.

The immune response and cytokine profiles in COVID-19 infections

Covid-19 infection results in monocyte, macrophage, and dendritic cell activation in two phases. The first phase is the incubation and non-severe one where the specific adaptive immune response eliminates the virus and prevents the disease from progressing further to the critical stage (Li et al., 2020; Shi et al., 2020). At this stage, anyone with a sound immune system will fight and eliminate the infection or any immune boosting intervention will help the host to fight the infection and eliminate it (Shi et al., 2020). The patients at this stage have mild symptoms or are completely asymptomatic as the case of the Germany patient 1, who did not show any symptoms but continued to infect others (Rothe et al., 2020).

The second phase is the severe disease symptoms such as fever, coughing, respiratory distress syndrome and pneumonia leading to tissue destruction and even mortality (Guan et al., 2020; Li et al., 2020; Wang et al., 2020a; Wölfel et al., 2020; Xu et al., 2020). This stage occurs if the protective immune system is impaired enabling the virus to propagate and multiply propelling the body to produce massive pro-inflammatory cytokines (Moore and June, 2020; Wang et al., 2020a). Once this occurs the remedy would be to suppress the inflammation and manage other disease symptom that sets in at this stage (Shi et al., 2020). The second phase is characterized by cytokines release syndrome (CRS) (Wang et al., 2020a) where the body produces massive pro-inflammatory cytokines such as IL-1, IL-6 and TNF (Huang et al., 2020) which leads to excessive tissue damage.

Excessive inflammatory immune response was confirmed in separate studies by presence of elevated levels of chemokines and Interleukin-6 (IL-6) in Covid-19 patients' serum (Huang et al., 2020). This indicates that the pathogenicity of Covid-19 is through induction of inflammatory cytokines. Earlier studies have reported that most Covid-19 patients had elevated levels of IL-6 confirming these assertions (McGonagle et al., 2020). Based on these studies, one would therefore hypothesize that any drug or treatment that suppresses the cytokine storms would be a good remedy for the management of Covid-19.

Chloroquine and hydroxychloroquine mode of action

Chloroquine (CQ) and hydroxychloroquine (HCQ) are old and very popular drugs which have been used for a long time for treatment of malaria. The drugs have long history of safe use and are readily available and affordable. Since there is no approved or recommended treatment for Covid-19, everybody is looking everywhere for any treatments that can help manage Covid-19 infections and these two drugs have been proposed as potential remedies. These two drugs have been shown to possess various immunomodulatory and immunosuppressive effects (Al-Bari, 2015); thus their role in the management of Covd-19 cannot be ignored.

Chloroquine and hydroxychloroquine have been demonstrated to contain strong antiviral effects against SARS-CoV when administered before and after infection (Chang et al., 2014; Keyaerts et al., 2004; Sun et al., 2020; Vincent et al., 2005). This means that it can serve as both a prophylactic and a treatment drug for SARS-CoV infections (Vincent et al., 2005). More studies also demonstrate that HCQ and CQ are effective against HIV, hepatitis B, HBV, influenza (Wang et al., 2015). HCQ and CQ mode of action has been proposed to be through the reduction of intracellular pH and inhibiting lysosomal activity in the antigen-presenting cells (APCs), Inhibition of pro-inflammatory cytokines pathways by interfering signaling pathways and transcriptional activity and interfering with terminal glycosylation of ACE2 resulting in blocking of virus-receptor binding and subsequent cell entry (Al-Bari, 2015; Vincent et al., 2005). The overall result is blocking the viral replication and subsequent infection and also reduced IL-1, IL-6 and TNF-α production (Al-Bari, 2015; Schrezenmeier and Doerner, 2020). It is through these mechanisms that many researchers hypothesize that HCQ and CQ are potential candidates for treatment of Covid-19 infections.

In their study, Sperber et al. (1993) demonstrated that CQ and HCQ acted by inhibition of interleukin 1 alpha (IL-1-alpha) and IL-6 by T cells. This was also demonstrated by later studies which all came into conclusion that IL-6 inhibition was a key mode of action of both CQ and HCQ (Chen et al., 2020; Dijkmans and Verweij, 1997; Ornstein and Sperber, 1996). Several recent studies concluded that HCQ treatment did not only significantly prolong life but also significantly reduce fatality of critically ill patients with COVID-19 and greatly lowered the levels of IL-6, one of the most inflammatory cytokines. The studies also demonstrated that when administration of HCQ was discontinued, levels of IL-6 went up significantly. This study is consistent with earlier studies both *in-vitro* and *in-vivo* (Wang et al., 2020b). Hjorton et al. (2018) also demonstrated that HCQ and CQ were able to inhibit cytokines production in patients with SLE and their results were consistent earlier observations where HCQ was able to inhibit the production of IFNα in patients with SLE (Willis et al., 2012). There are however very few clinical studies on application of HCQ and CQ on Covid-19 patients, although the few that exist indicate that treatment of patients with HCQ and CQ shows promising results (Gautret et al., 2020; Liu et al., 2020; Wang et al., 2020b). A study in France where patients with Covid-19 were treated with HCQ showed a complete elimination of virus although the treatment was reinforced with Azithromycin and had a small sample size (36) (Gautret et al., 2020). A few studies showed no effect on the treatment of Covid-19 patients with HCQ (Molina et al., 2020) although the cases in this study were severe unlike the other studies. Although there seems to be limited data on the efficacy of HCQ and CQ in terms of clinical applications, many countries have started to apply HCQ and CQ treatment as they await the results of a well-designed clinical trial. Since the drugs have a safe history of use and are very affordable they would form a cheaper option for the treatment of Covid-19. Their effects on the immune system has been studied extensively both *in-vivo* and *in-vitro* and all studies arriving at a conclusion that CQ and HCQ are effective anti-inflammatory and antivirals (Sperber et al., 1993; Vincent et al., 2005).

CONCLUSIONS

One way of combating disease is by understanding the host-microbe interaction. Understanding host-microbe interaction allows us to understand how the body reacts to certain infections. In this understanding we can then design drugs or administer drugs that target the reversal of the immune reaction causing the inflammation; hence controlling the disease. This is the case of Covid-19. Evidence exists supporting that the Covid-19 infection induces the excessive pro-inflammatory cytokines and that administration of CQ and HCQ reduces the production of pro-inflammatory cytokines. HCQ and CQ are likely to control infections as well as deter the progression of Covid-19 infections through he inhibition of cytokine storm which is a major characteristics of Covid-19 progression by modulating the T-cells. Since we are alive that it will take months if not years to get a recommended drug to treat Covid-19 while infections and deaths are increasing day by day; it would make sense to re-look at the existing drugs with history of safe use and screen them for Covid-19 treatment. In conclusion, from the reviewed literature; Chloroquine seems to be effective in limiting the replication of SARS-CoV-2 (virus causing COVID-19) *in vitro*. The rationale for use may be justified by their long time use. There is a wide existing knowledge supporting use of CQ and HCQ in treatment of Covid-19 although the evidence is supported by few studies involving very limited sample sizes that are not well controlled. We therefore recommend a further, well designed and randomized clinical trials on the efficacy of CQ and HCQ on Covid-19 as a treatment option.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

REFERENCES

Al-Bari MAA (2015). Chloroquine analogues in drug discovery: new directions of uses, mechanisms of actions and toxic manifestations from malaria to multifarious diseases. Journal of Antimicrobial Chemotherapy *70*(6):1608-1621.

Cao X (2020). COVID-19: immunopathology and its implications for therapy. Nature Reviews immunology 20(5):269-270.

Chang TH, Wang LF, Lin YS, Yang CS, Yu CY, Lin YL (2014). 25: Hydroxychloroquine activates host antiviral innate immunity. Cytokine70(1):33-34.

Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L (2020). Epidemiological and

clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. The Lancet *395*(10223):507-513.
Dijkmans BA, Verweij CL (1997). Chloroquine and hydroxychloroquine equally affect tumor necrosis factor-alpha, interleukin 6, and interferon-gamma production by peripheral blood mononuclear cells. The Journal of Rheumatology 24(1):55-60.
Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, Doudier B, Courjon J, Giordanengo V, Vieira VE, Dupont HT, Honoré S, Colson P, Chabrière E, La Scola B, Rolain J-M, Brouqui P, Raoulta D (2020). Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. International Journal of Antimicrobial Agents 105949.
Guan W-J, Ni Z-Y, Hu Y, Liang W, Ou C-Q, He J-X, Liu L, Shan H, Lei C-L, Hui DSC, Du B, Li L-J, Zeng G, Yuen K-Y, Chen R-C, Tang C-L, Wang T, Chen P-Y, Xiang J, Li S-Y, Wang J-L, Liang Z-J, Peng Y-X, Wei L, Liu Y, Hu Y-H, Peng P, Wang J-M, Liu J-Y, Chen Z, Li G, Zheng Z-J, Qiu S-Q, Luo J, Ye C-J, Zhu S-Y, Zhong N-S (2020). Clinical characteristics of coronavirus disease 2019 in China. New England Journal of Medicine 382(18):1708-1720.
Hjorton K, Hagberg N, Israelsson E, Jinton L, Berggren O, Sandling JK, Thörn K, Mo J, Eloranta M-L, Rönnblom L (2018). Cytokine production by activated plasmacytoid dendritic cells and natural killer cells is suppressed by an IRAK4 inhibitor. Arthritis Research and therapy 20(1):1-11.
Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. The Lancet 395(10223):497-506.
Keyaerts E, Vijgen L, Maes P, Neyts J, Van Ranst M (2004). In vitro inhibition of severe acute respiratory syndrome coronavirus by chloroquine. Biochemical and biophysical research communications *323*(1):264-268.
Li G, Fan Y, Lai Y, Han T, Li Z, Zhou P, Pan P, Wang W, Hu D, Liu X, Zhang Q, Wu J (2020). Coronavirus infections and immune responses. Journal of medical Virology 92(4):424-432.
Liu B, Li M, Zhou Z, Guan X, Xiang Y (2020). Can we use interleukin-6 (IL-6) blockade for coronavirus disease 2019 (COVID-19)-induced cytokine release syndrome (CRS)?. Journal of Autoimmunity 102452.
McGonagle D, Sharif K, O'Regan A, Bridgewood C (2020). Interleukin-6 use in COVID-19 pneumonia related macrophage activation syndrome. Autoimmunity reviews 102537.
Molina JM, Delaugerre C, Le Goff J, Mela-Lima B, Ponscarme D, Goldwirt L, de Castro N (2020). No evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection. M´edecine et Maladies Infectieuses 50(384):30085-30088.
Moore JB, June CH (2020). Cytokine release syndrome in severe COVID-19. Science 368(6490):473-474.
Ornstein MH, Sperber K (1996). The antiinflammatory and antiviral effects of hydroxychloroquine in two patients with acquired immunodeficiency syndrome and active inflammatory arthritis. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology 39(1):157-161.
Prompetchara E, Chutitorn K, Tanapat P (2020). Immune responses in COVID-19 and potential vaccines: Lessons learned from SARS and MERS epidemic. Asian Pacific Journal of Allergy Immunology 38(1):1-9.
Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, Zimmer T, Thiel V, Janke C, Guggemos W, Seilmaier M, Drosten C, Vollmar P, Zwirglmaier K, Zange S, Wölfel R, Hoelscher M (2020). Transmission of 2019-nCoV infection from an asymptomatic contact in Germany. New England Journal of Medicine 382(10):970-971.
Schrezenmeier E, Doerner T (2020). Mechanisms of action of hydroxychloroquine and chloroquine: implications for rheumatology. Nature Reviews Rheumatology 7:1-12.
Shi Y, Ying W, Changsun S (2020). COVID-19 infection: the perspective on immune response. Cell Death and Differentiation 27(5):1451-1454.
Sperber K, Quraishi HUMA, Kalb TH, Panja ASIT, Stecher V, Mayer L (1993). Selective regulation of cytokine secretion by hydroxychloroquine: inhibition of interleukin 1 alpha (IL-1-alpha) and IL-6 in human monocytes and T cells. The Journal of Rheumatology 20(5):803-808.
Sun X, Ni Y, Zhang M (2020). Rheumotologitsts' view on the use of hydroxychloroquine to treat COVID-19. Emerging Microbes and Infections 9(1):830-832.
Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, Ksiazek TG, Seidah NG, Nichol ST (2005). Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. Virology Journal *2*(1):69.
Wang LF, Lin YS, Huang NC, Yu CY, Tsai WL, Chen JJ, Kubota T, Matsuoka M, Chen S-R, Yang C-S, Lu R-W, Lin Y-L, Chang T-H (2015). Hydroxychloroquine-inhibited dengue virus is associated with host defense machinery. Journal of Interferon & Cytokine Research, 35(3):143-156.
Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z (2020a). Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus–infected pneumonia in Wuhan, China. Jama *323*(11):1061-1069.
Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, Shi Z, Hu Z, Zhong W, Xiao G (2020b). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell research 30(3):269-271.
Willis R, Seif AM, McGwin Jr.G, Martinez-Martinez LA, Gonzalez EB, Dang N, Papalardo E, Liu J, Vilá LM, Reveille JD, Alarcon G, Pierangeli SS (2012). Effect of hydroxychloroquine treatment on pro-inflammatory cytokines and disease activity in SLE patients: data from LUMINA (LXXV), a multiethnic US cohort. Lupus 21(8):830-835.
Wölfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Müller M A, Niemeyer D, Jones TC, Vollmar P, Rothe C, Hoelscher M, Bleicker T, Brünink S, Schneider J, Ehmann R, Zwirglmaier K, Drosten C, Wendtner C (2020). Virological assessment of hospitalized patients with COVID-2019. Nature pp. 1-5.
Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang F-S (2020). Pathological findings of COVID-19 associated with acute respiratory distress syndrome. The Lancet respiratory medicine 8(4):420-422.

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Full Length Research Paper

Genome codon bias analysis of dengue virus type 1

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Dengue viruses (DENV) are the most common mosquito-borne RNA virus with high variation and adaptation in tropical and subtropical regions. Exploration of codon usage bias of DENV can be significant to understand their genetic variation and adaptation. In the study, the codon usage pattern of dengue virus type 1 (DENV-1) was analyzed by using codonW, CUSP and SPSS. The extent of codon preference of DENV-1 is weak with a 50.57 mean value of ENC, indicating that the DENV-1 genome has low codon bias. Of the 18 optimal codons of DENV-1, 13 end in A/U, with A ending in the majority. The result shows that DENV-1 prefers A-ended codons, and their codon bias is influenced more by natural selection than by mutations selection, as revealed by ENC-plot and neutrality analysis. Furthermore, comparison of codon usage bias between DENV**-1 and host showed that codon usage pattern of DENV-1 is more similar to Home sapiens instead of *Aedes aegypti* or *A. albopictus*. Our findings contributed to understanding of the evolution of the DENV-1.**

Key words: Dengue virus type 1, Codon bias, RSCU, ENC-plot, neutrality-plot.

INTRODUCTION

Dengue virus (DENV) is a single-stranded positive-sense RNA virus belonging to the Flavivirus genus. DENV is divided into four serotypes (DENV-1, 2, 3, and 4), causing severe tropical and subtropical diseases such as dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Halstead, 2007). Dengue fever is the most important viral-borne disease in clinical practice, with 96 million cases of apparent infection each year among nearly four billion people at risk in 128 countries (Bhatt et al., 2013). Since 1978, the first outbreak of dengue fever in China, it has occurred every few years and has become a serious public health threat in Southern China (Sun et al., 2014; Hu et al., 2017). But the factors underlying the current spread of the virus and variation and adaptation remain largely unknown (Bhatt et al., 2013).

The genome of DENV-1 has 10735bp, which contains a 10179bp single open reading frame encoding Capsid protein, Membrane glycoprotein precursor, Membrane glycoprotein, Envelope protein and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Perera and Kuhn, 2008; Byk and Gamarnik, 2016).

Amino acids are coded by more than one synonymous codon; the preference of specific codons to synonymous codons is not equal, which leads to codon usage bias (Gustafsson et al., 2004). Variations in codon usage bias lead to a shift in the balance between mutation and natural selection (Morton, 2003). In addition, mutation pressure, natural selection, replication, and selective transcription can influence codon use patterns (Butt et al.,

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2014). The values of relative synonymous codon use (RSCU) may be virus-specific, independent of translation selection or gene length (Gu et al., 2004). And analysis of codon usage bias can reveal important information about the molecular evolution, regulation of gene expression, and the design of vaccine (Butt et al., 2014). In the present study, we analyzed the codon usage bias of DENV-1 and their influencing factors. We hope the comprehensive analysis of codon usage bias of DNEV-1 will provide help for understanding the evolution of DENV-1, provide some data to help research the vaccine and monitoring of the DENV-1 in the future.

