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ARTICLE

Antibiotic use in commercial broiler chicken farming and its consequential resistance development in root colonizing bacteria of carrot grown in manure-applied soils in a middle-income country

Warshi S. Dandeniya, Erandi M. Herath, Ayesh M. Lowe, Mathaniga Kasinthar, Rasika N. Jinadasa, Janak K. Vidanarachchi, and Thusith S. Samarakone

Abstract: Broiler chicken litter (BCL) is a cheap manure for vegetable crops in developing countries. Extensive antibiotic use in poultry production could increase antibiotic resistant bacteria (ARB) in manure and eventually in crop root microbiome. We investigated the prevalence of ARB in BCL from medium- and large-scale farms ($n = 33$) and in carrot (*Daucus carota*) grown in BCL-applied soils in Sri Lanka. All the BCL samples contained aerobic bacteria resistant to 10 $\mu g \cdot mL^{-1}$ of oxytetracycline or enrofloxacin. The abundance of ARB determined by viable plate-count method ranged from 0.05% to 30.10% of aerobic bacterial population. Soil from two fields applied with BLC for 3 yr (short history, SH) and 10 yr (long history, LH) were treated with BCL (10%, *w*/*w*) and oxytetracycline (10 and 100 $mg \cdot kg^{-1}$) in a pot experiment alongside an unamended control. Adding BCL and oxytetracycline had a significant ($P < 0.05$) effect on the abundance of oxytetracycline-resistant epiphytic and endophytic bacteria (EEB) in carrot roots at harvest. Both total and oxytetracycline-resistant EEB increased significantly ($P < 0.05$) with the application of BCL to LH soil but not to SH soil. Carrot sold at retailed markets ($n = 30$) contained epiphytic bacteria resistant to 1 $\mu g \cdot mL^{-1}$ oxytetracycline (4.13 ± 0.207 $\log_{10} CFU \cdot g^{-1}$ dry carrot). High minimum inhibitory concentrations for oxytetracycline (≥ 128 $\mu g \cdot mL^{-1}$) were observed in 83% and 50% of ARB isolates obtained from BCL ($n = 18$) and carrot ($n = 24$), respectively. Results confirmed that BCL acts as a carrier of ARB, and continuous application of BCL to soil increased the prevalence of ARB among EEB in carrot.

Key words: antibiotic resistance, broiler chicken litter, carrot, oxytetracycline, root colonizing bacteria.

Résumé : Dans les pays en développement, le fumier de poulet à griller (FPG) est un amendement peu coûteux pour les cultures maraîchères. Cependant, le recours abondant aux antibiotiques pour augmenter la production de volaille pourrait accroître la population de bactéries antibiorésistantes (BAB) dans le fumier et éventuellement dans le microbiome des racines. Les auteurs ont étudié la prévalence des BAB dans le FPG venant de moyens à gros élevages ($n = 33$) ainsi que dans les carottes (*Daucus carota*) cultivées dans des sols du Sri Lanka bonifiés avec du FPG. Tous les échantillons de FPG renfermaient des bactéries aérobies qui résistaient à 10 $\mu g \cdot mL^{-1}$ d'oxytétracycline ou d'enrofloxacine. La proportion des BAB, établie par numération des colonies viables sur plaque de gélose, variait de 0,05 % à to 30,10 % de la population des bactéries aérobies. Dans le cadre d'une expérience en pot, les auteurs ont traité le sol de deux champs amendés avec du FPG pendant trois ans (traitement court, TC) ou dix ans (traitement long, TL) avec un mélange de FPG (10 %, *p*/*p*) et d'oxytétracycline (10 ou 100 $mg \cdot kg^{-1}$). L'expérience comprenait un témoin non traité. L'addition de FPG et d'oxytétracycline a eu un effet significatif ($P < 0,05$) sur l'abondance des bactéries épiphytes et endophytes (BEE) résistant à l'oxytétracycline dans les racines de la carotte, à la récolte. La population totale de BEE et de BEE résistantes à l'oxytétracycline augmente significativement ($P < 0,05$) avec l'application de FPG au sol TC, mais pas au sol TL. Les carottes vendues sur le

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marché au détail ($n = 30$) renfermaient des bactéries épiphytes qui résistaient à 1 $\mu g \cdot mL^{-1}$ d'oxytétracycline (4,13 ± 0,207 log10 $CFU \cdot g^{-1}$ de carotte déshydratée). Les auteurs ont relevé une concentration inhibitoire minimale élevée pour l'oxytétracycline (≥ 128 $\mu g \cdot mL^{-1}$) dans respectivement 83 % et 50 % des BAB isolées dans le FPG ($n = 18$) et la carotte ($n = 24$). Ces résultats confirment que le FPG véhicule des BAB et qu'une application continue au sol augmente la prévalence des BAB chez les BEE, dans la carotte. [Traduit par la Rédaction]

Mots-clés : résistance aux antibiotiques, fumier de poulet à griller, carotte, oxytétracycline, bactéries colonisant les racines.

Introduction

The extensive use of antibiotics in human healthcare and animal husbandry increases the evolution and spread of antibiotic resistance (AR) in microbial communities (O'Neill 2016). Antibiotic residues and antibiotic resistant bacteria (ARB) are frequently detected in manures from farms that use antibiotics (Martinez 2009; Marti et al. 2013; Tasho and Cho 2016). Therefore, crops grown in soils amended with animal manure could be contaminated with ARB, and antibiotic residues could be taken up by the crops, both endangering the quality and safety of food (Marti et al. 2013; Tasho and Cho 2016; Pu et al. 2019; Zhang et al. 2019). Acquiring AR by human pathogens limits therapeutic options, often leading to extended hospital stays and the use of costly alternatives (O'Neill 2016; Boovaragamoorthy et al. 2019).

