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Profiles of antibiotic resistome and microbial community in groundwater of CKDu prevalence zones in Sri Lanka

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ABSTRACT

The chronic kidney disease of unknown etiology (CKDu) prevalent in certain regions of Sri Lanka poses a serious threat to human health. Previous epidemiological studies focused on the search of causative agents for CKDu etiology from the viewpoint of groundwater composition, but how CKDu prevalence affected the groundwater microbial composition, especially the antibiotic resistome, has never been illuminated. This study investigated the response of microbial community and antibiotic resistome to CKDu prevalence in the groundwater through the high throughput sequencing and qPCR (HT-qPCR), respectively. Results showed that CKDu prevalence significantly influenced the distribution of antibiotic resistome and microbial community composition. The *mexF* dominated in all the groundwater samples and could be considered as an intrinsic ARG, and the β-lactamase *cphA* was specially enriched and closely associated with the antibiotics used for CKDu patients. The *Acinetobacter* was a potential human pathogen common in the groundwater of CKDu affected regions, while CKDu prevalence specially enriched the *Aeromonas*. Statistical analysis indicated that CKDu prevalence impacted antibiotic resistome through the microbial community as a whole, and MGEs contributed to the occurrence of *mexF*, while the enrichment of *cphA* could be attributed to the increase of *Aeromonas*.

1. Introduction

In recent years, the inappropriate use of antibiotics worldwide has not only resulted in the environmental pollution but also increased inevitably the development and subsequent dissemination of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) (Zhao et al., 2019). In 2017, antibiotic resistance was listed as an emerging issue of the frontier environmental concern by United Nations Environment Programme (UNEP, Frontiers, 2017), and it was also estimated that annual ARGs related global deaths would increase from about 700 000 to 10 million by 2050 where effective antibiotics no longer existed (Roope et al., 2019).

Meanwhile, an increasingly serious global health concern was emerging as the chronic kidney disease of unknown etiology (CKDu) in agricultural communities of Sri Lanka, Central America, areas of India, and Egypt (Jayasinghe and Zhu, 2020), which presented as a kidney disease in patients who did not exhibit common causative factors, such as diabetes or hypertension (Cooray et al., 2019a). The CKDu was generally prevalent among agricultural workers, and an increasing number of Sri Lankans suffered from CKDu since the 1990s, e.g., more than 50,000 patients diagnosed with late-stage kidney disease (Makehelwala et al., 2020). CKDu patients of Sri Lanka included both men and women in between 17–70 years old, and the majority of them belonged to men aged 30–60, who were in their most productive era of the life (World Health Organization, 2013). Therefore, CKDu has become a major environmental health issue that affected both social and economic

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aspects of the people, even with the availability of free public health services to the entire nation (Jayatilake et al., 2013).

Although the CKDu became prominent and its causes were not yet precisely known, most of the external factors that triggered the disease were likely associated with the drinking water (Makehelwala et al., 2019). Therefore, the World Health Organization (WHO) recommended the provision of safe water to CKDu-affected zones, and the Government of Sri Lanka was committed to achieve the UN sustainable development goals by 2030, in which safe water and sanitation would be at high priorities (Cooray et al., 2019b). Nonetheless, to date, most of the CKDu-affected zones were not covered by the national grid of water-supply in Sri Lanka, and most of the CKDu affected areas were located in the dry zone regions of the country where surface water sources are limited (Cooray et al., 2019a). Therefore, groundwater has become the most important drinking-water source in the CKDu affected areas of Sri Lanka.

Nonetheless, antibiotic resistance can be transferred from the environmental compartments (e.g., sewage, animal faeces, soils) to drinking water sources posing a serious threat to human health, e.g., prolonged morbidity and increased mortality (Sanganyado and Gwenzi, 2019). Accordingly, antibiotic resistance has been reported in drinking water sources, including groundwater and surface water systems (Garner et al., 2019). However, the occurrence of antibiotic resistome in the groundwater of CKDu affected areas in Sri Lanka has not been investigated to date. How the prevalence of CKDu affected the antibiotic resistome in the groundwater required urgent attention, because antibiotics including beta-lactams, vancomycin etc., were widely used for the disease treatment, and antibiotic resistome found in drinking water might directly influence the treatment. Besides, the human activities on CKDu treatment was detrimental to environment which could be reflected by the presence of antibiotic resistome in groundwater. The role of CKDu prevalence on antibiotic resistome required to be examined in detail for successful prevention of the disease from Sri Lankan.

Most of the studies aiming to elucidate external factors for CKDu prevalence mainly focused on the chemical quality of groundwater such as pesticides, heavy metals, hardness, fluoride, micronutrient and dissolved organic matter (DOM) for disease etiology with inconclusive discussions (Cooray et al., 2019a; Makehelwala et al., 2020; Cooray et al., 2019b; Ranasinghe and Ranasinghe, 2015; Wickramarathna et al., 2017). However, the potent role of the microbial community for the CKDu etiology was not systematically pursed to date. It was vital to examine the potential human pathogens in groundwater of CKDu affected areas in Sri Lanka in the search of elucidating factors for disease etiology.