MATERIALS AND METHODS

Sequence

The complete genome of DENV-1 (ID:NC-001477.1) was retrieved from the GenBank database at the National Centre for Biotechnology Information (NCBI). The complete readability reading frame (10178bp), and 14 gene sequences were found after the anchor or precursor sequences were removed. The names are Capsid protein, Membrane glycoprotein precursor, Membrane glycoprotein, Envelope protein, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. Then they are used as samples for codon preference analysis.

Relative synonymous codon usage (RSCU) analysis

The RSCU value is the ratio of observed frequency to the predicted frequency in the synonymous codon family of a particular amino acid (Sharp and Li, 1987). To find the optimal codon for an amino acid, we used CodonW to calculate the sequence RSCU and define the optimal codon according to RSCU.

General analysis of genomic codon preference and its base composition

The frequencies of occurrence of nucleotides G+C at the first, second, and third base of codon (GC_1, GC_2, GC_3) and ENC (effective number of codons) of each gene in the genome of DENV-1 were calculated by codonW and CUSP of EMBOSS, and the relationship between each parameter was analyzed by SPSS 22.0 (https://www.ibm.com/analytics/spss-statistics-software).

ENC-plot analysis

The ENC-Plot (ENC vs GC_{3s}) is widely used to determine the effect of G+C compositional constraints on codon usage bias (Wright, 1990). CodonW was used to calculate the values of GC_{3s} and ENC, and a standard curve ($ENC=2+GC_{3s}+29/ (GC_{3s}^2+ (1-GC_{3s})^2)$) was added to the graph, indicating that the predicted value of the gene was determined only by the base composition. When the corresponding points fall near the expected curve, the mutation is the main force influencing the use of codon. And the points below the standard curve are more susceptible to natural selection (Morton, 2003).

Neutrality-plot analysis

By comparing GC_3 and GC_{12} (the mean value of GC_1 and GC_2), a neutrality-plot was drawn to illustrate the role of mutation-selection balance in codon usage disparity. An effect of mutation pressure on the biased usage of codons is indicated by the slope of a regression line of GC_{12} vs GC_3. If there is a significant correlation between the two, that is, the slope is close to 1, there is no significant difference in the cmomposition of the first two bases and the third base of the codon, that is, the mutation is the main factor affecting the use of the codon; on the contrary, it shows that the composition of the first two digits and the third digit of the codon are different, indicating that natural selection is the main factor affecting the use of codon (Sueoka, 1988; Zhao et al., 2016).

Comparison analysis

The RSCU of DENV-1 was compared with the RSCU of its host, including human (Homo sapiens) and mosquitoes (*A. aegypti* and *A. albopictus*). The codon usage data of DENV-1's hosts were retrieved from the codon usage database (http://www.kazusa.or.jp/codon). In our comparison, if the RSCU value of DENV-1 and that of the same codon of the host are both <0.6, >1.6, or between 0.6 and 1.6, then it is judged that the codon use pattern of both is similar (Wong et al., 2010; Ma et al., 2015).

RESULTS

RSCU of each gene of DENV-1

The RSCU value of DENV-1 was calculated by codonW. We can find that there are 23 codons in which RSCU>1, namely GCC, GCA, AGA, AGG, AAC, GAC, UGU, CAA, GAA, GGA, CAC, AUA, UUG, CUA, CUG, AAA, UUC, CCA, UCC, UCA, ACA, UAU, GUG (23 in total); those that end in A/U have 13 (56. 5%), and those that end with U only have 2; it explains the low frequency of codon that appears at the end of U. We plotted the optimal codon for each amino acid in *, and you can find that most of them end in A (Table 1 and Figure 1).

General analysis of genomic codon preference and its base composition

To determine whether codon bias exists in the genome of DENV-1, the effective codon usage (ENC) was measured. ENC is a simple and relatively direct method to estimate codon usage bias (Novembre, 2002). The ENC value of the genome gene of DENV-1 is 43. 33~57.21; the average is 50.57 (Table 2). It can be considered that the codon preference of DENV-1 is weak, that is, the use of each codon is more uniform. The difference between GC_1, GC_2, and GC_3 of codon of DENV-1 is small. The GC content of the genome is slightly lower than the AU content (Table 2). And according to the RSCU worthy of the conclusion is basically consistent.

ENC-plot

The ENC values of DENV-1 genomic genes were

Table 1. Relative synonymous codon usage (RSCU) of DENV-1

Amino acid	Codon	Number	RSCU	Amino acid	Codon	Number	RSCU
Phe	UUU	51	0. 95	Ser	UCU	37	1. 09
	UUC*	56	1. 05		UCC	30	0. 89
Leu	UUA	40	0. 75		UCA*	73	2. 16
	UUG	59	1. 11		UCG	11	0. 33
	CUU	36	0. 68		AGU	25	0. 74
	CUC	38	0. 71		AGC	27	0. 8
	CUA*	76	1. 43	Pro	CCU	25	0. 72
	CUG	70	1. 32		CCC	26	0. 75
Ile	AUU	55	0. 84		CCA*	72	2. 07
	AUC	52	0. 79		CCG	16	0. 46
	AUA*	90	1. 37	Thr	ACU	50	0. 77
Met	AUG	126	1		ACC	59	0. 91
Val	GUU	44	0. 78		ACA*	111	1. 71
	GUC	53	0. 93		ACG	39	0. 6
	GUA	34	0. 6	Ala	GCU	52	0. 87
	GUG*	96	1. 69		GCC*	81	1. 36
Tyr	UAU*	38	1. 06		GCA*	81	1. 36
	UAC	34	0. 94		GCG	25	0. 42
His	CAU	33	0. 92	Trp	UGG	96	1
	CAC*	39	1. 08	Arg	CGU	11	0. 35
Gln	CAA*	65	1. 18		CGC	12	0. 38
	CAG	45	0. 82		CGA	16	0. 51
Asn	AAU	51	0. 81		CGG	10	0. 32
	AAC*	75	1. 19		AGA*	100	3. 16
Lys	AAA*	137	1. 32		AGG	41	1. 29
	AAG	71	0. 68	Gly	GGU	37	0. 53
Asp	GAU	58	0. 81		GGC	36	0. 51
	GAC*	86	1. 19		GGA*	163	2. 33
Glu	GAA*	132	1. 21		GGG	44	0. 63
	GAG	87	0. 79	TER	UAA	1	3
Cys	UGU*	32	1. 08		UAG	0	0
	UGC	27	0. 92		UGA	0	0

calculated and plotted against GC_{3s} (Figure 2). From the figure, we can find that the ENC values of genomic genes of DENV-1 are distributed between 43 and 51, indicating that the preference of each gene to codon is not significantly different. When the ENC values of these genes are lower than the standard curve, it indicates that natural selection plays an important role in driving codon usage bias (Fuglsang, 2008). The ENC values of each

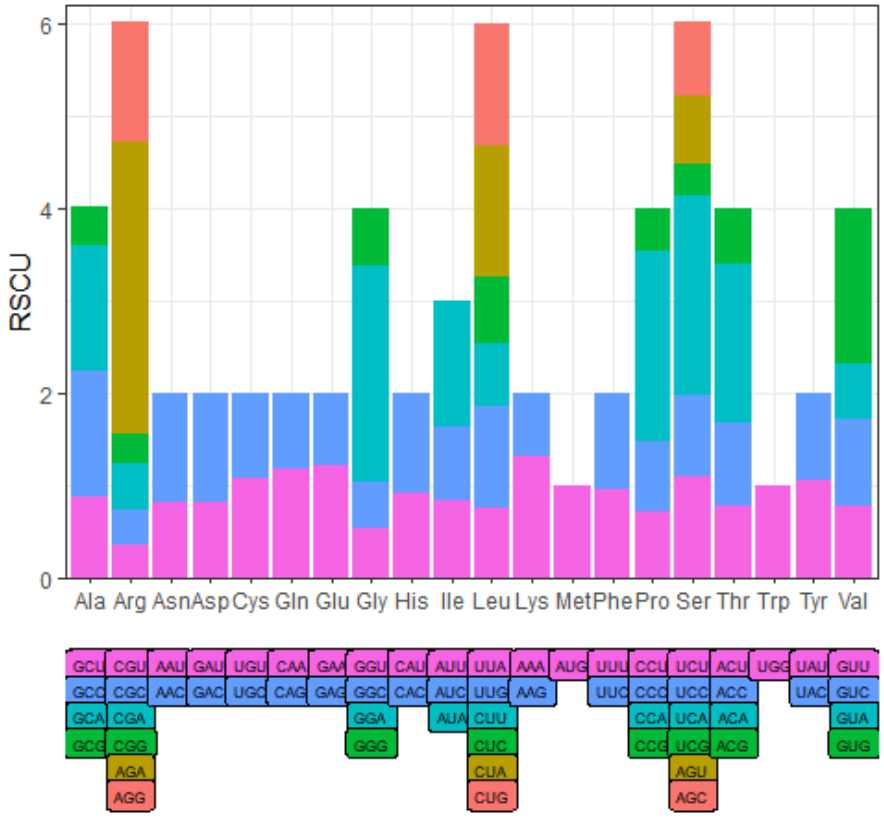

Figure 1. RSCU of DENV-1 Complete Genome. Each codon is plotted in a different color block; the proportions of different color blocks reflect the proportions of different codons in amino acids. X axis represents 18 synonymous codons of DENV-1 and their base combinations are listed; Y axis represents the RSCU value of each synonymous codon.

Table 2. The GC content of DENV-1 genome codon positions

Gene	GC content				ENC
	GC_1	GC_2	GC_3	GC	
C	0. 370	0. 430	0. 500	0. 433	48. 06
prM	0. 506	0. 476	0. 494	0. 492	57. 21
M	0. 533	0. 467	0. 480	0. 493	43. 33
E	0. 497	0. 438	0. 461	0. 465	53. 32
NS1	0. 460	0. 426	0. 474	0. 454	52. 75
NS2A	0. 431	0. 422	0. 431	0. 428	50. 03
NS2B	0. 562	0. 392	0. 415	0. 456	45. 33
NS3	0. 544	0. 444	0. 464	0. 484	53. 69
NS4A	0. 551	0. 386	0. 496	0. 478	52. 93
NS4B	0. 522	0. 458	0. 438	0. 473	51. 65
NS5	0. 497	0. 422	0. 457	0. 459	47. 96
Mean Value	0. 498	0. 433	0. 465	0. 465	50. 57

ENC-plot.

gene were basically below the curve, indicating that the genomic genes of DENV-1 were limited by mutations and more affected by natural selection.

Neutrality-plot

A neutrality-plot (GC_{12}-GC_3) was used to estimate the

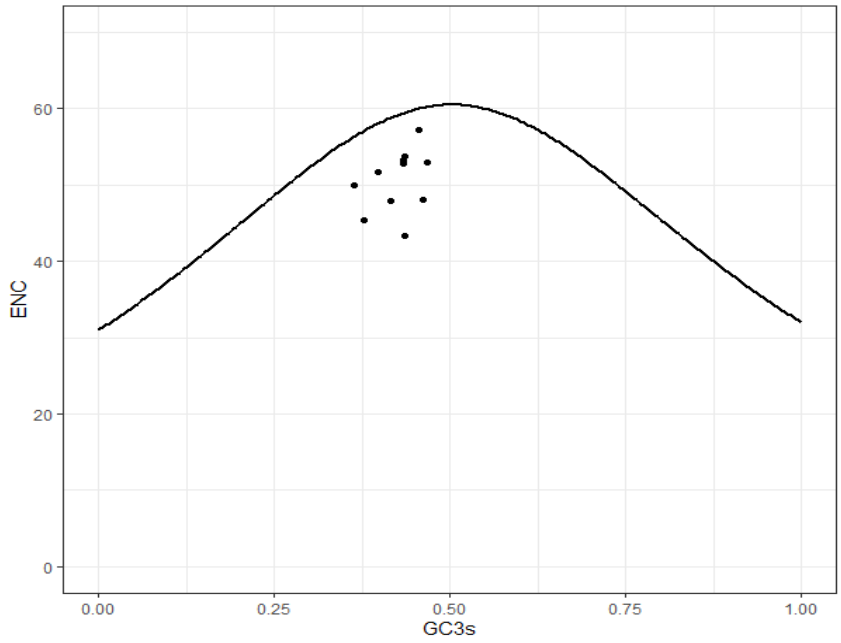

Figure 2. Analysis of ENC and GC3s relationship. The ENC of genomic gens were plotted against the GC3s. X axis represents the GC3s value of each gene of DENV-1 genome; Y axis represents its corresponding ENC value. The curve indicates the relationship between ENC and GC3s in absence of selection.

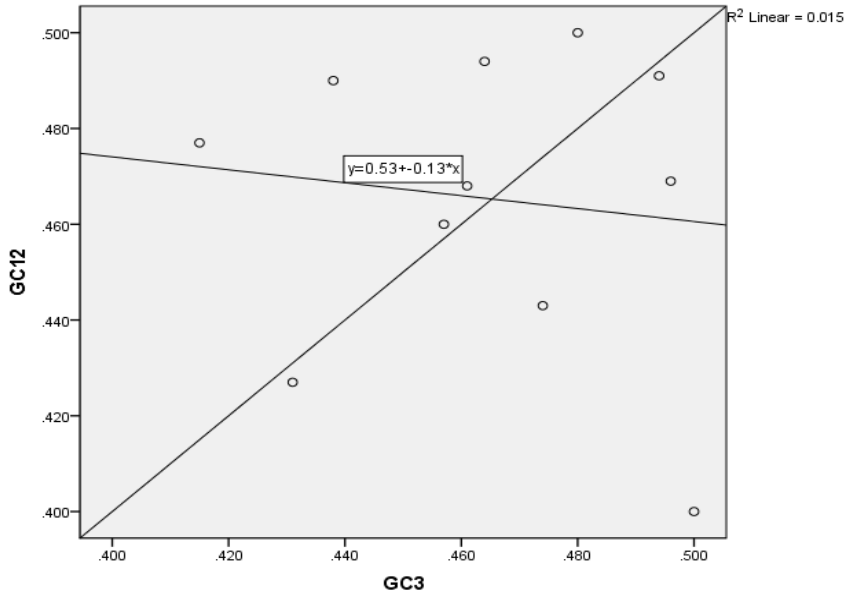

Figure 3. Neutrality plot analysis of GC12 and GC3. The regression curve is represented as y=0.53+0.13x, R^2=0.015. X axis represents the GC3 value of each gene of DENV-1 genome; Y axis represents the GC12 values corresponding GC3 value.

relationships among the genomic genes of DENV-1 (Figure 3). It can be seen from the figure that GC_3 content does not increase with the increase of GC_{12} content, and each point is far away from the diagonal of the figure, indicating that mutations play little role in codon bias. The regression curve of GC_{12} on GC_3 has a regression

Table 3. Correlation analysis of each genes relaxed parameters.

Item	GC1	GC2	GC3	GC
GC1	1			
GC2	-0. 067	1		
GC3	-0. 250	0. 246	1	
GC	0. 747**	0. 476	0. 314	1

**: Correlation is significant at the 0. 01 level *: Correlation is significant at 0. 05 level.

coefficient of -0. 13 and R^2=0. 015, indicating that GC_{12} and GC_3 are not correlated. This is the same with the correlation analysis of the preference parameters of the virus genome (Table 3). This indicates that the influence of natural selection on codon preference of DENV-1 is greater than that of mutation (Kumar et al., 2016).

Comparison analysis

To determine whether the codon usage pattern of DENV-1 is influenced by its hosts, the codon usage pattern of DENV-1 was compared with its natural hosts, including Home sapiens, A. aegypti, and A. albopictus. We found that 46 of 59 synonymous codons between DENV-1 and humans were considered similar, while only 38 or 28 were considered similar between DENV-1 and A. aegypti or A. albopictus (Table 4). As we can see, the codon usage pattern of DENV-1 is more similar to Home sapiens.

DISCUSSION

In the present study, we demonstrated that DENV-1 had a weak codon bias with an average ENC value of 54. 58. This indicates that the overall degree of codon usage bias in DENV-1 is low and the bias between genes is not significant, consistent with some previous reports (Jenkins and Holmes, 2003; Yohan et al., 2018).