When AR genes and antibiotic residues enter an environment, their fate and their impact on the evolution of resistance traits are determined by the diversity and background resistance levels of existing microbial communities (Dantas et al. 2008; Nesme and Simonet 2015; Pu et al. 2019). For example, in soil, heterotrophic bacteria in orders Burkholderiales, Pseudomonadales, and Actinomycetales may utilize some antibiotics as carbon sources, lowering the available antibiotic concentration that contributes to the evolution of AR (Dantas et al. 2008; Oz et al. 2014; Udikovic-Kolic et al. 2014). Increasing the frequency of exposure to antibiotics may build up the selection strength, accelerating the AR development (Fang et al. 2014). Thus, the repeated application of animal manures containing antibiotic residues and AR genes may alter the soil resistome (the pool of AR genes in soil microbiome) (Forsberg et al. 2014; Nesme and Simonet 2015). In addition, the application of animal manure to soil could facilitate the propagation and prevalence of AR genes by shifting microbial community composition and increasing the chances of horizontal gene transfer (HGT) (Fosberg et al. 2014; Zhang et al. 2019). Previous studies indicate that fresh vegetables cultivated on animal manure applied soils could be contaminated with ARB (Marti et al. 2013; Zhang et al. 2019). Lack of empirical evidence on AR spread hinders policy-level interventions to encourage the responsible use of antibiotics, particularly in low- and middle-income countries (Sivagami et al. 2020).

Managing farm waste is a major challenge for broiler chicken producers in Sri Lanka. Being a lower-middle-income country, the waste disposal regulations are relatively less strict in Sri Lanka compared with developed countries. The Nuisances Ordinance No.15 of 1862 (as amended in 1939 and 1946) provides the only regulations related to disposal of farm waste in the country (Lawnet 2016). There are no specific regulations requiring pretreatment of farm waste before applying to soil as manure in Sri Lanka. The bedding material (broiler chicken litter (BCL)) disposed after each production cycle (at 36–42 d) constitutes the bulk waste, which is sold as a manure directly to intensive vegetable growers, particularly in the Central Province of Sri Lanka (Herath et al. 2015). Nearly all broiler chicken producers rely on antibiotics heavily for disease prevention and control in Sri Lanka. Understanding the effect of antibiotic usage in commercial broiler chicken farming on AR spread to soil environment through BCL application and ultimately to crops could help to devise strategies for better managing antibiotic usage in poultry husbandry. We hypothesized that BCL could be a carrier of AR to agricultural fields, and high frequency of applying BCL as a manure to soil increases the prevalence of ARB in the roots of crops.

Oxytetracycline and enrofloxacin are among the most popular antibiotics used by the broiler chicken producers in Sri Lanka (Herath et al. 2015; Lowe et al. 2019). Therefore, these two antibiotics were considered for the present study. The aims of this study were to investigate the prevalence of oxytetracycline- and enrofloxacin-resistant bacteria in BCL obtained from commercial broiler chicken farms and to determine the effect of historical BCL application to soil on the prevalence of oxytetracycline-resistant bacteria in root environment of carrot. Further, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for oxytetracycline were assessed for selected bacterial isolates recovered from the BCL, soil, and carrot roots.

Materials and Methods

Description of poultry farms studied

Samples were collected from 33 commercial broiler chicken farms in Sri Lanka from September to November 2015 to study the prevalence of ARB in BCL. The farms were selected randomly from a list of farms ($n = 51$) used for information surveys on previous antibiotic use (Herath et al. 2015; Lowe et al. 2019). Considering the nature of management and scale of operation, the 33 farms were categorized into three

groups; closed-house farms and buy-back farms linked to large-scale companies and medium-scale farms operating independently (Supplementary Table S1[1], which details characteristics of different broiler chicken rearing systems in Sri Lanka considered for the present study). The farms used commercial broiler strains (Hubbard Classic, Cobb 500, or Indian River) originating from imported grandparent/parent stocks. In all these farms, using antibiotics for growth promotion and disease prevention was a common practice. Three popular commercial broiler feed brands had been used in all farms. The labels provided by feed manufacturer indicated that feed contains antibiotics as growth promoters, but the name of the antibiotic was mentioned only in some labels. It was a common practice to administer antibiotics to birds in drinking water during the first 18–24 d of age as a disease-preventive measure.

Broiler chicken litter sampling

The age of birds ranged from 35 to 40 d at the time of sampling. A representative composite sample per farm was obtained systematically. When there were more than one poultry-house per farm, the sample was collected from the house where the brooder was located. Approximately 100 g of BCL was collected and composited in a bucket at every 2 feet distance while walking in a transect diagonally in the poultry house and mixed well. A subsample of 150 g obtained from the mixed bulk sample was used for the analyses. All BCL samples were immediately transferred to the Department of Soil Science, University of Peradeniya, Sri Lanka, and stored at 4 °C until analyses, which were performed within 1 week. Electrical conductivity (EC) of each sample was analyzed using an electrical conductivity meter (Martini Mi306®) after suspending the BCL in distilled water at 1:10 ratio. The pH of each sample was determined using an electronic pH meter (EUTECH pH510®) after suspending the BCL in distilled water at 1:5 ratio. The gravimetric moisture content in each sample was determined by oven dry method. All these analyses were performed on two replicates per sample.

Enumeration of bacteria in BCL

Two threshold concentrations (1 $\mu g \cdot mL^{-1}$ and 10 $\mu g \cdot mL^{-1}$) of oxytetracycline and enrofloxacin were used to screen bacteria resistant to each antibiotic. These levels were established based on a preliminary investigation (Herath et al. 2016) and considering inhibitory concentrations reported in the literature (Sarmah et al. 2006; Panzenhagen et al. 2016; Trouchon and Lefebver 2016).

Each BCL sample was mixed well, and two subsamples were used for the enumeration of bacteria by spread plating and colony counting on selective media with a 10-fold serial dilution using 1% NaCl (You et al. 2012). Tryptic soy agar (TSA) (3 $g \cdot L^{-1}$ tryptic soy broth (TSB) and 15 $g \cdot L^{-1}$ agar from Hardy Diagnostics®) was used to grow total culturable bacteria. TSA spiked with 10 $\mu g \cdot mL^{-1}$ oxytetracycline or enrofloxacin (HPLC grade from Sigma Aldrich®) separately was used to grow bacteria resistant to respective antibiotics (You et al. 2012). Plates were incubated at 28 °C under aerobic conditions, and the total number of bacterial colony-forming units (CFUs) was observed 48 h after inoculation. Selected ARB colonies were subcultured for isolation using TSA medium at 28 °C.