Analysis of the ENC-GC3s plots indicated that the genomic genes of DENV-1 were more affected by natural selection, which is consistent with the codon preference of Flaviviridae viruses (Yao et al., 2019). Results of the neutrality analysis validated the results derived from ENC-GC3 plots and further suggested that natural selection pressures had a greater influence on the spread and mutation of DENV-1. Although we did not find any usage correlation between the first, second, and third positions of the codon of DENV-1, some relevant studies indicated that there was a correlation among all serotypes of DENV, and they put forward a viewpoint that all the codon sites are related to the geographical environment of the strain (Lara-Ramírez et al., 2014); their study found differences in codon expression between DENV-1 strain from America and DENV-1 from Asia. The content of A/U is higher than G/C; the RSCU analysis indicates that DENV-1 prefers A/U-ended codons, especially A-ended codons (Roy et al., 2019). This is similar to studies on codon preference of Flaviviridae viruses (Yao et al., 2019). And It is also similar to studies on codon preference of other RNA viruses such as Ebola virus (Cristina et al., 2015; Kustin and Stern, 2020).

The results of comparison analysis suggest that the codon usage pattern of DENV-1 is more similar to that of Home sapiens, instead of A. aegyptior and A. albopictus. The DENVs are known to be transmitted to humans by mosquitoes; the difference in codon usage bias between DENV-1 and its hosts might be caused by the different defense mechanisms of different hosts against DENV-1 infections (Sexton and Ebel, 2019). In addition, some relevant studies indicate that there were little correlation between mosquito vector index and human epidemic during the transmission of DENV (Bowman et al., 2014; Chadee, 2009). From this study, we can conclude that the codon usage pattern of DENV has more similarities with Home sapiens.

And as human genes are more biased to AT-ending codons (Alvarez-Valin et al., 2002), and DENV-1 have a similar pattern of codon usage bias, this may be related to the mechanism of human infection with DENV. The RSCU of all the codons in the genome of DENV-1 was used as the standard for screening, and we finally found the optimal codons of each amino acid. The discovery of optimal codon provides a way for viral expression of proteins, the development of viral vaccines for patients infected with DENV, and a theoretical basis for their selection of hosts (Kames et al., 2020). Due to the greater influence of natural selection on the preference of codon of DENV-1, there may be different codon usage bias of DENV in different regions. In the future, we should conduct specific analyses according to different regions to provide help for limiting the spread and development of DENV in different regions, such as America, Asia and Africa.

Conclusion

In summary, the combination of the ENC-plot and

Table 4. Comparison of RSCU between DENV-1 and its hosts

Amino acid	Codon	RSCU			
		DENV-1	Home sapiens	Aedes aegypti	Aedes albopictus
Phe	UUU	0. 95	0.87	0.56	0.48
	UUC	1. 05	1.13	1.44	1.52
Leu	UUA	0. 75	0.39	0.35	0.23
	UUG	1. 11	0.73	1.34	1.11
	CUU	0. 68	0.73	0.67	0.49
	CUC	0. 71	1.21	0.81	0.87
	CUA	1. 43	0.40	0.54	0.57
	CUG	1. 32	2.53	2.28	2.73
Ile	AUU	0. 84	1.03	1.00	0.74
	AUC	0. 79	1.52	1.59	1.86
	AUA	1. 37	0.44	0.40	0.40
Val	GUU	0. 78	0.69	1.05	0.88
	GUC	0. 93	1.00	1.09	1.30
	GUA	0. 6	0.42	0.60	0.51
	GUG	1. 69	1.90	1.26	1.31
Tyr	UAU	1. 06	0.84	0.64	0.55
	UAC	0. 94	1.16	1.36	1.45
His	CAU	0. 92	0.81	0.84	0.75
	CAC	1. 08	1.19	1.16	1.25
Gln	CAA	1. 18	0.51	0.81	0.60
	CAG	0. 82	1.49	1.19	1.40
Asn	AAU	0. 81	0.89	0.79	0.64
	AAC	1. 19	1.11	1.21	1.36
Lys	AAA	1. 32	0.82	0.79	0.58
	AAG	0. 68	1.18	1.21	1.42
Asp	GAU	0. 81	0.89	1.12	0.96
	GAC	1. 19	1.11	0.88	1.04
Glu	GAA	1. 21	0.81	1.15	1.11
	GAG	0. 79	1.19	0.85	0.89
Cys	UGU	1. 08	0.86	0.83	0.69
	UGC	0. 92	1.14	1.17	1.31
Ser	UCU	1. 09	1.11	0.67	0.54
	UCC	0. 89	1.39	1.20	1.40
	UCA	2. 16	0.84	0.68	0.48
	UCG	0. 33	0.33	1.41	1.70
	AGU	0. 74	0.84	0.93	0.79
	AGC	0. 8	1.50	1.11	1.08
Pro	CCU	0. 72	1.12	0.67	0.35
	CCC	0. 75	1.35	0.83	1.13
	CCA	2. 07	1.07	1.20	1.07
	CCG	0. 46	0.46	1.30	1.44

Table 4 Contd.

Thr	ACU	0. 77	0.94	0.80	0.64
	ACC	0. 91	1.52	1.48	1.79
	ACA	1. 71	1.07	0.70	0.58
	ACG	0. 6	0.46	1.01	0.99
Ala	GCU	0. 87	1.09	1.09	0.99
	GCC	1. 36	1.64	1.48	1.81
	GCA	1. 36	0.85	0.75	0.59
	GCG	0. 42	0.42	0.69	0.62
Arg	CGU	0. 35	0.51	1.36	1.50
	CGC	0. 38	1.20	1.25	1.32
	CGA	0. 51	0.63	1.17	0.97
	CGG	0. 32	1.20	1.05	1.22
	AGA	3. 16	1.20	0.64	0.58
	AGG	1. 29	1.26	0.53	0.41
Gly	GGU	0. 53	0.64	1.10	1.24
	GGC	0. 51	1.40	1.04	1.07
	GGA	2. 33	0.98	1.49	1.21
	GGG	0. 63	0.98	0.37	0.47

neutrality analysis proves that natural selection has a greater influence on the condon usage bias of DENV-1. We can consider that the geographic origin of dengue viruses has a strong influence on the formation of codon usage patterns (Lara-Ramírez et al., 2014). In addition, the preference of A-ended codon of DENV-1 may also be helpful for future research on DENV.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Alvarez-Valin F, Lamolle G, Bernardi G (2002). Isochores, GC3 and mutation biases in the human genome. Gene 300(1-2):161-168.

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI (2013). The global distribution and burden of dengue. Nature 496(7446):504-507.

Byk LA, Gamarnik AV (2016). Properties and Functions of the Dengue Virus Capsid Protein. Annual Review of Virology 3(1):263-281.

Bowman LR, Runge-Ranzinger S, McCall PJ (2014). Assessing the relationship between vector indices and dengue transmission: a systematic review of the evidence. PLOS Neglected Tropical Diseases 8(5):e2848.

Butt AM, Nasrullah I, Tong Y (2014). Genome-wide analysis of codon usage and influencing factors in chikungunya viruses. PLoS One 9(3):e90905.

Chadee DD (2009). Dengue cases and Aedes aegypti indices in Trinidad, West Indies. Acta Tropica 112(2):174-180.

Cristina J, Moreno P, Moratorio G, Musto H (2015). Genome-wide analysis of codon usage bias in Ebolavirus. Virus Research 196:87-93.

Fuglsang A (2008). Impact of bias discrepancy and amino acid usage on estimates of the effective number of codons used in a gene, and a test for selection on codon usage. Gene 410(1):82-88.

Gu W, Zhou T, Ma J, Sun X, Lu Z (2004). Analysis of synonymous codon usage in SARS Coronavirus and other viruses in the Nidovirales. Virus Research 101(2):155-161.

Gustafsson C, Govindarajan S, Minshull J (2004). Codon bias and heterologous protein expression. Trends in Biotechnology 22(7):346-353.

Halstead SB (2007). Dengue. Lancet 370(9599):1644-1652.

Hu TS, Zhang HL, Feng Y, Fan JH, Tang T, Liu YH, Zhang L, Yin XX, Chen G, Li HC, Zu J, Li HB, Li YY, Yu J, Zhang FQ, Fan QS (2017). Epidemiological and molecular characteristics of emergent dengue virus in Yunnan Province near the China-Myanmar-Laos border, 2013-2015. BMC Infectious Diseases 17(1):331.

Jenkins GM, Holmes EC (2003). The extent of codon usage bias in human RNA viruses and its evolutionary origin. Virus Research 92(1):1-7.

Kames J, Holcomb DD, Kimchi O, DiCuccio M, Hamasaki-Katagiri N, Wang T, Komar AA, Alexaki A, Kimchi-Sarfaty C (2020). Sequence analysis of SARS-CoV-2 genome reveals features important for vaccine design. Scientific Reports 10(1):15643.

Kumar N, Bera BC, Greenbaum BD, Bhatia S, Sood R, Selvaraj P, Anand T, Tripathi BN, Virmani N (2016). Revelation of Influencing Factors in Overall Codon Usage Bias of Equine Influenza Viruses. PLoS One 11(4):e0154376.

Kustin T, Stern A (2020). Biased mutation and selection in RNA viruses. Molecular Biology and Evolution 28:msaa247.

Lara-Ramírez EE, Salazar MI, López-López Mde J, Salas-Benito JS, Sánchez-Varela A, Guo X (2014). Large-scale genomic analysis of codon usage in dengue virus and evaluation of its phylogenetic dependence. BioMed Research International 2014:851425.

Ma YP, Liu ZX, Hao L, Ma JY, Liang ZL, Li YG, Ke H (2015). Analysing codon usage bias of cyprinid herpesvirus 3 and adaptation of this virus to the hosts. Journal of Fish Diseases 38(7):665-673.

Morton BR (2003). The role of context-dependent mutations in generating compositional and codon usage bias in grass chloroplast DNA. Journal of Molecular Evolution 56(5):616-629.

Novembre JA (2002). Accounting for background nucleotide composition when measuring codon usage bias. Molecular Biology and Evolution 19(8):1390-1394.
Perera R, Kuhn RJ (2008). Structural proteomics of dengue virus. Current Opinion in Microbiology 11(4):369-377.
Roy P, Dey S, Nandy A, Basak SC, Das S (2019). Base Distribution in Dengue Nucleotide Sequences Differs Significantly from Other Mosquito-Borne Human-Infecting Flavivirus Members. Current Computer-Aided Drug Design 15(1):29-44.
Sexton NR, Ebel GD (2019). Effects of Arbovirus Multi-Host Life Cycles on Dinucleotide and Codon Usage Patterns. Viruses 12-11(7):643.
Sharp PM, Li WH (1987). The codon Adaptation Index--a measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Research 15(3):1281-1295.
Sun J, Wu D, Zhou H, Zhang H, Guan D, He X, Cai S, Ke C, Lin J (2014). The epidemiological characteristics and genetic diversity of dengue virus during the third largest historical outbreak of dengue in Guangdong, China, in 2014. Journal of Infection 72(1):80-90.
Sueoka N (1988). Directional mutation pressure and neutral molecular evolution. Proceedings of the National Academy of Sciences of the United States of America 85(8):2653-2657.
Wong EH, Smith DK, Rabadan R, Peiris M, Poon LL (2010). Codon usage bias and the evolution of influenza A viruses. Codon Usage Biases of Influenza Virus. BMC Evolutionary Biology 10:253.
Wright F (1990). The 'effective number of codons' used in a gene. Gene 87(1):23-29.
Yao H, Chen M, Tang Z (2019). Analysis of Synonymous Codon Usage Bias in Flaviviridae Virus. BioMed Research International 2019:5857285.
Yohan B, Wardhani P, Trimarsanto H, Aryati A, Sasmono RT (2018). Genomic analysis of dengue virus serotype 1 (DENV-1) genotypes from Surabaya, Indonesia. Virus Genes 54(3):461-465.
Zhao Y, Zheng H, Xu A, Yan D, Jiang Z, Qi Q, Sun J (2016). Analysis of codon usage bias of envelope glycoprotein genes in nuclear polyhedrosis virus (NPV) and its relation to evolution. BMC Genomics 17(1):677.

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Full Length Research Paper

Antibiotic resistance of enterobacteria (*Escherichia coli, Klebsiella spp. and Salmonella spp*) isolated from healthy poultry and pig farms in peri-urban area of Lome, Togo

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This study aimed to determine for the first time the levels and patterns of antimicrobial resistance of enterobacteria isolated from poultry and pigs farms in southern Togo. A cross-sectional study was conducted in south Togo in 70 and 47 poultry and pig farms, respectively. Fecal samples were collected once in each farm and enterobacteria isolated according to recommended techniques. Isolates from each sample were tested for susceptibility to 14 antibiotics by disc diffusion method. A total of 109 and 85 strains were recovered from 72.7% (n=64) and 87.93% (n=50) poultry and pig samples respectively. Respectively for *Escherichia coli, Klebsiella* spp. and *Salmonella* spp. strains, the most important resistances were observed in poultry/pig farms against tetracycline antibiotic (93.1%/67.6%; 96.2%/78.7% and 100%/100%) and the association sulfoxide-trimethoprim (72.4%/81.1%; 66.7%/78.7% and 100%/100%). In general, resistances were higher against penicillin antibiotics like ampicillin (55.17%/54.05%, 46.15%/38.3% and 50.00%/100%) than cephalosporin antibiotics like ceftazidime (0.00%/0.00%, 5.13%/0.00% and 0.00%/0.00%) resistances where very low or absent. Also, resistance to nalidixic Acid (31.03%/16.22%, 33.33%/29.79, 0.00%/0.00%), first generation quinolones, was relatively high than resistance to norfloxacin (10.3%/10.81%; 20.5%/2.13%; 50%/0.00%) a second generation fluoroquinolone. In poultry, 44.83% of *E. coli*, 50% of *Klebsiella* spp. and 100% of Salmonella strains were multi-resistant while in pigs, 37.83% and 27.65% of *E. coli* and *Klebsiella* spp. strains showed multi-resistance. In many farms, farmers managed the health of their animals on their own. All surveyed poultry farmers and the majority of pig farmers indicated that they used antibiotics in their farms. This study showed that antimicrobial resistance in animal production in Togo portends a serious problem.

Key words: Antibiotic resistance, Enterobacteria, poultry, pig, Lomé, Togo.

INTRODUCTION

Rapid increase in income and urbanization over the past three decades, combined with population growth have led to increased demand for meat and other animal products in many developing countries (FAO, 2009). To meet increasing daily food demand, economic and technology changes are transforming the livestock sector especially

in Africa. Indeed, in Africa and other developing countries, shift in animal production from small holder, mixed crop to intensive, large-scale, and specialized commercialization farms have been observed (Schar et al., 2018). Production of livestock, especially pigs and poultry, is becoming more intensive, geographically concentrated around big towns, linked to supply chains and supported by the use of veterinary drugs like antibiotics (Mensah et al., 2014). Antibiotics used either as curative or preventive treatment against the onset of certain diseases, or even, in extreme cases, to offset poor animal production hygiene in intensive productions is leading to the development of drug resistance. Antibiotic resistance (ABR) is today a worldwide public health concern, with economic, and societal repercussions (Schar et al., 2018). Animals are proofed to be key reservoirs of antibiotic-resistant bacteria that can spread to human through direct contact or food chain.

In Togo, like other Sub-Saharan Africa countries, the use of antibiotics in animal production remains largely undocumented. However, poor control of the use of veterinary pharmaceutical products due to absence or poorly applied legislation to guarantee the quality and the holding of the products released onto the market is reported (Mensah et al., 2014). Inappropriate use of antibiotics as growth promoters by untrained farmers, especially in intensive poultry and pig farms to combat low productivity and high mortality caused by infectious diseases is common due to inadequate legislation. This situation may promote the development of resistance to the antibiotics commonly used in farm animals in these countries. Unfortunately, there is limited data concerning antimicrobial resistance in West African countries due to the absence of monitoring systems (Founou et al., 2018). Recent studies by Vounba et al. (2018, 2019a and b) and Sidibé et al. (2019) in Senegal and Mali respectively, has shown the resistance of some enterobacteria namely *Escherichia coli* and Salmonella, of avian origin to antibiotics.

In Togo, the poultry sector can be categorized into two: traditional poultry farming, and modern poultry farming. Traditional poultry farming has undergone a remarkable development over the past twenty years as a result of several interventional programs. The results of these programs are reflected in real emergence of a category of farmers adopting improved farming practices (vaccination, housing, improved nutrition, etc.) According to FAO (2015), modern poultry farming is dominated by laying hens for production of eggs for consumption. Poultry farms involve in eggs production account for approximately 95% of current poultry establishments. As in poultry production, pig farming can also be categorized into traditional farming and the modern or semi-intensive farming. Under the traditional farming, pigs are allowed to roam freely. Farmers generally associate livestock with their agricultural or commercial activities. Modern semi-intensive farms exploit exotic breeds (Large White and Landrace), and are characterized by keeping animals in enclosures with rational feeding, and health management including the use of antibiotics (Lhoste, 2009). To date, there are no data on the antibiotic resistance of enterobacterias to antibiotics commonly used in poultry and pig farms in Togo. Thus, the aim of the present study was to provide data on the phenotypic antibiotic resistance of enterobacterias isolated from poultry and pig farms in southern Togo.

MATERIALS AND METHODS

Sampling area and sample collection

Sampling area

A cross-sectional study was conducted on private poultry and pig farms located in the peri-urban area of Lomé, in Maritime region (South Togo). The Maritime region is the area of excellence for modern poultry and pig production where commercial poultry and pig farms are mainly located. With more than 80% of modern poultry farms established, this region accommodates more than 90% of the national laying hens' farms. The maritime region accounts for 50% of the country's urban population with an annual growth of 6.1%. This demographic importance which encourages poultry and pig production in peri-urban areas is due to the concentration of industry and administrative services. Indeed, the Maritime region hosts more than 90% of industrial activity, the largest university and all political institutions.

Sample collection

Fecal samples (88 and 58) were collected (September – October 2019) from 70 and 47 modern poultry and pig farms respectively, based on willingness of the farm owners to participate in the study and accessibility of the farms in the Maritime region of Togo. The sample size is based on the unknown population size (the exact number of poultry and pig farms is unknown in the Maritime region), an expected prevalence of farms with non-susceptible isolates of 50%, a precision of 10% and a confidence level of 90%. The required sample size for prevalence estimation is then estimated to 68 farms using the online WinEpiscope 2.0 (http://www.winepi.net/uk/sample/indice.htm). This 50% expected prevalence of non-susceptible isolates to at least one antibiotic at farm level was used as a conservative approach as no studies had previously estimated the prevalence of poultry or pig farms harbouring resistant enterobacterias. When a farm consisted of one poultry (chicken) house or one building with less than five pig pens, samples were taken from this chicken house or pig building whereas, when there were at least two chicken houses or two pig buildings or one building with more than 5 pens, two samples were collected in two separate chicken houses or pens. In each chicken house, one sample of fresh feces was collected. Each sample consisted of a pool of five samples taken in different parts of the

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chicken house. In pig's farm, each pooled fecal sample was obtained from a pen and consisted of five different fecal samples, one collected from each of the four corners and one from the center of the pen. The pens from which fecal samples were collected were randomly selected from each building. Each farm was visited once. A questionnaire (available in French on request) was completed in each farm at the time of sampling, to collect data relating to biosecurity measures and use of antibiotics on the farm.

Isolation and Identification of targeted bacteria

Necessary laboratory equipment and required media were used to culture the target enterobacteria. The isolation of *E.coli* was done by the method previously described by Vounba et al. (2019a) and identified by classical gallery tests and API 20 E (Biomerieux). For Salmonella isolation and identification, the method described by Bada-Alambedji et al. (2006) was used. The isolates, which tested positive for *E.coli*, *Klebsiella* spp and *Salmonella* spp, were sub-cultured on nutritive agar for antimicrobial susceptibility testing.

Antimicrobial susceptibility testing

All isolated strains were tested against 14 antibiotics commonly used in veterinary medicine belonging to 06 different antibiotics classes: aminoglycosides [Streptomycin, Gentamicin], Penicillin's [Ampicillin, Ticarcillin, Amoxicillin + Clavulanic Acid] Cephalosporin's [Cefuroxime, Cefotaxime, Ceftazidime, Ceftriaxone], Quinolone [Nalidixic Acid; Norfloxacin], and tetracycline's [Doxycycline; tetracycline], Sulfoxides and Folate pathway inhibitor [Sulfamethazine + Trimethoprim]. Disc diffusion method was performed and interpreted according to the recommendations of the Antibiogram Committee of the French Society of Microbiology (CA-SFM/EUCAST) (Bonnet et al., 2019). Isolates were categorized as susceptible or non-susceptible to each antimicrobial. An isolate was considered susceptible, if it was sensitive to the entire antibiotic tested and non-susceptible if it was resistant or intermediate to this particular antibiotic. The isolate was Multi Drug Resistant (MDR) when it was non-susceptible to at least 1 agent in more than 3 antimicrobial categories as listed by (Magiorakos et al., 2012) when defining multi-drug resistance. Then according to antibiotics tested, 10 antibiotics belonging to 07 categories were used to classify strains as multi-resistant. Indeed a strain was considered multi-resistant when it was resistant to three (03) or more antibiotics belonging to at least three of the following categories: Cephalosporin 2nd generation (Cefuroxime); Cephalosporin 3rd generation (Cefotaxime; Ceftazidime; Ceftriaxone); β-Lactam 3rd generation (Ampicillin) ; β-lactam+ (Amoxicillin + Ac. Clavulanic); Foliate pathway inhibitor (Sulfamethazine + Trimethoprim); Tetracyclines (Tetracycline; Doxycycline); Aminoglycosides (Gentamicin).

Data analyses

Data were entered into Excel 2013 sheet and the prevalence of antibiotic resistance among different groups was calculated by dividing the number of resistant isolates in the group to the number of isolates tested.

RESULTS

Bacterial isolation and antibiotic susceptibility

Number of strains isolated

A total of 109 bacterial strains were identified from poultry samples as described above including 29 *E. coli*, 78 *Klebsiella* spp, and 2 *Salmonella.* Bacterial Strains were recovered from 72.7% of all samples. *Klebsiella*, *E. coli* and *Salmonella* spp isolation rates was 67.1, 20.5 and 2.3%, respectively. In the samples from pig farms, 85 strains were isolated including 37 *E. coli*, 47 *Klebsiella* and 01 *Salmonella*. Global isolation rate of target enterobacteria strains from pig samples was 87.93% with 56.89, 48.28, and 1.72%, respectively for *Klebsiella* spp, *E. coli* and *Salmonella* spp.

Resistance to antibiotics

Resistance to Beta-lactam antibiotics

In this family, resistance was more observed in penicillin antibiotics than cephalosporin antibiotics (Table 1). Indeed, in poultry, *E. coli*, *Klebsiella Spp* and *Salmonella Spp* resistances were high respectively for ampicillin (55.17, 46.15 and 50.00%), ticarcilline (48.28, 41.03 and 50.00%) and amoxicillin+ clavulanic acid (13.79, 21.79 and 0.00%) compared to cefuroxime (17.24, 20.51 and 50.00%), ceftriaxone (0.00, 1.28 and 0.00), ceftazidime (0.00, 5.13 and 0.00%) and cefotaxime (3.45; 1.28 and 0.00%) where relatively low resistance were observed. The same range of resistance was obtained in pigs with resistance being relatively high to ampicillin (54.05; 38.30 and 100%), ticarcilline (35.14; 27.66 and 0.00%) and amoxicillin + clavulanic acid (13.79; 21.79; 0.00%) compared to cefuroxime (2.70; 10.64 and 0.00%), ceftriaxone (5.41; 4.26 and 0.00%), ceftazidime (0.00; 0.00 and 0.00%) and cefotaxime (0.00; 4.26 and 0.00%).

Resistance to quinolones

Either in poultry or pigs, resistance was more observed for first generation quinolone than second generation quinolone. Indeed, in poultry, resistance of *E. coli, Klebsiella* spp and *Salmonella* spp to nalidixic acid was respectively, 31.03, 33.33 and 0.00% while resistance to norfloxacin was 10.3, 20.5, and 50.00%. In pigs resistance to nalidixic acid was 16.22, 29.79 and 0.00% higher than resistance observed for norfloxacin which was 10.81, 2.13, 0.00%, respectively for *E. coli*, *Klebsiella* spp and *Salmonella* (Table 2).

Resistance to aminoglycosides

In this class, resistance was more observed for Streptomycin than for Gentamycin. In poultry, resistance *for E. coli*, *Klebsiella* spp and *Salmonella* spp strains to streptomycin was 55.17, 51.28, and 0.00%, respectively; while resistance to gentamycin was 0.00, 6.41, and 0.00%, respectively. Similarly, in pigs, resistance was high to streptomycin (45. 95, 27.66, and 0.00%) than to gentamicin (0.00, 2.13, and 0.00%) (Table 3).

Table 1. Antibiotic resistance of enterobacterias from poultry and pig farms in Togo to β -lactam antibiotics.

Poultry	β-lactam/Céphalosporines						
	Ampicilline	**Ticarcilline**	**Amoxicilline +ac.clavulinique**	**Céfuroxime**	**Ceftriaxone**	**Ceftazidime**	**Céfotaxime**
E. coli (N=29)	16 (55.2%)	14 (48.3%)	4 (13.8%)	5 (17.2%)	0 (0.00%)	0 (0.00%)	1 (3.5%)
Klebsiella spp (N=78)	36 (46.2%)	32 (41%)	17 (21.8%)	16 (20.5%)	1 (1.3%)	4 (5.1%)	1 (1.2%)
Salmonella spp (N=2)	1 (50.00%)	1 (50.00%)	0 (0,00%)	1 (50.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Pig	**β-lactam/Céphalosporines**						
	Ampicilline	**Ticarcilline**	**Amoxicilline +ac.clavulinique**	**Céfuroxime**	**Ceftriaxone**	**Ceftazidime**	**Céfotaxime**
E.coli (N=37)	20 (54.1%)	13 (35.1%)	5 (13.5%)	1 (2.7%)	2 (5.4%)	0 (0.00%)	0 (0.00%)
Klebsiella spp (N=47)	18 (38.3%)	13 (27.7%)	4 (8.5%)	5 (10.6%)	2 (4.3%)	0 (0.00%)	2 (4.3%)
Salmonella spp (N=1)	1 (100%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)

Table 2. Antibiotic resistance of enterobacterias from poultry and pig farms in Togo to quinolone antibiotics.

Poultry	Quinolone		Pigs	Quinolone	
	Nalidixic acid	**Norfloxacine**		**Nalidixic acid**	**Norfloxacine**
E.coli (N=29)	9 (31%)	3 (10.3%)	*E. coli* (N=37)	6 (16.2%)	4 (10.8%)
Klebsiella spp (N=78)	26 (33.3%)	16 (20.5%)	*Klebsiella* spp (N=47)	14 (29.8%)	1 (2.1%)
Salmonella spp (N=2)	0 (0.00%)	1 (50.00%)	*Salmonella* spp (N=1)	0 (0.00%)	0 (0.00%)

Resistance to other class of antibiotics

The most important resistance was observed to cyclin antibiotics (tetracycline; doxycycline) and the association sulfoxide – trimethoprim. In poultry, resistance of *E.coli*, *Klebsiella* spp and Salmonella strains was the same for tetracycline and doxycycline. Resistance to the association sulfoxide –trimethoprim was 72.41, 66.67, and 100.00%, respectively for *E. coli*, *Klebsiella* spp and Salmonella. In pigs, similar resistance was also observed to tetracycline (65.67, 78.72, and 100%), doxycycline (81.08, 89.36, and 0.00%) and Association sulfoxide –trimethoprim (48.65, 38.30, and 0.00%) (Table 4).

Multi –resistance to antibiotics

Tables 5 and 6 show multi-Drug resistance pattern of enterobacteria strains. In poultry, 44.83% of *E.coli*, 50% of *Klebsiella* spp and 100% of Salmonella strains were multi-resistant. In Pigs, 37.83 and 27.65% of *E. coli* and *Klebsiella* spp strains were multi-resistant. Being in bacteria from poultry or pig farms, the most frequent MDR pattern was simultaneous resistance to ampicillin – sulfoxide + trimethoprim -tetracycline.

Antibiotic use in farms

According to the results of the questionnaire reported in Table 7, being in poultry or in pigs farms (56.25 and 53.66% respectively), farmers managed the health of the animals on their own. Only 29.69 and 7.32%, for poultry and pig farms, respectively engaged the services of a veterinarian. All surveyed poultry farmers (100%)

Table 3. Antibiotic resistance of enterobacterias from poultry and pig farms in Togo to aminoglycoside antibiotics.

Poultry	Aminoglycoside		Pigs	Aminoglycoside	
	Streptomycin	Gentamicin		Streptomycin	Gentamicin
E. coli (N=29)	16 (55.2%)	0 (0.00%)	*E. coli* (N=37)	17 (45.9%)	0 (0.00%)
Klebsiella spp (N=78)	40 (51.3%)	5 (6.4%)	*Klebsiella* spp (N=47)	13 (27.7%)	1 (2.1%)
Salmonella spp (N=2)	0 (0.00%)	0 (0.00%)	*Salmonella* spp N=1)	0 (0.00%)	0 (0.00%)

Table 4. Antibiotic resistance of enterobacterias from poultry and pig farms in Togo to Cyclins and sulfoxide antibiotics.

Poultry	Tetracyclines/Sulfonamide+DI			Pigs	Trétracyclines/Sulfonamide+DI		
	tetracycline	Doxycycline	Co-Trimoxazole		tetracycline	Doxycycline	Co-Trimoxazole
E. coli (N=29)	27 (93.1%)	27 (93.1%)	21 (72.4%)	*E. coli* (N=37)	25 (67.6%)	30 (81.1%)	18 (48.7%)
Klebsiella spp (N=78)	75 (96.2%)	75 (96.2%)	52 (66.7%)	*Klebsiella Spp* (N=47)	37 (78.7%)	42 (89.4%)	18 (38.3%)
Salmonella spp (N=2)	2 (100.00%)	2 (100.00%)	2 (100.00%)	*Salmonella Spp (*N=1)	1 (100.00%)	0 (0.00%)	0 (0.00%)

and the majority (65%) of pig farmers indicated that they used antibiotics in their farms. Among farmers who used antibiotics, 6.25% (for poultry) and 25.93% (for pig farms) were not able to define an antibiotic or did not know exactly what an antibiotic was among veterinary drugs they commonly used. In addition, 68.75 and 62.96% of poultry and pig farmers indicated that they mainly used antibiotics for prevention. All the antibiotics used in poultry and 88.89% used in pigs were purchased from a veterinary drug store but in the majority of the case without prescription. Hundred percent of pig farmers and 88.89% of poultry farmers had never sent samples for analysis in a laboratory. Forty nine percent and 29.17% of poultry and pig farms experienced a treatment failure after an antibiotic use and only 55.56% of poultry farmers and 36.67% of pig farmers heard about antibiotic resistance. Interestingly, 26.56 and 44.12% of farmers said they used traditional medicine (herbs) sometimes to treat their animals.

DISCUSSION

Antibiotics are widely used in both humans and livestock and have greatly contributed to better human and animal health. As a consequence, animal health, welfare and productivity have been improved in the livestock sector, and ultimately food safety, food security and nutrition and economic growth have shown positive development. However, the achievements in modern medicine and in the livestock sector due to the discovery and development of antibiotics are threatened by the global emergence of antimicrobial resistance (AMR) (FAO, 2019). Antibiotic use in food animals is highly increasing in many parts of the world (Van Boeckel et al., 2015) and It has been shown that antimicrobial resistance can be transmitted from animals to humans (Manishimwe et al., 2017). Given the context of a One Health approach (that is the perspective that the health of people, animals and the environment are interconnected), the emergence of resistance to antibiotics (antibacterial) in the primary production is an issue and a key task for all livestock sectors is to reduce the inappropriate use of antibiotics, as such use is closely linked to development of AMR in humans (Ozawa et al., 2012).

The focus of this study was to investigate the prevalence of antibiotic resistance of enterobacterias and to assess the use of antibiotics in poultry and pig farms in the peri-urban area of Lome in southern Togo. Although the poultry industry is rapidly evolving in Togo as in other West African countries, knowledge and skills related to biosafety management in poultry production are still low among poultry and pig farmers. This may be the cause of high rates of Enterobacterias strains obtained in the poultry and pig samples are 72.72 and 87.7% respectively. This high prevalence of enterobacterias obtained in farms is a concern as these farms can be the

Table 5. Multi-resistance patterns of enterobacterias strains from poultry farms in Togo.