Pot experiment using carrot grown in soils with different histories of manure application

A controlled experiment was conducted in November, 2015 with oxytetracycline as the target antibiotic and carrot as the test crop to assess the response of root colonizing bacteria in two soils with different historical BCL exposure to new inputs of antibiotic and BCL (which may contain antibiotic residues and ARB). Soil samples (Typic Paleudults) were collected at 0–20 cm depth from two adjacent fields, which had been intensively cultivated for over 20 yr in the Nuwara Eliya district in Central province, Sri Lanka (6.91307°N 80.75301°E, elevation: 1699 m above mean sea level, WU3 agro-ecological zone). The two fields varied with respect to the history of BCL application (Supplementary Table S2[1], which details land-use history and soil characteristics of two fields with different history of broiler chicken litter (BCL) application (3 years (SH) and 10 years (LH)) selected for collecting soil samples for pot experiment). Broiler chicken litter had been applied as manure during the past 3 consecutive yr (short history, SH), while the other field had BCL applied for the past 10 yr (long history, LH). Soil samples were transported to a greenhouse facility at the University of Peradeniya, Sri Lanka. Soils were analyzed for basic soil properties using standard protocol (Supplementary Table S2[1]). Microbiological analyses were performed on field moist soil, and air-dried sieved (2 mm sieve) soil was used for physical and chemical properties and preparation of potting mixture. The soils were mixed with sterilized sand (2–4 mm) at 1:1 (*v*/*v*) ratio in plastic trays and moistened to reach 40% (*w*/*w*) gravimetric water content. One week later, each potting mixture was divided into four subsamples. These three subsamples were treated once with 4.5 $g \cdot kg^{-1}$ (equivalent to 10 $tons \cdot ha^{-1}$) BCL, 10 $mg \cdot kg^{-1}$ oxytetracycline (OTC10), and 100 $mg \cdot kg^{-1}$ oxytetracycline (OTC100) separately before filling the potting mixture to pots. The remaining untreated potting mixture served as the control. Pots were prepared from each treatment as 250 g of potting mxture per pot in four replicates. Samples of BCL were analyzed for the abundance of ARB, availability of nutrients, pH, and EC

[1]Supplementary data are available with the article at https://doi.org/10.1139/cjss-2021-0001.

using previously described standard protocols (Herath et al. 2016). Pots were fertilized with nutrient solution according to the recommendations of the Department of Agriculture, Sri Lanka, for carrot (N-180 kg·ha^{-1}, P-90 kg·ha^{-1}, and K-120 kg·ha^{-1}). Fertilizers were applied equalizing nutrient levels among treatments considering the nutrient availability in potting mixture and nutrient inputs from amendments. Pots were irrigated using bottom-up watering method, and after 4 d, surface-sterilized carrot seeds were sowed in pots. One week later, seedlings were thinned to retain four healthy plants per pot and maintained for 3 mo. Pots were arranged in a Completely Randomized Design (CRD). At the end of the experiment, carrot plants were uprooted for the enumeration of EEB.

Survey of ARB in carrot sold at retail markets

To discuss the results of carrot root colonization (in the pot experiment) by ARB in context, we analyzed the abundance of ARB in carrot samples collected from retail markets. Carrot samples (each weighing approximately 250 g) were collected from 30 vegetable sales points in Kandy, the major municipality in the Central Province, Sri Lanka, including supermarkets, small boutiques, and roadside vendors. The sampling locations were distributed in an area of nearly 5 km^2, which had approximately 100 permanent sales points of vegetables. Sampling from the market was performed over a period of 2 weeks in September 2015 to ensure representation of different batches of vegetables brought into the market and considering resource availability in the laboratory for timely processing of samples. The samples were stored at 4 °C, and epiphytic bacteria were enumerated within 24 h from sampling.

Enumeration of epiphytic and endophytic bacteria

Carrot leaves were discarded, and the roots were rinsed with sterilized distilled water to remove lightly attached soil particles. Then 5 g of fresh carrot root was added to a conical flask containing 25ml of sterilized phosphate buffered saline (PBS) at pH 7.4 and shaken at 150 rpm for 1 h to extract epiphytic bacteria (Yrjala et al. 2010). The same root samples that were used to isolate epiphytes were then used for isolating endophytes, according to a previously described method (Surette et al. 2003). Briefly, carrot roots were surface disinfected by immersion in commercial bleach (5.25% available chlorine) for 3 min and then in 3% peroxide solution for 3 min. Disinfected samples were then rinsed three times with sterilized distilled water and finally with sterilized rinsing solution, which contained a surface-tension depressant, polyoxyethylene sorbitan monolaurate (Tween 20) at 0.005% (*v*/*v*). The success of surface disinfection was confirmed by plating water from the final rinse on Petri plates containing 0.3% TSA. Successfully surface-disinfected carrot roots were weighed, crushed, and homogenized in 25 ml of three strength (3×) Ringer's solution (215 mg NaCl, 7.5 mg KCl, 12 mg $CaCl_2$, 50 mg $Na_2S_2O_3$ in 100 mL distilled water) at pH 6.6 and shaken for 1 h at 150 rpm to extract endophytes. Extraction of epiphytes and endophytes was performed in two replicates per sample.

Epiphyte and endophyte extracts were serially diluted with PBS and plated on TSA spiked with 0, 1, and 10 μg·mL^{-1} oxytetracycline. Plates were incubated at 28 °C under aerobic conditions, and CFUs were counted 24 h after inoculation. Morphologically distinct CFUs of oxytetracycline-resistant EEB were isolated for further characterization. Based on the results from the pot experiment, epiphytic bacteria resistant to 1 μg·mL^{-1} oxytetracycline were responsive to BCL addition. Hence, only the epiphytic bacteria resistant to 1 μg·mL^{-1} oxytetracycline were enumerated from carrot roots sampled from market in triplicates following the procedure described earlier (Yrjala et al. 2010).

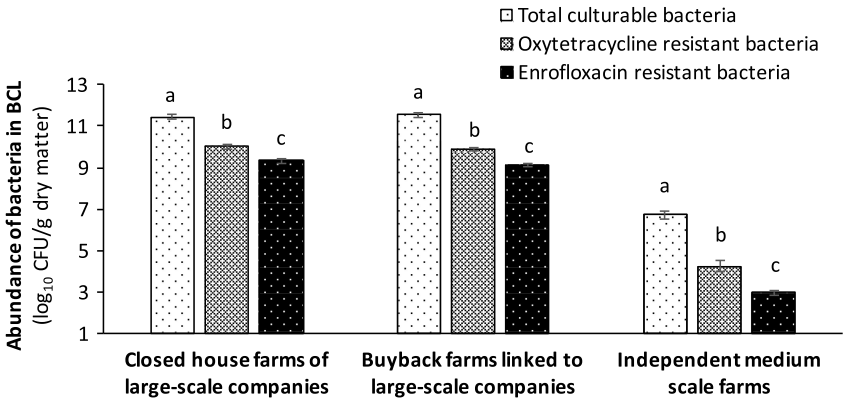
Characterizing MIC and MBC for oxytetracycline

Along with bacteria isolated from BCL ($n = 18$) and carrot roots [epiphytic bacteria ($n = 20$) and endophytic bacteria ($n = 4$)] from the current study, a few selected bacterial isolates obtained from BCL piled at agricultural fields ($n = 12$) and BCL-treated soils ($n = 12$) from Kandy District in a previous study (Herath et al. 2016) were included for comparison purpose. Gram staining was performed on all isolates, and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oxytetracycline for each isolate were determined as described previously (Andrews 2001). An *Escherichia coli* clinical isolate obtained from the Microbiology Laboratory at the Faculty of Veterinary Medicine & Animal Science, University of Peradeniya with known resistance to oxytetracycline was used as the positive control. A dilution series of oxytetracycline was prepared in 0.1% TSB to include concentrations 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 750, and 1024 μg·mL^{-1}. Each bacterial isolate was grown in in 0.3% TSB for 15–18 h to achieve 10^5 bacteria·mL^{-1} density, and this culture was used to inoculate the dilution series of oxytetracycline in triplicate. Cultures were incubated at 28 °C for 24 h. The lowest concentration of oxytetracycline that inhibits the visible growth of bacteria was recorded as MIC. The dilution levels that did not result in visible growth for each isolate were plated on TSA plates, and the oxytetracycline concentration where colony counts became zero was recorded as MBC.

Identification of selected bacterial isolates

Twelve isolates were randomly selected from the 66 bacterial isolates for identification. Each isolate was cultured in 0.3% TSB at 28 °C for 24 h and centrifuged at 10 000 rpm for 1 min. Then supernatant was removed, and pellet was frozen at −20 °C for 24 h. DNA was extracted using the Promega Wizard SV genomic

Fig. 1. Mean abundance of total culturable, oxytetracycline-resistant (10 μg·mL^{-1}), and enrofloxacin-resistant (10 μg·mL^{-1}) aerobic bacteria populations (enumerated on 0.3% agar medium) in broiler chicken litter (BCL) collected from closed-house farms (n = 3) and buy-back farms (n = 22) associated with large-scale companies and independent medium-scale farms (n = 8). Error bars represent standard error. Means followed by same letter in a given farm category are not significantly different ($P > 0.05$).



DNA purification system® following manufacturer's instructions. The 16S rRNA gene was amplified using an Applied Biosystem veriti® 96-well thermal cycler. Universal primers specific for bacterial 16S rRNA gene (27F forward primer: 5′-AGAGTTTGATCCTGGTTC-3′ and 1492R reverse primer: 5′-GGTTACCTTGTTACGACTT-3′) were used with a primer-specific thermal program (Rahmani et al. 2006). PCR products were sequenced using Genetic Analyzer 3500 series® sequencer at the Faculty of Science, University of Peradeniya, Sri Lanka. Each Flowgram was manually checked, and 50–700 bp region was used for sequence alignment using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) using NCBI GenBank database.