Number of different class	MDR Pattern of *Klebsiella* spp. strains	Number of *Klebsiella spp* strains (%)	MDR pattern of *E. coli* strains	Number of *E. coli* strains (%)	MDR pattern of *Salmonella* spp. strains	Number of *Salmonella* spp. strains (%)
3	AMP-STX-TET	9 (45.00%)	AMP-STX-TET	8 (88,89%)	AMP-STX-TET	1 (50.00%)
	CXM-STX-TET	6 (30.00%)	CTX-STX-TET	1 (11,11%)	CXM-STX-TET	1 (50.00%)
	AMC-STX-TET	2 (10.00%)				
	STX-GEN-TET	2 (10.00%)				
	CAZ-AMP-TET	1 (5.00%)				
Total strains resistant to three different antibiotics		**20**		**9**		
4	AMC-AMP-STX-TET	7 (53.85%)	AMC-AMP-STX-TET+	2 (66.67%)		
	CXM-AMP-STX-TET	4 (30.77%)	CXM-AMP-STX-TET+	1 (33.33%)		
	AMC-CAZ-STX-TET	1 (7.69%)				
	CXM-STX-GEN-TET	1 (7.69%)				
Total strains resistant to four different antibiotics		**13**		**3**		
5+	AMC-CXM-AMP-STX-TET	2 (33.33%)	AMC-AMP-CXM-STX-TET	1 (100.00%)		
	CAZ-CRO-CXM-AMP-STX-TET+	1 (16.67%)				
	AMC-AMP-STX-GEN-TET	1 (16.67%)				
	AMC-CEF-AMP-STX-TET	1 (16.67%)				
	CXM-AMP-STX-GEN-TET	1 (16.67%)				
Total strains resistant to five or more different antibiotics		**6**		**1**		
Total multi resistant strains		**39 (100.00%)**		**13 (100.00%)**		**2 (100.00%)**

principal source of contamination of poultry or pig meat (Bada-Alambedji et al., 2006).

In general, enterobacterias strains exhibited very high level of resistance to tetracyclines, sulfoxide-trimethoprim corresponding to antibiotics commonly used in veterinary practice in Togo according to survey conducted during sampling followed by increasing resistances to streptomycin, ampicillin and nalidixic acid similar to the finding of Yassin et al. (2017) in China. The level of resistance observed in this study are also similar to other findings reported by some authors in different countries. In Senegal for example, Vounba et al. (2019a) investigated resistance of *E.coli* strains in poultry and reported high prevalence of non-susceptibility to tetracycline (92.2%), sulfisoxazole (80.8%), trimethoprim-sulfamethoxazole (76.7%), streptomycin (47.7%) and nalidixic acid (44.0%) very close to our findings and those of Sidibé et al. (2019) and Chen et al. (2004) in Mali and China respectively for Salmonella strains.

Among beta-lactam antibiotics tested, like in the study of Yassin et al. (2017) in China, third generation cephalosporins and the association amoxicillin + clavulanic acid remained very active on enterobacterias with low resistance rates recorded. Indeed, high resistances were observed for ampicillin, ticarcilline and cefuroxime compared to amoxicillin+ clavulanic acid, ceftriaxone, ceftazidime, and cefotaxime. This may be due to the fact that third generation cephalosporin's are not commonly used by farmers. This is a good indicator as third generation cephalosporins constitutes antibiotic of critical importance in veterinary and human health (OIE, 2014; WHO, 2018). Concerning resistance to quinolones and aminoglycosides which also are important antibiotics for veterinary and human health, resistance level was low for second generation quinolone (norfloxacin) and for gentamycin due to the fact that this antibiotics are more expensive and also less used by farmers (Sidibé et al., 2019).

Resistance to at least one antibiotic was common in this study (100% of isolates from

Table 6. Multi-resistance profiles of Enterobacterias strains from swine farms in Togo.

Number of different class	MDR pattern of *Klebsiella* spp. strains	Number of Klebsiella spp. strains (%)	MDR pattern of *E. coli* strains	Number of *E. coli* strains (%)
3	AMP-STX-TET	5 (50.00%)	AMP-STX-TET	8 (80.00%)
	AMC-STX-TET	2 (20.00%)	AMC-STX-TET	2 (20.00%)
	CXM-STX-TET	2 (20.00%)		
	CRO-AMP-TET	1 (10.00%)		
Total strains resistant to three different antibiotics		**10**		**10**
4	AMC-AMP-STX-TET	**1**	AMC-AMP-STX-TET	3 (75.00%)
			CXM-AMP-STX-TET	1 (25.00%)
Total strains resistant to four different antibiotics		**1**		4 (100.00%)
5+	AMC-CTX-CXM-AMP-STX-TET-GEN	1 (50%)		
	AMC-CEF-AMP-STX-TET	1 (50%)		
Total strains resistant to five or more diffrent antibiotics		**2**		
Total		**13 (100%)**		**14 (100%)**

poultry). Multi-drug resistance defined by Magiorakos (Magiorakos et al., 2012) as resistance to at least 03 antibiotics belonging to 03 different categories or classes of antibiotics was high and most frequently observed in poultry, where 44.83% of *E. coli*, 50% of *Klebsiella* spp and 100% of Salmonella strains were multi-resistant. Potential selection factor for multiple resistance observed may be co-selection, as this is found in other studies (Ozawa et al., 2012). Indeed, it is shown that the usage of antibiotics in livestock promotes the development of antibiotic resistance in farm environments (Heuer et al., 2011). In this context, the resistance detected in enterobacterias isolates from poultry and pigs in this study may have been caused by the selection pressure due to antibiotic use in farms. Indeed, the survey during sampling showed that 100% of poultry farmers and 65.85% of pig farmers used antibiotics. As most of the farmers managed the health of their animals on their own and only few had a veterinarian, antibiotic use without prescription was high with some farmers who used antibiotic without knowing what exactly an antibiotic was. This is of concern because this indicates that veterinary drug shops sell antibiotics to farmers without prescription. The quality of the antibiotics used is another reason for antibiotic resistance as it was observed that there was no adequate measure in place to guarantee the quality of antibiotics imported into the country (Hestbjerg et al., 2002). In the present survey, it was found out that majority of farmers used the manure from livestock for crop production. Manure is a reservoir of resistant bacteria and antibiotic compounds, and its application on agricultural soils is assumed to significantly increase selection of resistant bacteria harboring antibiotic resistance genes in soil (Quaik et al., 2020). The genome location of resistance genes is sometime mobile genetic elements such as plasmids, integrons, and transposable elements and their horizontal transfer to bacteria adapted to soil and their environmental transmission to human without animal's contact can represent a serious threat to human's health (Heuer et al., 2011).

Conclusion

This is the first study in Togo to provide information on antibiotic resistance of enterobacterias isolated from different poultry and pig farms. The prevalence of antibiotic resistance of Enterobacterias to tetracyclins and sulfoxide-trimethoprim (more than 50%) was generally high and very low to gentamycin and third generation cephalosporin's (less than 5%). Use of antibiotic without veterinary prescription among poultry and pig farmers was practiced as farmers managed the health of their animals on their own and ignorantly chose any antibiotic for their animals. Despite its limitations, this study showed that the antimicrobial resistance in the poultry and pig farms in Togo is a serious problem. For this

Table 7. Antibiotic use in farms and knowledge of farmers about Antibiotic resistance.

	Poultry		Pigs	
Person in charge of animal's health	**N**	**% (Number of response/ Total number of respondents) (%)**	**N**	**% (Number of response / Total number of respondents) (%)**
vet	19	29.7	3	7.3
technician	9	14	16	39.0
Breeder himself	36	56.3	22	53.7
Total	64	100.00	41	100.00
Use of antibiotics				
Yes	64	100.00	27	65.9
No	0	0.00	14	34.2
Total	64	100.00	41	100.00
Know what is an antibiotic				
Yes	60	93.8	20	74.1
No	4	6.2	7	25.9
Total	64	100.00	27	100
Raison for Antibiotic use				
Prevention of disease	44	68.8	17	63
Treatment	20	31.3	10	37
Total	64	100.00	27	100
Place of antibiotics purchase				
In a vet drug store	64	100	25	88.9
Other (with a technician or in the market)	0	0.00	2	11.1
Total	64	100	27	100.00
Have ever send samples to laboratory for analysis				
Yes	7	11.1	0	0.00
No	56	88.8	27	100
Total	63	100.00	27	100.00
Failure of treatment after using an antibiotic				
Yes	30	49.2	7	29.2
No	31	50.8	17	70.8
Total	61	100.00	24	100.00

Table 7. Contd.

Heard about Antibiotic Resistance				
Yes	35	55.6	11	36.7
No	28	44.4	19	63.3
Total	63	100.00	30	100.00
Use of traditional medicine to treat animals				
Yes	17	26.6	15	44.1
No	47	73.4	19	55.9
Total	64	100.00	34	100.00

reason, studies of virulence genes and antimicrobial resistance at molecular level in multi-resistant strains are needed and should be the next step of this preliminary investigation. This will help assess the threat posed by antimicrobial resistance in animal's production to human health. It is recommended that policies and regulations promoting controlled use of antibiotics be established and enforced in Togo. Food and Agriculture Organization of the United Nations (FAO, 2019) and Word Organization for animal health have provided guidance toward a responsible and prudent use of antimicrobial in pigs and poultry. This guidance can be used as a baseline to sensitize veterinarians and establish contextualized policies and regulations controlling the import, distribution and responsible use of antibiotics in animal production in Togo.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Bada-Alambedji R, Fofana A, Seydi M, Ayayi Akakpo J (2006). Antimicrobial resistance of salmonella isolated from poultry carcasses in Dakar (Senegal). Brazilian Journal of Microbiology *37*:510-515.

Bonnet R, Bru J-P, Caron F, Cattoir V, Courvalin P, Dubreuil L, Jarlier V, Lina G, Merens A, Plesiat P, Ploy MC, Soussy CJ, Varon E (2019). Comité de l'antibiogramme de la Société Française de Microbiologie. www.sfm-microbiologie.org

Chen S, Zhao S, White DG, Schroeder CM, Lu R, Yang H, McDermott P F, Ayers S, Meng J (2004). Characterization of Multiple-Antimicrobial-Resistant Salmonella Serovars Isolated from Retail Meats. Applied and Environmental Microbiology 70(1):1-7.

Food and Agriculture Organization (FAO) (2015). revues nationales de l'élevage SECTEUR AVICOLE Togo. www.fao.org/publications

Food and Agriculture Organization (FAO) (2009). The state of agriculture and Food, Livestock and the environment. https://doi.org/10.18356/6e4ebb75-en

Food and Agriculture Organization (FAO) (2019). Prudent and efficient use of antimicrobials in pigs and poultry FAO Animal production and health manual. www.fao.org/

Founou LL, Amoako DG, Founou RC, Essack SY (2018). Antibiotic Resistance in Food Animals in Africa: A Systematic Review and Meta-Analysis. Microbial Drug Resistance 24(5):648-665.

Hestbjerg L, Aarestrup F, Johannes S (2002). Quantification of bioavailable chlortetracycline in pig feces using a bacterial whole-cell biosensor. Veterinary Microbiology 87:51-57.

Heuer H, Schmitt H, Smalla K (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. Current Opinion in Microbiology 14(3):236-243.

Lhoste P (2009). L'élevage africain, source possible d'une révolution alimentaire attendue ? http://www.inter-reseaux.org/IMG/pdf_p33_34_Conclu_Dossier.pdf

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas MY, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology and Infection 18(3):268-281.

Manishimwe R, Buhire M, Uyisunze A, Turikumwenayo JB, Tukei M (2017). Caractérisation d'Escherichia coli résistant aux antibiotiques dans différents systèmes avicoles de la province de l'Est et de la ville de Kigali au Rwanda. Revue d'élevage et de Médecine Vétérinaire Des Pays Tropicaux 70(1).

Mensah SEP, Koudandé OD, Sanders P, Laurentie M, Mensah GA, Abiola FA (2014). Antimicrobial residues in foods of animal origin in Africa: public health risks. Revue scientifique et technique (International Office of Epizootics) 33:3.

OIE (2014). Critères utilisés pour la catégorisation: Liste OIE des agents antimicrobiens importants. Organisation mondiale de la santé animale 33(0):1-9.

Ozawa M, Makita K, Tamura Y, Asai T (2012). Associations of

antimicrobial use with antimicrobial resistance in Campylobacter coli from grow-finish pigs in Japan. Preventive Veterinary Medicine 106(3-4):295-300.
Quaik S, Embrandiri A, Ravindran B, Hossain K, Al-Dhabi NA, Arasu M V, Ignacimuthu S, Ismail N (2020). Veterinary antibiotics in animal manure and manure laden soil: Scenario and challenges in Asian countries. Journal of King Saud University-Science 32(2):1300-1305.
Schar D, Sommanustweechai A, Laxminarayan R, Tangcharoensathien V (2018). Surveillance of antimicrobial consumption in animal production sectors of low- and middle-income countries: Optimizing use and addressing antimicrobial resistance. PLoS Medicine 15(3):1-9.
Sidibé S, Traoré B, Sidi Y, Broulaye A, Afou D, Oumar B (2019). Antibiorésistance des souches de Salmonella gallinarum isolées en aviculture moderne en zones périurbaines au Mali. Revue Elevage et de Medecine Veterinaire Pays Tropicaux 72(4):1-6.
Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson T P, Teillant A, Laxminarayan R (2015). Global trends in antimicrobial use in food animals. https://doi.org/10.1073/pnas.1503141112
Vounba P, Arsenault J, Bada-Alambédji R, Fairbrother JM (2019a). Prevalence of antimicrobial resistance and potential pathogenicity, and possible spread of third generation cephalosporin resistance, in Escherichia coli isolated from healthy chicken farms in the region of Dakar, Senegal. PLoS ONE 14(3).
Vounba P, Rhouma M, Arsenault J, Bada Alambédji R, Fravalo P, Fairbrother JM (2019b). Prevalence of colistin resistance and mcr-1/mcr-2 genes in extended-spectrum β-lactamase/AmpC-producing Escherichia coli isolated from chickens in Canada, Senegal and Vietnam. Journal of Global Antimicrobial Resistance 19:222-227.
Vounba P, Yaghouba K, Ndiaye C, Arsenault J (2018). Molecular Characterization of Escherichia coli Isolated from Chickens with Colibacillosis in Senegal. Foodborne Pathogens and Disease 15(8):1-9.
World Health Organization (WHO) (2018). WHO list of Critically Important Antimicrobials (CIA). http://www.who.int/foodborne_disease/resistance/cia/en/
Yassin AK, Gong J, Kelly PLG, Guardabassi L, Wei L, Han X, Qiu H, Price S, Cheng D, Wang C (2017). Antimicrobial resistance in clinical Escherichia coli isolates from poultry and livestock, China. PLoS ONE 12(9):1-8.

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Antibacterial abilities of spray sanitizer solutions formulated with chitosan and acid complexes at pH 3 on broiler carcass surfaces inoculated with selected pathogenic bacteria before refrigeration

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Single acid (acetic acid, lactic acid, propionic acid and phosphoric acid) and acid complex solutions at the ratio 1:1 or 2:1 at pH 3 were investigated their antimicrobial activities against three selected foodborne pathogens (Escherichia coli, Salmonella typhi and Staphylococcus aureus). The influences of the deacetylation degrees (DD) (80% and 95%), concentrations (500, 1000, and 2000 µg/mL) and contact time (10, 20, 30, 40, 50 and 60 min) on the antimicrobial activity of chitosan against three bacteria were also studied. The better condition of chitosan and acid complex solutions were selected to use as sanitizers sprayed on the broiler carcass surfaces (breast and thigh) to determined their antimicrobial activities. The results showed that acid complex solutions with the ratio 2:1 had the better inhibiting efficiency against pathogens than the single acid and acid complex solutions at the ratio 1:1. The antimicrobial activity of chitosan against bacteria significantly increased as the contact time and chitosan concentrations increased. Acetic acid+lactic acid or acetic acid+propionic acid (2:1) were dissolved with/without chitosan solution (1000 µg/mL with DD 95 %) and sprayed on the broiler carcass surfaces against pathogens. The results displayed that acetic acid + lactic acid sprayed with chitosan significantly reduced S. aureus, E. coli and S. typhi counts on the surface of the breast (2.73, 2.84 and 2.71 log CFU/cm2, respectively) and the thigh (2.56, 2.85and 2.43 log CFU/cm2, respectively). Conclusion, acid complex solutions mixed with chitosan can be used to avoid the deterioration of slaughtered meat quality.