Statistical analysis

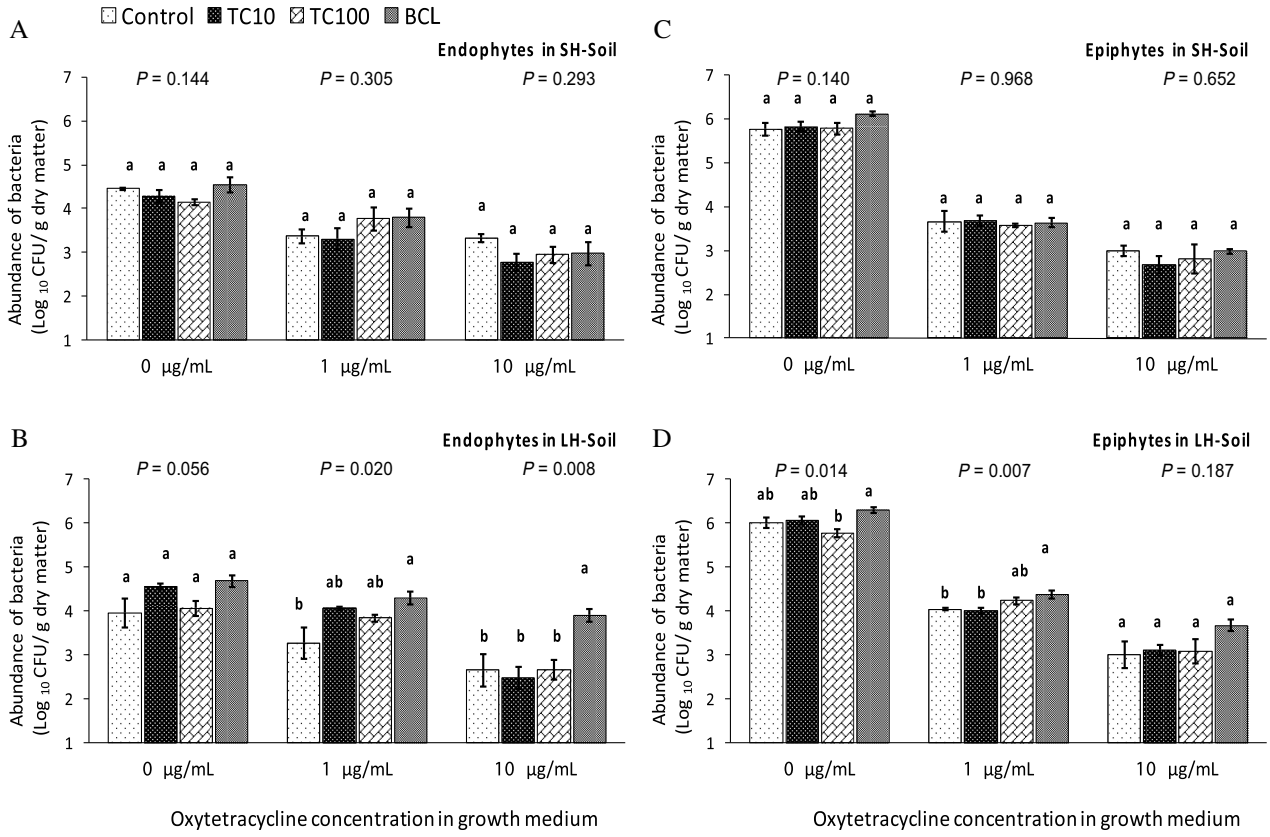
Statistical analyses were performed using MINITAB (version 16) software. All bacterial abundance data were log transformed and expressed as $\log_{10}$CFU·g^{-1} prior to statistical analysis. Data generated on bacterial abundance and chemical properties of BCL were first tested for normality using Anderson Darling Test and homogeneity using Bartlett's test (at P value of 0.05). Assessing the impact of management practices on prevalence of ARB in BCL is beyond the scope of the study, and the experiment design does not warrant such an analysis. Thus, the prevalence of ARB in BCL from the three farm categories was not compared statistically. Instead, total aerobic bacteria and ARB populations within each farm category were compared using the paired sample t test at P value of 0.05. Pearson's correlation analysis was performed to analyze correlations between measured microbiological and chemical characteristics of BCL from buy-back farms and medium-scale farms separately and to analyze the relationship between MIC and MBC of bacteria isolates. Data generated from the pot experiment were tested for normality (Anderson Darling Test at P value of 0.05) and used in analysis of variance (ANOVA) in three-factor, fixed-effect design with generalized linear model (GLM) procedure to determine the significance of the effect of manure application history and the treatments on the abundance of epiphytic and endophytic bacteria populations in carrot. The history of BCL application to soil (SH and LH), antibiotic input treatment (control, BCL, OTC10, and OTC100), and bacteria type (epiphytic bacteria and endophytic bacteria) were used as the grouping factors. Considering the significance of interaction effects, one-way ANOVA was performed to assess the effect of antibiotic input treatment for a bacteria type in SH and LH separately. Tukey's mean comparison was performed at a P value set at 0.05. Descriptive statistics are presented in the text as mean ± standard deviation.

Results

All the BCL samples contained aerobic bacteria resistant to 10 μg·mL^{-1} oxytetracycline or enrofloxacin (Fig. 1). Aerobic bacterial populations resistant to oxytetracycline in BCL from all three farm categories were significantly larger ($P < 0.001$) than those resistant to enrofloxacin (Fig. 1). The prevalence of oxytetracycline-resistant bacteria (at 10 μg·mL^{-1}) in BCL from farms operating under large-scale companies (closed-house and buy-back) ranged from 0.25 to 30.10% (average, 5.14%) of the aerobic bacterial population, whereas the prevalence in medium-scale farms ranged from 0.05% to 3.60% (average, 1.28%).

Broiler chicken litter from closed houses, buy-back farms, and independent medium-scale farms had high EC levels (2.78 ± 0.935, 2.90 ± 1.037 dS·m^{-1}, and 3.11 ± 0.366 dS·m^{-1}, respectively) and alkaline pH (8.57 ± 0.371, 8.62 ± 0.308 and 9.11 ± 0.105, respectively). Significant positive correlations ($P < 0.05$) were observed between pH and the abundance of oxytetracycline- and enrofloxacin-resistant bacteria populations

Fig. 2. The mean abundance of total and oxytetracycline-resistant (1 μg·mL^{-1} and 10 μg·mL^{-1}) endophytic bacteria (A and B) and epiphytic bacteria (C and D) from the root environment of carrot grown in two soils with different histories of BCL application (3 yr (SH) and 10 yr (LH)) and treated with no amendments (control), oxytetracycline at 10 mg·kg^{-1} (OTC10), and 100 mg·kg^{-1} (OTC100) rates and BCL at 4.5 g·kg^{-1} (BCL). Error bars represent standard error (n = 4). P value indicates the calculated probability for the significance of treatment effect on bacteria abundance at each oxytetracycline concentration in growth medium. Means followed by same letter for a given oxytetracycline concentration in growth medium are not significantly different ($P > 0.05$).



in BCL from buy-back farms (Supplementary Table S3[1], which details correlation coefficient (R) for relationships between measured parameters of broiler chicken litter analyzed separately for samples collected form buy-back farms operated under large-scale companies and the medium-scale farms that operated independently). In this category, the abundance of total culturable bacteria and oxytetracycline-resistant bacteria had a significant ($P < 0.05$) positive correlation with moisture content of BCL (Supplementary Table S3[1]). Interestingly, the abundance of two ARB populations did not have a significant relationship ($P > 0.05$) with the abundance of total culturable bacteria in BCL from buy-back and medium-scale farms (Supplementary Table S3[1]).

From among the root colonizing bacteria isolated from carrot grown in the pot experiment, the abundance of total culturable epiphytic bacterial population was significantly higher ($P < 0.001$) than the total culturable endophytic bacterial population (Fig. 2 and Supplementary Table S4[1], which details summary statistics from analysis of variance (ANOVA) factorial analysis of total culturable and oxytetracycline resistant bacteria populations in carrot roots considering the history of manure application to soil (soil type), antibiotic input treatment (treatment) and bacteria type as the main grouping factors). The prevalence of endophytic bacteria resistant to oxytetracycline at 1 μg·mL^{-1} and 10 μg·mL^{-1} concentrations was 26.9 ± 17.17% and 8.3 ± 10.72%, respectively, whereas the prevalence of epiphytic bacterial counts was 1.1 ± 0.87% and 0.2 ± 1.89% at the same antibiotic concentrations. Therefore, the relative abundance of ARB compared with total bacterial population was significantly higher ($P < 0.05$) among the endophytic population than the epiphytic population (Fig. 2). The input of BCL to the soil with an LH of manure application significantly increased the abundance of oxytetracycline-resistant bacteria among epiphytic and endophytic bacterial populations compared with the soil with an SH of exposure to manure (SH) (Fig. 2). In BCL used for the pot experiment, total aerobic bacteria, those resistant to oxytetracycline at 1 μg·mL^{-1} and at 10 μg·mL^{-1} concentrations were 8.24 ± 0.054, 6.19 ± 0.035, and 5.39 ± 0.028 $\log_{10}$ CFU·g^{-1} dry wt., respectively.

Fig. 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oxytetracycline for 66 bacteria isolates originated from endorhizosphere of carrot (ENC), rhizoplane of carrot (RC), broiler chicken litter (BCL), BCL collected from piles in crop fields (BCL_{field}), and BCL-amended soils from intensively vegetable cultivated fields (MS). Oxytetracycline supplemented (0 to 1024 $\mu g \cdot mL^{-1}$) 0.1% TSB was used to assess MIC. The dilution levels that did not result in a visible growth were plated on 0.3% TSA medium, and the antibiotic concentration where colony counts become zero was recorded as MBC.

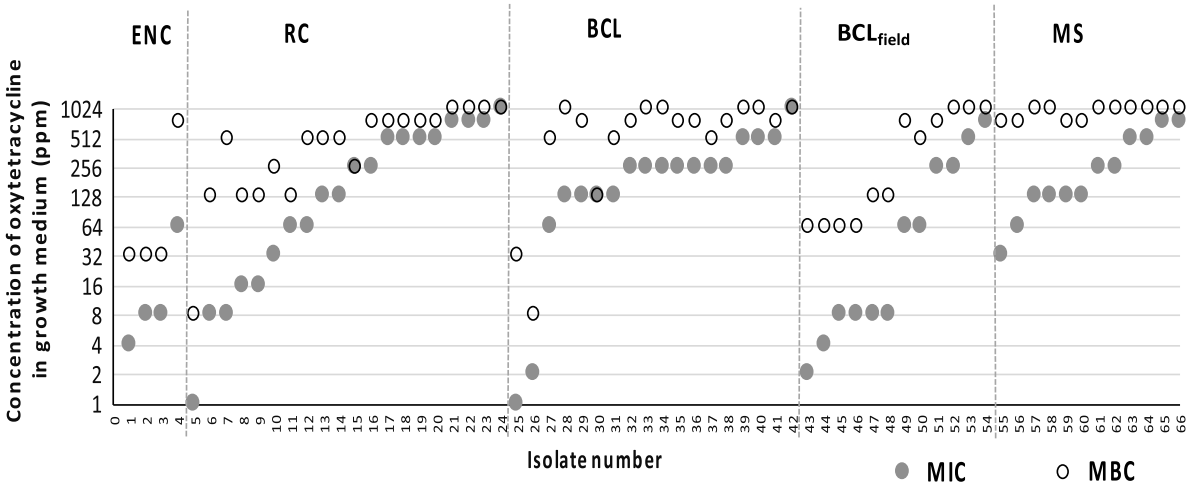


Table 1. The identity of bacterial isolates obtained from different environmental samples based on 16S rRNA gene sequence similarity.

Isolate No.[a]	Isolated environment[b]	Bacterial species	Identity	NCBI GenBank accession No.
1	ENC	*Bacillus* spp.	99%	MZ126956
4	ENC	*Stenotrophomonas* spp.	99%	MZ126962
8	RC	*Stenotrophomonas maltophilia*	99%	MZ126958
15	RC	*Bacillus* spp.	99%	MZ126957
28	BCL	*Bacillus* spp.	99%	MZ126959
31	BCL	*Bacillus* spp.	100%	MZ127653
49	BCL_{field}	*Stenotrophomonas* spp.	92%	MZ126961
57	MS	*Bacillus* spp.	99%	MZ126960
61	MS	*Bacillus* spp.	99%	MZ126963
63	MS	*Serratia marcescens*	97%	MZ126953
65	MS	*Bacillus* spp.	100%	MZ126954
66	MS	*Staphylococcus saprophyticus*	97%	MZ126955

[a]Isolate number corresponds to isolate number in Fig. 3.
[b]ENC, endorhizosphere of carrot; RC, rhizoplane of carrot; BCL, broiler chicken litter; BCL_{field}, BCL collected from piles in crop fields; MS, BCL amended soils from intensively vegetable cultivated fields.

The average abundance of total epiphytic bacteria and bacteria resistant to 1 $\mu g \cdot mL^{-1}$ oxytetracycline in carrot sampled from retail markets in Kandy municipality was 6.61 ± 0.122 and 4.13 ± 0.207 $\log_{10}$ CFU$\cdot g^{-1}$ dry carrot, respectively. Accordingly, the prevalence of epiphytic bacterial population resistant to 1 $\mu g \cdot mL^{-1}$ oxytetracycline ranged from 0.1% to 0.6% of the total epiphytic bacterial population. These values are within the range of respective observations (0.1%–3.1%) made on epiphytic bacterial populations in the pot experiment.