Key words: Foodborne pathogens, chitosan, organic acid, sanitizer, broiler carcass.

INTRODUCTION

During the slaughtering process for poultry and livestock, several methods, such as hot water washes, acid sprays, chemical sanitizers or flames, etc., can be used to reduce microbial contamination on the surface of the carcass before chilling or refrigeration. The use of synthetic chemical sanitizers is generally effective at reducing post-

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harvest microbes. Chlorine is the decontaminating agent generally used as a sanitizer to eradicate pathogenic microorganisms in the poultry slaughtering system. But, chlorine can cause severe irritation to the nose, throat and upper respiratory tract. Chlorine exposure at high concentrations results in severe respiratory tract damage, causing bronchitis and pulmonary edema and possibly be deadly (Chaiyakosa et al., 2007).

Organic acids are generally recognized as safe (GRAS) antimicrobial agents approved by USDA Food Safety and Inspection Service and they have been used as sanitizers for slaughtered carcasses with good sterilizing effects (Acuff et al., 1987; Sallam et al., 2020; FDA, 2003). Organic acids have the antimicrobial action by reducing environmental and cellular pH values and increasing anion accumulation (Carpenter and Broadbent, 2009). Moreover, the antimicrobial activities of organic acids are dependent on the pKa value and the effect is greater under acidic condition (Nguyen et al., 2020). Organic acid dilutions (1-3%) can effectively reduce the number of bacteria on an animal carcass before chilling, refrigeration or processing (Raftari et al., 2009). A high level of organic acid with low pH is highly effective in reducing microorganisms, but higher concentrations of these acids result in defects, such as bad flavor and color fading, which affect the quality of the product when applied in the poultry slaughtering system during storage or marketing (Smulders and Greer, 1998; Sohaib et al., 2016). Garbutt (1997) reported that the optimum growth pH of bacteria at neutral pH (6.8-7.2) and the minimum growth pH is nearer to 4.0-4.5. This study also found that growth of food poisoning bacteria, such as *Staphylococcus aureus*, *Salmonella* species and *Listeria monocytogenes* could retard when the pH adjusted lower than 4.0 with organic acids, such as lactic acid, citric acid and acetic acid. Many research found that the organic acids, such as acetic acid, citric acid and lactic acid decreased the microbial populations of *Escherichia coli*, *Salmonella*, psychrotrophic Gram-negative and Enterobacteriaceae when sprayed on pork, poultry and beef carcass or use as wash (Laury et al., 2009; Harris et al., 2012; Dan et al., 2007). Therefore, it is important to determine the optimal acidic pH for bacterial inhibition and also to meet the meat quality requirements (indicated by the least amount of discoloration, off-flavor and drip loss).

Lactic acid (2-hydroxypropanoic acid) is a natural organic acid (pKa 3.79) produced by microbial fermentation. It is commonly used in the food production as food preservative, flavor agent and acidulant (Wee et al., 2006; Lipnizki, 2010). Lactic acid is classified as GRAS for use as an antimicrobial agents for decontamination of meat carcass. It is approved for use as part of a carcass wash at level <5% acid for pre- and post-chilling, 2-3% for sub-primal cuts and 2-2.8% in washing systems for trimings and beef head and tongues (Ba et al., 2018; Mani-López et al., 2012). It can interfere with cell membrane permeability and cell functions (Chauret, 2014).

Acetic acid is a monocarboxylic and also known as vinegar, which formed naturally due to spoilage of wine. Acetic acid has a limit to use in foods due to a pungent, vinegar-like odor and sour taste. It is highly water soluble and found in pickled products (Mani-López et al., 2012).

Propionic aicd is a naturally carboxylic acid with a pungent odour, colorless and miscible with water. Propionic acid is a commonly organic acid produced through microbial fermentation (*Propionibacterium* species). In food industry, it is commonly used as food preservative, antimold, antirope agent and flavouring agent (Gonzalez-Garcia et al., 2017; Haque et al., 2009).

Phosphoric acid is an inorganic acid acquired by chemical reaction of phosphorous rock. It is a colorless, odourless and viscous liquid. It is an important chemical for the manufacture of fertilizers, detergents, toothpastes and alimentary supplies for cattle. In food, it is used as a sequenstrant, an antioxidant and flavor enhancer in beverages and fruit procucts (Awwad et al., 2013; Kandil et al., 2017).

Chitosan is a nontoxic natural polymer. It can be synthesized via the deacetylation of chitin which is major component of the shells of crustaceans, such as crab, shrimp and crawfish (Hong et al., 2002). The chemical structure of chitosan is a linear polysaccharide composed with β-(1-4)-linked 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose. Chitosan is a natural cationic polysaccharides and it has been applied for several purposes, including antimicrobial, food, chemical engineering, pharmaceutical, nutrition and environmental protection applications (Kahya, 2019). Many reports have shown evidence that an edible chitosan film or coating on pork, sausage or ground meat can be used to control the growth of spoilage bacteria during storage or marketing and prolong the shell life (Sagoo et al., 2002; Roller et al., 2002; Lucera et al., 2012). Chitosan has also been shown to inhibit some pathogenic bacteria, including *E. coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Vibrio* species, *Salmonella* Typhi and *S. aureus* (Sudarshan et al., 1992; Tepe et al., 2004; 1992, 1992; 1992, et al., 1992, 2011) and the reported minimum inhibitory concentrations (MIC) vary widely from 0.01 to 1.0% (Zheng and Zhu, 2003).

Although many studies have shown evidence for the antimicrobial activities of chitosan and acids, no published studies have combined chitosan with organic acids at pH 3. Thus, the aim of this study was to look for an optimum formula of the single organic/inorganic acid and their acid complex solutions at different ratios at 1:1 and 2:1 at pH 3, and the combination with chitosan on their antibacterial inhibition and the lowest amount of damage on meat quality (discoloration, off-flavor and drip loss). In this study, the single acid (acetic acid, lactic acid, propionic acid and phosphoric acid) and acid complex solutions at the ratio 1:1 or 2:1 at pH 3 investigated their

antimicrobial activities against three selected foodborne pathogens including *E. coli, S.* Typhi and *S. aureus* for 1 h. Besides, the influences of the deacetylation degrees (DD) (80 and 95%), concentrations (500, 1000, and 2000 µg/mL), and the contact time (10, 20, 30, 40, 50 and 60 min) on the antimicrobial activity of chitosan against three selected foodborne pathogens were also studied. The better condition of acid complex solutions and chitosan were selected to be used as sanitizers sprayed on the broiler carcass surfaces (breast and thigh) to determine their antimicrobial activities.

MATERIALS AND METHODS

Raw materials

Chitosan, with a molecular weight (MW) of 100-300 kDa and a deacetylation degree (DD) of 95%, was purchased from Lytone Enterprise Inc. (Taipei, Taiwan). Three strains of pathogenic microorganisms (*E. coli* BCRC 10675, *S.* Typhi BCRC 10746 and *S. aureus* BCRC 10781) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan).

Preparation of acid and chitosan

Propionic acid (Merck, Darmstadt, Germany), acetic acid (Union Chemical Work Ltd., Hsinchu, Taiwan), lactic acid (Wako Inc., Japan) and phosphoric acid (Union Chemical Work Ltd., Hsinchu, Taiwan) separately prepared the single acid solution at pH 3 in sterilized distilled water. For the acid complex, solutions (pH 3) were prepared by the mixtures of propionic acid + acetic acid, phosphoric acid＋propionic acid, acetic acid+phosphoric acid or lactic acid+lactic acid at the ratio of 1:1 or 2:1 (v/v) in sterilized distilled water.

Preparation of chitosan

Chitosan acidic solution was prepared according to the modified method of Sudarshan et al. (1992). A 500, 1000, or 2000 µg/mL chitosan acidic solutions was prepared by dissolved chitosan powder in distilled water and adjusted to pH 5 with glacial acetic acid.

Microbial culture and growth conditions

According to the protocol of the Food Industry Research and Development Institute (Hsinchu, Taiwan), *S.* Typhi and *E. coli* were separately cultured in a nutrient broth (Acumedia, Michigan, USA) and then incubated at 37°C for 24 h. *S. aureus* was cultured in tryptic soy broth (Acumedia, Michigan, USA) at 37°C for 24 h. Then, *S.* Typhi, *E. coli* and *S. aureus* cultures were collected.

Antimicrobial activity of the acid solution

Evaluations of antimicrobial activity of acid solutions were performed as follows: 1 mL of bacterial suspension (10^8 CFU/mL) was mixed with 9 mL of various acid solutions and incubated at 37°C for 60 min. These mixtures were then serially diluted to 10^6 CFU/mL and incubated at 37°C for 24 h. Colony numbers were determined using the plate count method. The initial colonies number of *S.* Typhi, *E. coli* and *S. aureus* was 4.5×10^6, 6.1×10^6 and 5.4×10^6 CFU/mL, respectively. The inhibition efficiency was defined as: reduced count (log CFU/mL) = N_1 - N_2, where N_1 and N_2 represent the colony numbers on the plates before and after treatment.

Antimicrobial activity of the chitosan solution

Antimicrobial activity of the chitosan solution was evaluated as previously described: 1 mL of bacterial suspension was mixed with 1 mL of chitosan solutions and 8 mL of lactic acid to the final chitosan concentrations at 500, 1000 and 2000 µg/mL. Then, the suspension with chitosan was incubated at 37°C for 10, 20, 30, 40, 50 or 60 min. The mixtures were then serially diluted to 10^7 CFU/mL and incubated at 37°C for 24 h. Colony numbers were counted using the plate count method. The initial colony numbers of *E. coli*, *S.* Typhi and *S. aureus* were 7.1×10^7, 5.2×10^7 and 4.5×10^7 CFU/mL, respectively. The inhibition efficiency was defined in the same way as described for the acid treatments.

Preparation of sanitizing spray

Acetic acid+lactic acid and acetic acid+propionic acid solutions at pH 3 were separately prepared at the ratio 2:1 (v/v). Then, chitosan was added and dissolved completely to the final concentration at 1000 µg/mL.

Treatment of spray

A total of 15 broiler carcasses (average weight 1.67 kg) were purchased from Charoen Pokphand Enterprise (Taiwan) Co., Ltd. and divided into 3 treatment groups of 5 birds; each group was inoculated with *S. aureus, E. coli* or *S.* Typhi. The procedure was repeated three times for the experiment. Approximately, 5 log CFU/cm^2 bacteria were inoculated on the surface of the breast and leg areas by cotton swab, as described by Dubal et al. (2004) and carcasses were maintained at 10°C for 2 h. The bacterial counts for *S. aureus, E. coli* and *S.* Typhi inoculated on the carcass surfaces were 3.4×10^5, 4.1×10^5 and 2.4×10^5 CFU/cm^2, respectively. The spraying procedure was performed as follows: 100 mL sanitizer was sprayed on the whole surface of each bird, which was then maintained at 10°C for 1 h. Solutions formulated only with organic acid complexes without chitosan were used as the controls.

At the end of treatment, a sterilized albumin foil (5 × 5 cm) was placed on the breast and leg of each bird and the swab method was used to take samples to determine colony counts. The inhibition efficiency was defined in the same way as described previously.

Statistical analysis

Data were analyzed using the Statistical Analysis System's Procedures (SAS) (Institute Inc., Cary, NC) software package with a 5% level of significance. The GLM system was applied to determine the significance of the treatments; when significant (P<0.05) differences were found, the means were determined by the Duncan's multiple range test.

RESULTS AND DISCUSSION

Antimicrobial ability of single acids at pH 3

Garbutt (1997) stated that strong inorganic acids are not

Table 1. Effect of acids with various proportions of different organic acids at pH3 on the antibacterial activity for *S. aureus*.

Proportions	Different acids	Reduced log (CFU/mL)
single acid	Propionic acid	1.03^{ab}
	Acetic acid	0.35^{c}
	Lactic acid	0.58^{bc}
	Phosphoric acid	0.18^{d}
1:1 combined acids	Acetic acid+propionic acid	1.35^{a}
	Lactic acid+propionic acid	0.75^{b}
	Lactic acid+acetic acid	0.76^{b}
	Phosphoric acid+propionic acid	0.48^{c}
	Phosphoric acid+acetic acid	0.31^{c}
	Phosphoric acid+lactic acid	0.44^{c}
2:1 combined acids	Propionic acid+acetic acid	1.42^{a}
	Propionic acid+lactic acid	1.31^{a}
	Propionic acid+phosphoric acid	1.02^{ab}
	Acetic acid+propionic acid	0.97^{ab}
	Acetic acid+lactic acid	1.13^{a}
	Acetic acid+phosphoric acid	0.82^{b}
	Lactic acid+propionic acid	1.14^{a}
	Lactic acid+acetic acid	1.22^{a}
	Lactic acid+phosphoric acid	0.83^{b}
	Phosphoric acid+propionic acid	0.77^{b}
	Phosphoric acid+acetic acid	0.61^{b}
	Phosphoric acid+lactic acid	0.68^{b}
SEM	-	0.11

$^{a-d}$Different superscripts at the same column indicate significantly different ($P < 0.05$).

often included in processed foods, but hydrochloric and phosphoric acids are used in the manufacturing of carbonated drinks and non-carbonated drinks (for example, cola) contain phosphoric acid. Therefore, in this study, 3 organic acids (acetic acid, propionic acid and lactic acid) and 1 inorganic acid (phosphoric acid) were evaluated for the ability to inhibit three selected pathogens (*S. aureus, E. coli* and *S*. Typhi); the results are presented in Tables 1 to 3. For single acids at pH 3, propionic acid had the best and most highly significant inhibition (approximately reduced 1.03 log CFU/mL) against *S. aureus* when compared with all organic acids or the inorganic acid. Moreover, the reduced bacterial count for all organic acids was 0.35-1.03 log CFU/mL and significantly higher than that of the inorganic acid (phosphoric acid: 0.15 log CFU/mL). For *E. coli*, the reduction in bacterial counts for all single acids was below 0.5 CFU/mL, indicating that the antimicrobial ability of single acids was less efficacious at inhibiting *E. coli* regardless of whether the acid was organic or inorganic. However, acetic acid exhibited the best ability to inhibit *S.* Typhi, reducing growth by 0.69 CFU/mL. The data also indicated that organic acids were better than the inorganic acid on inhibit *Salmonella* bacteria. This result may be due to *Salmonella* having an inorganic acid resistance mechanism and acid tolerance response. Brenneman et al. (2013) reported that the RpoS is an essential regulator in *Salmonella* for the acid tolerance response. Moreover, PhoP, PhoQ and Flu also play an important role in acid response. PhoP and PhoQ protect against inorganic stress. Mani-López et al. (2012) also reported that the lethal effects of organic acid on *Salmonella* depended on concentration, pH of the environment and the dissociation constant of each acid. According to the data described earlier, single organic acids can be used to inhibit one specific type of bacteria; for example, propionic acid is suitable to use against *S. aureus* and acetic acid is suitable for *S.* Typhi*.* Acid has effect on the minimum pH for microorganism. The organic acids (acetic, lactic, citric and tartaric) have better activities than inorganic acids and the order of acids according to the level of their antimicrobial activity is as follows: propionic > acetic > lactic >citric> phosphoric > hydrochloric (Buchanan and Golden, 1994; Garbutt, 1997).

Table 2. Effect of acids with various proportions of different organic acids at pH3 on the antibacterial activity for *E. coli*.

Proportions	Different acids	Reduced log (CFU/ml)
Single acid	Propionic acid	0.27^{cd}
	Acetic acid	0.46^{c}
	Lactic acid	0.29^{cd}
	Phosphoric acid	0.11^{d}
1:1 combined acids	Acetic acid+propionic acid	0.78^{a}
	Lactic acid+propionic acid	0.61^{ab}
	Phosphoric acid+propionic acid	0.33^{c}
	Lactic acid+acetic acid	0.84^{a}
	Phosphoric acid+acetic acid	0.20^{cd}
	Phosphoric acid+lactic acid	0.26^{cd}
2:1 combined acids	Propionic acid+acetic acid	0.71^{a}
	Propionic acid+lactic acid	0.78^{a}
	Propionic acid+phosphoric acid	0.42^{c}
	Acetic acid+propionic acid	0.73^{a}
	Acetic acid+lactic acid	0.86^{a}
	Acetic acid+phosphoric acid	0.42^{c}
	Lactic acid+propionic acid	0.33^{c}
	Lactic acid+acetic acid	0.66^{ab}
	Lactic acid+phosphoric acid	0.38^{c}
	Phosphoric acid+propionic acid	0.34^{c}
	Phosphoric acid+acetic acid	0.33^{c}
	Phosphoric acid+lactic acid	0.36^{c}
SEM		0.27^{cd}

$^{a-d}$Different superscripts at the same column indicate significantly different ($P<0.05$).