Figure 3 presents MIC and MBC of oxytetracycline observed for bacterial isolates studied. The MIC and MBC values significantly correlated ($r = 0.70$, $P < 0.001$). From the 66 isolates used for characterization, 60% were Gram-negative bacteria, which included all endophytic bacteria isolated from carrot roots. The 12 bacterial isolates that we randomly selected for identification were represented by five different genera with *Bacillus* being the most common but with different MIC and MBC levels (Table 1 and Fig. 3).

Discussion

Antibiotics are routinely used in many countries including Sri Lanka for commercial broiler chicken production (Sarmah et al. 2006). While therapeutic doses are administered orally (most common practice) or intramuscularly, subtherapeutic doses are incorporated into feed (<200 mg·kg^{-1} of feed) for growth enhancement and improving feed conversion efficiency (Chopra and Roberts 2001; Sarmah et al. 2006). The repeated exposure of bacteria to high concentration of antibiotics supports the evolution of AR (Fang et al. 2014; Oz et al. 2014). Hence, despite its beneficial effects, the administration of antibiotics to poultry affects microbial diversity and contributes to AR development among the commensal bacteria in BCL (Boovaragamoorthy et al. (2019).

Antibiotic resistant bacteria in broiler chicken litter

In the present study, we used 10 μg·mL^{-1} as the critical concentration to enumerate oxytetracycline- and enrofloxacin-resistant bacteria from BCL. According to Sarmah et al. (2006), the concentration of oxytetracycline that inhibited the growth of bacteria isolated from soil and sewage sludge by more than 50% ranged from 0.12 to 10 μg·mL^{-1}. The MIC of enrofloxacin for common pathogenic bacteria ranged from 0.02 to 64 μg·mL^{-1} (Panzenhagen et al. 2016; Trouchon and Lefebver 2016). Therefore, the critical concentration of 10 μg·mL^{-1} of oxytetracycline and enrofloxacin should facilitate the screening of respective ARB populations from BCL. The total number of microorganisms in BCL could exceed 10^{10} cells·g^{-1} (Bolan et al. 2010; Brooks et al. 2016). According to a study conducted using qPCR technique targeting 16S rRNA gene and tetracycline resistance gene *tetA*, the prevalence of oxytetracycline-resistant bacterial populations in BCL ranged from 0.001% to 0.01% (Brooks et al. 2016). The same study reported that nearly 80% of the bacteria isolates obtained from BCL were resistant to at least one of the antibiotics tested. In soil, the prevalence of oxytetracycline-resistant bacterial population has been reported as less than 0.7% from the total bacterial population (viable counts or as genomic units) (Marti et al. 2013; Herath et al. 2016). The prevalence of ARB observed in the majority of samples in our study is much higher than these values reported in literature. This could be partly due to methodological differences in molecular-based and culture-based approaches as previously reported (Brooks et al. 2016). We observed that only the medium-scale farms operated independently of large-scale companies used oxytetracycline during the study period (Supplementary Table S1[1]). Closed-house and buy-back farms of large-scale companies discontinued the use of tetracycline nearly 1 yr prior to sampling as it was no longer effective in prevention and control of bacterial infections in their production systems (Lowe et al. 2019). In BCL from these farms, ARB represented a relatively high proportion of the total bacterial community, indicating a build-up of AR in the environment of broiler chicken houses.

We observed that despite the similarities in housing and some management practices between buy-back and medium-scale farms (Supplementary Table S1[1]), the abundance of total aerobic bacteria and ARB populations tested were lower in BCL from medium-scale farms than those in buy-back farms (Fig. 1). The differences with respect to climatic conditions, density of birds in broiler chicken houses, BCL management practices (single use vs. multiple use), quality and quantity of feed, frequency and the types of antibiotics administered, cleaning protocols of poultry houses in-between two flocks, and the number of production cycles (flocks) per year lead to differences in bacterial abundance and the prevalence (loading) of ARB in BCL (Bolan et al. 2010; Brooks et al. 2016). Keeping BCL dry and reducing pH and nutrient availability would help to reduce proliferation of bacteria (Bolan et al. 2010). Brooks et al. (2010) observed that the bacterial population densities increased in BCL and aerosols as the flock of birds progressed from pre-flock to late flock. Therefore, removal of litter followed by a disinfection process is important to reduce ARB from building up in poultry houses. However, supplying materials and managing workload for the frequent replacement of litter and the disposal of BCL waste are a significant financial overhead for the farmers. As a result, bedding material is replaced only partially during some production cycles. Further, a complete floor to ceiling disinfection of poultry houses after removal of one flock of birds was not practiced in any of the studied farms. Moreover, the removed BCL is either collected in bags or directly heaped unsheltered until being sold or burnt. Therefore, through aerosols and the activities of insects and small animals, ARB could spread in the farm environment (Graham et al. 2009; Zurek and Ghosh 2014).

Pretreatment of animal manures through heat treatments, thermophilic composting, or other stabilization processes prior to land application could lower the levels of ARB and antibiotic residues (Marti et al. 2013; Herath et al. 2015; Khadra et al. 2019; Staley et al. 2021). Heating beef manure on concrete slabs significantly reduced ARB and AR genes in manure (Staley et al. 2021) while thermophilic composting caused tetracycline degradation (Khadra et al. 2019). In addition, the adsorption of antibiotics by small organic molecules such as fulvic acids in compost and soil reduces the mobility of the antibiotics in the environment (Chen et al. 2017; Cycoń et al. 2019). The success of composting as a method to eliminate ARB and antibiotic residues varies widely depending on the type of antibiotics and composting conditions (Khadra et al. 2019; Pu et al. 2019). Composting of BCL is less commonly practiced in Sri Lanka due to many limitations in policies and processes related to waste handling (Dandeniya and Caucci 2020). Vegetable growers in Sri Lanka generally

heap BCL in unsheltered piles in cropping fields where it is allowed to cure for approximately 1 mo before use (Herath et al. 2016). There is a high possibility for antibiotic residues and ARB in BCL to spread in the environment with leaching and runoff water passing through unsheltered manure piles (Martinez 2009; Joy et al. 2013; Liyanage and Pathmalal 2017).