The results also signed to support this notion.

Antimicrobial abilities of acid complexes with different acids and formula ratios

The results showing the inhibitory effects of acid complex solutions (pH 3) with different acids and component proportions on three selected pathogens (*S. aureus, E. coli* and *S.* Typhi) are displayed in Tables 1 to 3. These data indicate that all acid complexes using inorganic acid (phosphoric) had the least ability to inhibit microorganisms, regardless of the ratio, when compared with organic acids. Conversely, for the microorganisms examined, acid complexes were adjusted with different acid ratios and organic acids in fact improved antibacterial ability.

For *S. aureus,* the result showed that all 2:1 acid complexes had better antibacterial ability than all 1:1 acid complexes and all single acids. These results also indicated that propionic acid combined with the other organic acids (lactic and acetic) had the best bacterial inhibition efficiency. Although the acid complexes using acetic acid and lactic acid were not better than propionic acid, there were no differences by statistical analysis in this study. The antimicrobial activity of organic acids is attributed with the ability of undissociated acid molecules to enter the bacteria cell and the lower pH value than the growth range of bacteria (Yu et al., 2010; Sallam et al., 2020). Dubal et al. (2004) found that spraying with the mixture of acetic acid + proionic acid (1.5 + 1.5%) on sheep/goat forequarters surfaces was completely inhibited in the inoculated pathogens, *Salmonella* Typhimurium (10^3 CFU/g). Yang et al. (1998) indicated that 2% lactic acid (pH 2.2) could reduce *S. aureus* by approximately 1 log CFU/mL. However, there has been some research suggesting that 2% or even 1% organic acid is responsible for the presence of detrimental effects on meat quality (Smulders and Greer, 1998). The bacterial inhibition of lactic acid (pH 3) for *S. aureus* in this experiment was 0.35 log CFU/mL. Moreover, better count reductions for *S. aureus*, 1.22-1.35 log CFU/mL, were observed in acetic acid complexes using propionic acid (1:1) and lactic acid (2:1) in this study. Thus, *S. aureus* count reduction can be achieved with a pH 3 acetic acid complex, which may also reduce damage to quality.

Table 3. Effect of acids with various proportions of different organic acids at pH3 on the antibacterial activity for *S. typhi*.

Proportions	Different acids	Reduced log (cfu/ml)
Single acid	Propionic acid	0.51^{c}
	Acetic acid	0.69^{c}
	Lactic acid	0.63^{c}
	Phosphoric acid	0.21^{d}
1:1 combined acids	Acetic acid+propionic acid	0.73^{bc}
	Lactic acid+propionic acid	0.65^{c}
	Phosphoric acid+propionic acid	0.46^{cd}
	Lactic acid+acetic acid	0.96^{ab}
	Phosphoric acid+acetic acid	0.31^{d}
	Phosphoric acid+lactic acid	0.44^{cd}
2:1 combined acids	Propionic acid+acetic acid	0.92^{ab}
	Propionic acid+lactic acid	0.88^{b}
	Propionic acid+phosphoric acid	0.65^{c}
	Acetic acid+propionic acid	1.27^{a}
	Acetic acid+lactic acid	1.43^{a}
	Acetic acid+phosphoric acid	0.72^{bc}
	Lactic acid+propionic acid	0.84^{b}
	Lactic acid+acetic acid	0.96^{ab}
	Lactic acid+phosphoric acid	0.63^{c}
	Phosphoric acid+propionic acid	0.54^{c}
	Phosphoric acid+acetic acid	0.66^{c}
	Phosphoric acid+lactic acid	0.47^{cd}
SEM		0.51^{c}

$^{a-d}$Different superscripts at the same column indicate significantly different ($P < 0.05$).

For *E. coli,* the results showed that all acid complexes (1:1 or 2:1) adjusted with organic acids had better antibacterial ability than all acid complexes using inorganic acids and all single acids. Moreover, these results also indicated that acid complexes using lactic and acetic acid had the best inhibition efficiency. Although acid complexes using acetic acid and lactic acid were better than propionic acid, there were no differences by statistical analysis in this study. Another study (Bracket et al., 1994) also noted that the compound use of organic acids had better inhibition effects than the use of a single organic acid against *E. coli*. Skřivanová and Marounek (2007) stated that the antimicrobial effect of organic acids on *E. coli* is depended on pH. At low pH, organic acids are undissociated. These undissociated forms are lipophilic and could permit through the cell membrane and inhibited microbial growth. Stivarius et al. (2002) applied 5% lactic acid to wash beef trimmings inoculated with a mixture of *S.* Typhimurium and *E. coli* before grinding and the results showed that higher concentration of lactic acid was effective for reducing the growth of all inoculated pathogens and increasing the shelf-life. Dorsa et al. (1997) indicated that 2% of acetic acid and lactic acid had high inhibition effects against *E. coli*.

However, this experiment results showed that all acids exhibited the poorest inhibition effects with *E. coli* and thus, these data do not agree with the results of the previous study. The reason for this discrepancy may be because a pH 3 acid solution was used in this study and the percentage of acid was significantly lower than 2%, which was used in the aforementioned review. Smulders and Greer (1998) also indicated that *E. coli* O157:H7 had better resistance to organic acids (lactic acid or acetic acid). When they used organic acid alone in treatment, the inhibition effect was lower than 1 log CFU/cm^{2}.

For *S.* Typhi*,* the results showed that all acetic acid complexes (1:1 or 2:1) adjusted using lactic acid and propionic acid had better antibacterial abilities (reduced count was 1.27-1.43 log CFU/mL) than other acid complexes and all single acids. These results also indicated that acetic acid combined with lactic acid had the best inhibition efficiency. The acid complexes using acetic acid and propionic acid were not better than lactic acid and there was no difference by statistical analysis in

Table 4. Effects of deacetylation degree (DD), concentration, and contact time of chitosan on the antibacterial activity (reduced log CFU/mL) against *E. coli*.

DD (%)	Concentration (µg/ml)	Time (min)						SEM
		10	20	30	40	50	60	
80	500	0.55^{fF}	0.92^{eF}	1.28^{dF}	1.84^{cF}	2.46^{bF}	2.85^{aF}	0.12
	1000	0.62^{fE}	0.95^{eE}	1.39^{dE}	1.89^{cE}	2.54^{bE}	2.99^{aE}	0.14
	2000	0.68^{fD}	1.02^{eD}	1.46^{dD}	1.99^{cD}	2.7^{bD}	3.07^{aD}	0.14
95	500	1.18^{fC}	1.41^{eC}	1.91^{dC}	2.34^{cC}	2.8^{bC}	3.43^{aC}	0.15
	1000	1.26^{fB}	1.49^{eB}	2.01^{dB}	2.44^{cB}	2.95^{bB}	3.54^{aB}	0.15
	2000	1.32^{fA}	1.54^{eA}	2.11^{dA}	2.50^{cA}	3.02^{bA}	3.64^{aA}	0.17
	SEM	0.03	0.02	0.03	0.02	0.03	0.03	-

$^{a-f}$Different superscripts at the same row indicate significant difference ($P < 0.05$). $^{A-F}$Different superscripts at the same column indicate significant difference ($P < 0.05$).

this study. Smulders and Greer (1998) demonstrated that spraying 1-3% lactic acid or 2% acetic acid on a slaughtered body could reduce *S.* Typhi 1-2 log CFU/cm^2. Xiong et al. (1998) also indicated that spraying 2% lactic acid or compound acids on chicken skin could reduce *S*. Typhi by 0.52 and 1.16 log CFU/cm^2, respectively.

In this experiments, all single and complex acids displayed better antibacterial action against *S. aureus* (reduced count 0.18-1.42, log CFU/mL) and *S.* Typhi (reduced count 0.21-1.43, log CFU/mL) than *E. coli* (reduced count 0.11-0.86, log CFU/mL) when the results in Tables 1 and 3 are compared to those in Table 2. However, the results might be due to different microbe sensitivities to different acids and the coordination effect with organic acids. Different groups of microbes have different optimum inhibitions (Liu et al., 2001). Furthermore, the results also showed that pH 3 acetic acid complexes using propionic or lactic acid enhanced bacterial inhibition and prevented the deterioration of slaughtered animal carcasses. Therefore, the researcher decided to use 2:1 acid complexes with acetic acid + lactic acid and acetic acid + propionic acid, combined with an optimum level of chitosan, to create a sterilization solution that we could then apply in a poultry slaughtering site to evaluate antimicrobial action against *E. coli*, *S.* Typhi and *S. aureus*, as in the last experiment.

Antimicrobial ability of chitosan with different deacetylation degrees and concentrations

Table 4 illustrates the influence of deacetylation degree (DD), concentration and contact time of chitosan on antibacterial activity against *E. coli*. The results showed that the inhibition effects of chitosan against *E. coli* increased significantly as chitosan concentration increased ($P < 0.05$) at any contact time and with the same DD. For example, the bacterial count reduction increased significantly from 2.85 to 3.07 log CFU/mL when the chitosan concentration (80% DD) increased from 500 to 2000 µg/mL with contact for 60 min. These results agreed with the study conducted by Zheng and Zhu (2003) who reported that chitosan (305 kDa molecular weight) had a 0% inhibition rate at a concentration of 0.25%, whereas it had a 40% inhibition rate against *E. coli* when the chitosan concentration increased to 0.5%. This inhibition rate further increased to 100% when the chitosan concentration increased to 1.0%. Dorsa et al. (1997) also explained that higher NH_3^+ concentration, which was due to a higher chitosan concentration in the medium, contributed to increased chitosan antibacterial activity. Liu et al. (2004) reported that chitosan at the higher concentration of 0.5% caused more cell membrane damage to *E. coli* than chitosan at the lower level concentration of 0.25%.

In this study, the reduction in *E. coli* bacterial counts also significantly increased ($P < 0.05$) as the contact time increased at the same DD and concentration of chitosan. For example, the bacterial count reduction increased significantly from 1.32 to 3.64 log CFU/mL when contact time increased from 10 to 60 min for 2000 µg/mL (95% DD) chitosan solution. Liu et al. (2004) found that the permeability of the outer and inner membranes of *E. coli* increased with increased chitosan contact time. A significant reduction in the numbers of *Vibrio parahaemolyticus*, which was artificially inoculated in shrimp, was observed when the chitosan exposure time increased (Chaiyakosa et al., 2007). Similarly, the growth of *E. coli* was inhibited when the chitosan exposure time increased (Liu et al., 2004). A study performed by Chung et al. (2003) also illustrates that the antibacterial activity of chitosan inhibits *E. coli* and *S. aureus* increased with the contact time. Moreover, chitosan with low molecular weight possesses a grander flexibility to bind more than one cell. This situation causes the bridge between polymer chains of chitosan and bacteria cells rapidly formed and inhibits bacteria (Wu et al., 2006). Helander et al. (2001) reported that chitosan displays stronger

Table 5. Influences of deacetylation degree (DD), concentration, and contact time of chitosan on the antibacterial activity (reduced log CFU/mL) against *S. typhi*.

DD (%)	Concentration (µg/ml)	Time (min)						SEM
		10	20	30	40	50	60	
80	500	0.76^{fF}	1.26^{eE}	1.46^{dF}	2.09^{cE}	2.52^{bD}	2.92^{aE}	0.17
	1000	0.85^{fE}	1.31^{eD}	1.60^{dE}	2.20^{cD}	2.70^{bC}	3.04^{aD}	0.14
	2000	0.95^{fD}	1.36^{eD}	1.72^{dD}	2.28^{cC}	2.77^{bC}	3.19^{aC}	0.16
95	500	1.47^{fC}	1.64^{eC}	2.23^{dC}	2.87^{cB}	3.34^{bB}	3.58^{aB}	0.14
	1000	1.53^{fB}	1.77^{eB}	2.33^{dB}	3.01^{cA}	3.40^{bA}	3.71^{aA}	0.15
	2000	1.62^{fA}	1.86^{eA}	2.44^{dA}	3.06^{cA}	3.44^{bA}	3.79^{aA}	0.16
	SEM	0.02	0.03	0.03	0.03	0.04	0.04	-

$^{a-f}$Different superscripts at the same row indicate significant difference ($P < 0.05$). $^{A-F}$Different superscripts at the same column indicate significant difference ($P < 0.05$).

Table 6. Effects of deacetylation degree (DD), concentration, and contact time of chitosan on the antibacterial activity (reduced log CFU/mL) against S. aureus

DD (%)	Concentration (µg/ml)	Contact time (min)						SEM
		10	20	30	40	50	60	
80	500	0.67^{fF}	0.79^{eF}	1.14^{dF}	1.51^{cF}	2.02^{bF}	2.35^{aF}	0.17
	1000	0.97^{fE}	1.13^{eE}	2.02^{dE}	2.19^{cE}	2.76^{bE}	3.12^{aE}	0.15
	2000	1.03^{fD}	1.20^{eD}	2.30^{dD}	2.53^{cD}	3.04^{bD}	3.31^{aD}	0.18
95	500	1.82^{fC}	2.02^{eC}	2.93^{dC}	3.15^{cC}	3.63^{bC}	4.05^{aC}	0.19
	1000	1.87^{fB}	2.22^{eB}	3.12^{dB}	3.34^{cB}	3.72^{bB}	4.18^{aB}	0.16
	2000	1.98^{fA}	2.38^{eA}	3.21^{dA}	3.43^{cA}	3.83^{bA}	4.31^{aA}	0.19
	SEM	0.03	0.04	0.03	0.03	0.03	0.04	

$^{a-f}$Different superscripts at the same row indicate significant difference ($P < 0.05$).
$^{A-F}$Different superscripts at the same column indicate significant difference ($P < 0.05$)

antimicrobial activty in acid condition. The activity decreases with the increasing pH.

In this experiment, it was found that contact time (that is, 10-60 min) had a greater influence on *E. coli* inhibition than the concentration (that is, 500-2000 µg/mL) of chitosan. For example, count reduction increased by approximately 2.35 log CFU/mL (that is, from 0.62 to 2.97 log CFU/mL) when the chitosan contact time increased from 10 to 60 min at all chitosan concentrations (80% DD) from 500 to 2000 µg/mL. However, the count reduction only increased by approximately 0.22 log CFU/mL (that is, from 2.85 to 3.07 log CFU/mL) when the contact time was 60 min and when the concentration increased from 500 to 2000 µg/mL. Liu et al. (2004) stated that the permeability of the outer and inner membranes of *E. coli* increased with increased chitosan contact time. Another study by Chung et al. (2003) illustrates that an increase of the contact time increases the antibacterial activity of chitosan on *E. coli* and *S. aureus*.

Moreover, with regard to DD bacterial count, reduction with 95% DD was higher than for 80% DD when chitosan concentrations and contact time were maintained at the same conditions. For example, chitosan with 95% DD resulted in a significantly higher count reduction for *E. coli* (1.18 to 1.32 log CFU/mL) than for 80% DD (that is, 0.55 to 0.68 log CFU/mL) when contact time was 10 min at concentrations varying from 500 to 2000 µg/mL. This higher inhibition efficiency due to higher deacetylation degrees of chitosan solutions was also observed for different contact times in this study, which agrees with Liu et al. (2001) who reported that the antibacterial activities of chitosan against *E. coli* increased when the DD increasing from 74 to 96%. Similar increases in antibacterial activities with increased DD were also reported by Hongpattarakere and Riyaphan (2008).

The antibacterial effects of chitosan with different DD concentrations and contact time for *S.* Typhi and *S. aureus* are shown in Tables 5 and 6. The inhibition effects of chitosan against *S.* Typhi and *S. aureus* increased significantly as the concentrations and contact time increased ($P < 0.05$) and these results were similar to *E. coli* in the previous experiment. However, antibacterial activity of the same DD concentrations and contact time was higher for *S. aureus* and *S.* Typhi than for *E. coli.* For example, a 1000 µg/mL chitosan solution with 95% DD

Table 7. Effect of chitosan dissolved in different organic acid on the antibacterial activity to *S. aureus, E. coli and S.* typhi

		Control		Acetic acid + lactic acid + chitosan	Acetic acid + propionic acid + chitosan	SEM
		Acetic acid＋lactic acid	Acetic acid＋propionic acid			
Part		**Reduced log CFU/ cm^2**				
Breast skin	*S. aureus*	0.64^{b}	0.58^{b}	2.73^{a}	2.74^{a}	0.12
	E. coli	0.57^{c}	0.61^{c}	2.84^{a}	2.63^{b}	0.15
	S. typhi	0.72^{c}	0.65^{c}	2.71^{a}	2.58^{b}	0.18
Thigh skin	*S. aureus*	0.59^{b}	0.67^{b}	2.56^{a}	2.46^{a}	0.18
	E. coli	0.66^{b}	0.79^{b}	2.85^{a}	2.31^{b}	0.16
	S. typhi	0.71^{b}	0.65^{b}	2.43^{a}	2.54^{a}	0.21

$^{a\text{-}c}$Different superscripts at the same row indicate significantly different ($P < 0.05$)

and a contact time of 60 min utilized against *E. coli*, *S.* Typhi and *S. aureus* reduced bacterial counts by 3.54, 3.71 and 4.18 log CFU/mL, respectively. In summary, the data in this study demonstrate that better antibacterial activity was achieved against *S. aureus*, regardless of DD concentration and contact time. Zheng and Zhu (2003) showed that chitosan (305 kDa molecular weight) had a 99% inhibition rate against *S. aureus* at a concentration of 0.25% and a 100% inhibition rate when the concentration increased to 0.5%. In this study, antibacterial efficiency was more profound with increases in chitosan contact time compared with increased concentrations of chitosan. Moreover, for the same concentrations and contact times, chitosan with higher DD resulted in higher antibacterial efficiency against *S.* typhi and *S. aureus.*

Antibacterial efficiency of sanitizers with chitosan and organic acids at pH 3

Four sanitizers, including: acetic acid+lactic acid (2:1), acetic acid+propionic acid (2:1), acetic acid + lactic acid (2:1) + chitosan 1000 µg/mL and acetic acid + propionic acid (2:1) + chitosan 1000 µg/mL was separately prepared. Broiler carcasses were individually inoculated with selected bacteria (*S. aureus, E. coli* and *S.* Typhi) and then, the 4 sanitizers were applied by spraying on the broiler carcass surfaces (breast and thigh). The bacterial inhibition for *S. aureus, E. coli* and *S.* Typhi when the sanitizers were sprayed individually are shown in Table 7. The results showed that sanitizers formulated with 1000 µg/mL chitosan and organic acids (acetic acid + lactic acid or acetic acid + propionic acid) significantly inhibited the growth of *S. aureus, E. coli* and *S.* Typhi on breast and thigh surfaces of broiler carcasses when compared with sanitizers formulated only with organic acids. However, the sanitizer with the best inhibition efficiency for *S. aureus, E. coli* and *S.* Typhi was formulated with 1000 µg/mL chitosan and organic acid (acetic acid + lactic acid). The reduced counts for *S. aureus, E. coli* and *S.* Typhi were 2.73, 2.84 and 2.71 log CFU/cm^2, respectively, on the breast surface and 2.56, 2.85 and 2.43 log CFU/cm^2, respectively, on the thigh surface. It was determined that the bacterial inhibition efficiency was the same for all parts of the broiler carcasses examined in this study. Many reviews have also indicated that chitosan with acids has better antibacterial activity in foods. For example, chitosan (0.6%) mixed with a low concentration of sulfide (170 ppm) significantly inhibited growth of lactic acid bacteria and yeast, as determined by total plate count (Roller et al., 2002). Coma et al. (2003) reported that the addition of chitosan to cheese did not significantly affect the product's components. Kanatt et al. (2008) reported that chitosan added to ground lamb and salami sausage can significantly increase shelf-life when stored at 0-3°C. Fruits with high commercial value can be corrupted when fruit frostbite, water loss and microbial contamination occur due to storage at low temperatures.

Some reports have shown that juiced fruit, mango, strawberry, orange and longan whose surfaces were covered with chitosan had significantly increased storage time and reduced drip loss (Chien et al., 2007; Jiang and Li, 2001; Pilar et al., 2008). Moreover, the chitosan layer can effectively inhibit bacterial contamination of the fruit. In this study, a sanitizer solution formulated with chitosan and organic acid at pH 3 effectively controlled and reduced the bacterial counts for *S. aureus, E. coli* and *S.* Typhi on the

surface of broiler carcasses.

Conclusion

All food chemicals were considered to improve the microbial quality of food according to cost, safety and antibacterial ability. Although phosphoric acid was cheaper, all the organic acids in this study showed better bacterial inhibition capabilities than the inorganic acid, regardless of whether the acids were single or complex. The most effective acids for solutions formulated with chitosan were found in the acid complexes (2:1), such as acetic acid + lactic acid and acetic acid + propionic acid and these acid complexes were utilized to treat the breast and thigh surfaces by spraying and to determine the greatest sanitizer formulation. The solution consisting of 1000 µg/mL chitosan and an acid complex with acetic acid + lactic acid with ratio at 2:1 and pH 3 was the paramount optimal according to the antibacterial results shown in Table 7. Organic acid and chitosan are not only very safe and have good sterilization ability, but the pH of the solution (pH 3) was also shown to have similar antibacterial abilities when compared with 2% organic acids in this study. Therefore, this new formulation of organic acid and chitosan can be recommended as a sanitizer for use in the poultry slaughtering system.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Acuff GR, Vanderzant C, Savell JW, Jones DK, Griffin DB, Ehlers JG (1987). Effect of acid decontamination of beef subprimal cuts on the microbiological and sensory characteristics of steaks. Meat Science 19(3):217-226.

Awwad NS, El-Nadi YA, Hamed MM (2013). Successive processes for purification and extraction of phosphoric acid produced by wet process. Chemical Engineering and Processing Process Intensification 74:69-74.

Ba HV, Seo HW, Seong PN, Kim YS, Park BY, Moon SS, Kang SJ, Choi YM, Kim JH (2018). The effects of pre-and post-slaughter spray application with organic acids on microbial population reductions on beef carcasses. Meat Science 137:16-23.

Bracket RE, Hao YY, Doyle MP (1994). Ineffectiveness of hot acid sprays to decontaminate *E. coli* O157:H7 on beef. Journal of Food Protection 57(3):198-203.

Brenneman KE, Willingham C, Kong W, Curtiss R, Roland KL (2013) low-pH rescue of acid-sensitive *Salmonella enterica* serovar typhi strain by a rhamnose-regulated arginine decarboxylase system. Journal of Bacteriology 195(13):3062-3072.

Buchanan RL, Golden MH (1994). Interaction of citric acid concentration and pH on the kinetics of *Listeria monocytogenes* inactivation. Journal of Food Protection 57(7):567-570.

Carpenter CE, Broadbent JR (2009). External concentration of organic acid anions and pH: key independent variables for studying how organic acids inhibit growth of bacteria in mildly acidic foods. Journal of Food Science 74(1):R12-15.

Chaiyakosa S, Charernjiratragul W, Umsakul K, Vuddhakul V (2007). Comparing the efficiency of chitosan with chlorine for reducing *Vibrio parahaemolyticus* in shrimp. Food Control 18(9):1031-1035.

Chauret CP (2014). Sanitization. Encyclopedia of Food Microbiology (2ed) pp. 360-364.

Chien PJ, Sheu F, Lin HR (2007). Coating citrus (*Murcott tangor*) fruit with low molecular weight chitosan increases postharvest quality and shelf life. Food Chemistry 100(3)*:*1160-1164.

Chung YC, Wang HL, Chen YM, Li SL (2003). Effect of abiotic factors on the antibacterial activity of chitosan against waterborne pathogens. Bioresource Technology 88(3):179-184.

Coma V, Deschamps A, Martial-Gros A (2003). Bioactive packaging materials from edible chitosan polymer antimicrobial activity assessment on dairy related contaminants. Journal of Food Science 68(9):2788-2792.

Dan SM, Mihaiu M, Rotaru O, Dalea I (2007). Microbial changes on the surface of pork carcasses due lactic and acetic acids decontamination. USAMV-CN 64(1-2):403-408.

Dorsa WJ, Siragusa GR, Cutter CN, Berry ED, Koohmaraie M (1997). Efficacy of using a sponge sampling method to recover low levels of *Escherichia coli* O157:H7*, Salmonella typhimurium,* and aerobic bacteria from beef carcass surface tissue. Food Microbiology 14(1):63-69.

Dubal ZB, Paturkar AM, Waskar VS, Zende RJ, Latha C, Rawool DB, Kadam MM (2004). Effect of grade organic acids on inoculated *S. aureus, L. monocytogenes, E. coli and S. typhimurium* in sheep/goat meat stored at refrigeration temperature. Meat Science 66(4):817-821.

FDA (2003). Code of federal regulations title 21. Government Printing Office, USA. https://www.fda.gov/medical-devices/medical-device-databases/code-federal-regulations-title-21-food-and-drugs

Garbutt J (1997). Essentials of food microbiology. Hodder Headline Group, London UK pp. 135-174.

Gonzalez-Garcia RA, McCubbin T, Navone L, Stowers C, Nielsen LK, Marcellin E (2017). Microbial propionic acid production. Fermentation 3(2):21.

Haque MN, Chowdhury R, Islam KMS, Akbar MA (2009). Propionic acid is an alternative to antibiotics in poultry diet. Bangladesh Journal of Animal Science 38(1-2):115-122.

Harris D, Brashears MM, Garmyn AJ, Brooks JC, Miller MF (2012). Microbiological and organoleptic characteristics of beef trim and ground beef treated with acetic acid, lactic acid, acidified sodium chlorite, or sterile water in simulated commercial processing environment to reduce *Escherichia coli* O157:H7 and *Salmonella*. Meat Science 90(3):783-788.

Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. International Journal of Food Microbiology 71(2-3):235-244.

Hong KN, Park NY, Lee SH, Meyers SP (2002). Antibacterial activity of chitosan and chitosan oligomers with different molecular weights. International Journal of Food Microbiology 74(1-2):65-72.

Hongpattarakere T, Riyaphan O (2008). Effect of deacetylation conditions on antimicrobial activity of chitosans prepared from carapace of black tiger shrimp (*Penaeus monodon*). *Songklanakarin* Journal of Science and Technology 30:1-9.

Jiang Y, Li Y (2001). Effect of chitosan coating on postharvest life and quality of longan fruit. Food Chemistry 73(2):139-143.

Kandil AT, Mira HI, Taha MH, Kamel MF (2017). Production of pure phosphoric acid from El-Sebaeya low–grade phosphate ore. Separation Science and Technology 52(4):679-690.

Kahya N (2019). Water soluble chitosan derivatives and their biological activities: a review. Polymer Sciences 5(3):1-11.

Kanatt SR, Chander R, Arun S (2008). Chitosan glucose complex – A novel food preservative. Food Chemistry 106(2):521-528.

Laury AM, Alvarado MV, Nace G, Alvarado CZ, Brook JC, Echeverry A, Brashears MM (2009). Validation of a lactic acid- and citric acid-based antimicrobial product for chicken carcass. Journal of Food Protection 72(10):2208-2211.

Lipnizki F (2010). Basic Aspects and Applications of Membrane Processes in Agro-Food and Bulk Biotech Industries. Comprehensive Membrane Science and Engineering pp. 165-194. DOI: 10.1016/B978-0-08-093250-7.00035-9

Liu H, Du YM, Wang XH, Sun LP (2004). Chitosan kills bacteria through cell membrane damage. International Journal of Food Microbiology

95(2):147-155.
Liu X, Yun L, Dong Z, Zhi L, Kang D (2001). Antibacterial action of chitosan and carboxymethylated chitosan. Journal of Applied Polymer Science 79(7):1324-1335.
Lucera A, Costa C, Conte A, Del Nobile MA (2012). Food applications of natural antimicrobial compounds. Frontiers in Microbiology 3(287):1-13.
Mani-López E, García HS, López-Malo A (2012). Organic acids as antimicrobials to control Salmonella in meat and poultry products. Food Research International 45:713-721.
Nguyen DH, Seok WJ, Kim IH (2020). Organic acids mixtures as a dietary additive for pigs-a review. Animals 10(952):1-12.
Pilar HM, Eva A, Valeria DV, Dinoraz V, Rafael G (2008). Effect of chitosan coating combined with postharvest calcium treatment on strawberry (*Fragaria ananassa*) quality during refrigerated storage. Food Chemistry 110:428-435.
Raftari M, Jalilian FA, Abdulamir AS, Son R, Sekawi Z, Fatimah AB (2009). Effect of organic acids on *Escherichia coli* O157:H7 and *Staphylococcus aureus* contaminated meat. The Open Microbiology Journal 3:121-127.
Roller S, Sagooa S, Boarda R, O'Mahonyb T, Capliceb E, Fitzgeraldb G, Fogdenc M, Owenc M, Fletcherc H (2002). Novel combinations of chitosan, carnocin and sulphite for the preservation of chilled pork sausages. Meat Science 62:165-177.
Sagoo S, Board R, Roller S (2002). Chitosan inhibits growth of spoilage micro-organisms in chilled pork products. Food Microbiology 19:175-182.
Sallam KI, Abd-Elghany SM, Hussein MA, Imre K, Morar A, Morshdy AE, Sayed-Ahmed MZ (2020). Microbial decontamination of beef carcass surfaces by lactic acid, acetic acid, and trisodium phosphate sprays. BioMed Research International 2324358:1-11.
Skřivanová E, Marounek M (2007). Influence of pH on antimicrobial activity of organic acids against rabbit enteropathogenic strain of Escherichia coli. Folia Microbiology 52(1):70-72.
Smulders FJM, Greer GG (1998). Integrating microbial decontamination with organic acids in HACCP programs for muscle foods: prospects and controversies. International Journal of Food Microbiology 44(3):149-169.
Sohaib M, Anjum FM, Arshad MS, Rahman UU (2016). Postharvest intervention technologies for safety enhancement of meat and meat based on products; a critical review. Journal of Food Science and Technology 53(1):19-30.
Stivarius MR, Pohlman FW, McElyea KS, Waldroup AL (2002). Effects of hot water and lactic acid treatment of beef trimmings prior to grinding on microbial, instrumental color and sensory properties of ground beef during display. Meat Science 60(4):327-334.
Sudarshan NR, Hoover DG, Knorr D (1992). Antibacterial action of chitosan. Food Biotechnology 6(3):257-272.
Tepe B, Daferera D, Sokmen M, Polissiou M, Sokmen A (2004). In vitro antimicrobial and antioxidant activities of the essential oils and various extracts of *Thymus eigii* M. Zohary et PH Davis. Journal of agricultural and food chemistry 52(5):1132-1137.
Wang GH (1992). Inhibition and inactivation of five species of foodborne pathogens by chitosan. Journal of Food Protection 55(11):916-919.
Wang L, Liu F, Jiang Y, Chai Z, Li P, Cheng Y, Jing H, Leng X (2011). Synergistic antimicrobial activities of natural essential oils with chitosan films. Journal of agricultural and food chemistry, 59(23):12411-12419.
Wee YJ, Kim JN, Ryu HW (2006). Biotechnological production of lactic acid and its recent applications. Food Technology and Biotechnology 44(2):1-11.
Wu XY, Zeng QX, Mo SF, Ruan Z (2006). Antibacterial activities of chitosan with different degree of deacetylation and molecular masses. Journal of South China University Technology 34:58-62.
Xiong X, Hirata M, Takanashi H, Lee MG, Hano T (1998). Analysis of acclimation behavior against nitrification inhibitors in activated sludge processes. Journal of Fermentation and Bioengineering 86(2):207-214.
Yang ZP, Li YB, Slavik M (1998). Use of antimicrobial spray applied with an inside-outside birdwasher to reduce bacterial contamination on prechilled chicken carcasses. Journal of Food Protection 61(7):829-832.
Yu G, Huang GH, Zhang XD, Li Y (2010). Inhibitory effects of organic acids on bacteria growth during food waste composting. Compost Science & Utilization 18(1):55-63.
Zheng LY, Zhu JF (2003). Study on antimicrobial activity of chitosan with different molecular weights. Carbohydrate Polymers 54(4):527-530.

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