The effect of historical BCL application on endophytic and epiphytic bacteria in carrot

In the present study, we observed ARB in carrot roots originating from the pot experiment as well as from the retail markets. Vegetables and potato contaminated with *Clostridium botulinum*, *Salmonella* spp., *Bacillus cereus*, *Campylobacter jejuni*, *Escherichia coli,* and *Clostridium perfringens* have been associated with a number of outbreaks and illnesses from 2000 to 2007 in the United States (Erickson 2010). Therefore, the consumption of bacterial-contaminated raw vegetables originating from manure amended soils represents a route of human exposure to AR (Center for Disease Control and Prevention 1994; Erickson 2010; Marti et al. 2013; Zhang et al. 2019).

Antibiotic residues that enter into soil undergo processes such as sorption, degradation, and transformation as explained in the review by Cycoń et al. (2019). Thus, soil could act as a filter to incoming antibiotic residues and AR genes limiting the spread of AR along the food chain (Dantas et al. 2008; Nesme and Simonet 2015; Cycoń et al. 2019). However, the nature of anthropogenic disturbances may influence the degree of change in soil microbial community structures and ultimately the level of AR spread in the soil environment (Forsberg et al. 2014; Udikovic-Kolic et al. 2014; Nesme and Simonet 2015). We hypothesized that soils receiving repeated applications of BCL over a long period of time will have a larger ARB population more responsive to inputs of oxytetracycline or BCL compared with the ARB population in a soil exposed to fewer BCL application events. We further predicted that the two soils would behave differently in regulating the spread of AR to the microbiome of the food crops grown therein. Although soil physical and chemical properties and the population size of total and oxytetracycline EEB populations of carrot were not different between LH and SH soils (Supplementary Table S2[1] and Fig. 2), the ARB populations were responsive to the new input of BCL only in the LH soil (Fig. 2), suggesting the importance of history of manure application on the functionality of the plant microbiome. Therefore, results only partially support our hypothesis because BCL application history in the studied system did not affect the total abundance of ARB, while it changed the responsiveness of ARB population to new inputs of BCL. Zhang et al. (2019) applied composted poultry manure to a soil that had no known history of organic fertilizer application for the preceding 5 yr, and they observed a significant increase in AR genes in the plant microbiome of lettuce grown in composted-manure-treated soil. The repeated application of BCL may have affected compositional and functional characteristics of the soil microbiome creating a "soil memory," which leads to changes in responsiveness of root colonizing bacteria to the input of BCL. Recent reviews on the concept of "soil memory" indicate the importance of anthropogenic disturbances such as agronomic practices and historical interactions between plants and microorganisms on driving the functional and compositional characteristics of soil microbial communities that are important for maintaining soil functions (Lapsansky et al. 2016; Grzadziel 2017).

Antimicrobial resistance traits of bacteria isolates

The MIC and MBC values can be used to infer the acquired AR in bacteria (EFSA 2012). If a bacterial strain acquired resistance, it may demonstrate higher levels of resistance than other strains of the same taxonomic unit (EFSA 2012). Microbiological cutoff values of tetracycline concentration to define resistance for *Bacillus* spp., *Enterococcus* spp., and most of the Gram-positive bacteria are 8, 4, and 2 $\mu g \cdot mL^{-1}$, respectively (EFSA 2012). The majority of isolates from the current study were of *Bacillus* spp. and had MIC values exceeding these cutoff values (Table 1 and Fig. 3). Acquiring resistance through HGT is not common in soil environments compared with clinical settings (Forsberg et al. 2014; van Goethem et al. 2018). However, frequent inputs of antibiotics and ARB through animal manures could strengthen the selection pressure and increase the abundance of AR genes, thus facilitating HGT and accelerating AR evolution (Udikovic-Kolic et al. 2014; Nesme and Simonet 2015; Boovaragamoorthy et al. 2019). The genera *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Enterococcus,* and *Staphylococcus* contain potential human pathogens, and some members can easily acquire AR due to high transmissibility of plasmids among strains (Sievert et al. 2002; Miller et al. 2002; Erickson 2010; Brookes 2012).

It is important to phase-out nontherapeutic use of antibiotics to reduce the spread of AR in the environment and to decrease the upregulation of virulence factors of pathogenic bacteria (Verbrugghe et al. 2016; Sivagami et al. 2020). The government of Sri Lanka has taken actions to increase awareness among different stakeholder groups on risks associated with AR development and to promote the responsible use of antibiotics in recent years. Accordingly, the use of growth-promoter antibiotics in poultry feed has been banned since 2018 (NSP 2017). Our findings will support the development of regulations and policy framework for responsible use of antibiotics in low- and middle-income countries.

Conclusion

The study confirmed that BCLs originating from commercial-scale farms are carriers of ARB to soils. Our findings showed that endophytic ARB in carrot grown

in the soil with an LH of BCL application proliferated with new inputs of BCL, while a similar trend was not observed in the soil with an SH of BCL application. Thus, the history of BCL application influences the response of root colonizing ARB in carrot to new inputs of BCL. Therefore, it is important to take measures to reduce the introduction of antibiotic residues and ARB to agricultural soils to limit the spread of AR within the soil food web. We advocate regulation of antibiotic use in broiler chicken production and encourage the development of regulations requiring pretreatment of BCL prior to being released to the environment and used in vegetable cultivation.

Conflicts of Interest

We declare that there is no conflict of interest.

Author Contributions

- Study concept and design: Dandeniya
- Analysis and interpretation of data: Dandeniya, Herath, Jinadasa, Lowe, Kasinthar,
- Drafting of the manuscript: Dandeniya, Herath, Jinadasa, Lowe
- Critical revision of the manuscript for important intellectual content: Dandeniya, Herath, Jinadasa, Lowe, Samarakone, Vidanarachchi
- Statistical analysis: Dandeniya, Herath
- Obtained funding: Dandeniya
- Study supervision: Dandeniya, Jinadasa, Samarakone, Vidanarachchi

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