The occurrence of antibiotic resistome has been investigated worldwide focusing on drinking water treatment plants (Ma et al., 2019; Liu et al., 2018); however analogous data related to groundwater resources were limited. Sri Lanka is a developing country with a Buddhist culture with limited livestock industry particularly in rural sector where CKDu was dominant. Therefore, it provided an ideal situation to assess the background data of antibiotic resistome in groundwater affected by the human activities. Besides, the mechanisms of the distribution of antibiotic resistome in groundwater need a detailed investigation. Horizontal gene transfer, pertinent microbial community, heavy metals and external environmental factors contribute to ARGs distribution (Allen et al., 2010; Berendonk et al., 2015). The underlying mechanism of the CKDu prevalence on the antibiotic resistome need further investigation.

The groundwater samples collected from high, moderate, and non-prevalence areas of CKDu in Sri Lanka were examined through high-throughput quantitative PCR (HT-qPCR) and high throughput sequencing method to determine 1) the distribution patterns of antibiotic resistome and microbial community; 2) possible correlations between the CKDu prevalence with antibiotic resistance and microbial community, 3) the biomarkers from the ARGs and microbes present in the CKDu affected zones; 4) the potential mechanisms of the distribution of antibiotic resistome focusing on mobile genetic elements, microbial community, environmental factors, human pathogens and co-selection from heavy metals, which contributed to better understanding the occurrence of CKDu from the perspective of its mutual interactions with the consumption of drinking water.

2. Materials and methods

2.1. Sampling

The groundwater samples were collected from the divisional secretariat divisions (DSD) of Anuradhapura district (endemic CKDu area), Monaragala district (none-endemic CKDu area) and Kandy district (No CKDu area) according to the CKDu epidemiologic data of Ministry of Health, Sri Lanka at May 2017 (dry season), and the sampling was finished in one week. The DSDs of Wellawaya, Thanamalwila, Buttala and Monaragala were selected as the non-endemic CKDu prevalence divisional secretariats in Monaragala district according to 2017 epidemiologic data. Anuradhapura district has the highest number of CKDu patients, and DSDs of Kebithigollawa and Kahatagasdigiliya were selected for high CKDu prevalence divisional secretariats, Mihintale and Talawa were selected for moderate CKDu prevalence divisional secretariats, while Galnewa, Palagale, and Kahatagasdigiliya were selected for non-endemic CKDu prevalence divisional secretariats. For comparison, six samples were collected from Kandy district (No CKDu area; control area). There were 64 samples in total, and the detailed information of sampling coordinates is shown in Fig. S1 and Table S1.

Communities in all selected areas were relying on groundwater for drinking requirements, which was extracted from dug wells (maximum depth 15 m) and tube wells (maximum depth 60 m) of variable depth depending on the region and underlying geological conditions. Thus, all sample points were classified as shallow (5–10 m deep dug wells) or medium deep (20–30 m deep hand pump tube wells) wells used for groundwater extractions. In some areas, natural springs were treated as the drinking water sources, thus, two samples were also collected from natural springs. 3 L of water samples were collected using the sterile glass bottles from each location, and then transported to the laboratory within 24 h in cold condition using ice boxes, where 0.5 L of water samples were vacuum-filtered (0.22-μm filters) and the filters were stored under -20℃ before the DNA extraction. Triplicate filters were collected from each sample.

2.2. DNA extraction

The filters were cut into pieces for DNA extraction using the FAST DNA Spin Kit for Soil (MP Biomedicals, USA). Extracted genomic DNA was detected and quantified using 1% agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific, USA), respectively, and then stored at −20 °C before use. The three resulting extracts from the same sample were composited to get a representative DNA sample for further analysis.

2.3. Microbial community analysis

The microbial communities were evaluated for all the samples using the primers (515F: GTGYCAGCMGCCGCGGTAA/806R: GGACTACNVGGGTWTCTAAT) targeting the bacterial and archaeal microbes of 16S rRNA genes. Paired-end library was constructed using NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA) according to the product manual. Adapters containing the full complement of sequencing primer hybridization sites were ligated to the blunt-end of fragments. Sequencing was conducted at the Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) sequencing center through pair-end Illumina sequencing (Illumina Miseq PE × 300, USA). The raw pair-end reads were firstly merged (PEAR: -x, 0.1) and assigned to each sample according to the unique barcode (Zhang et al., 2014); the merged reads were quality controlled (PRINSEQ) and chimers filtered (USEARCH) to get the clean sequences (Schmieder and Edwards, 2011; Edgar et al., 2011); the clean sequences were normalized at the depth of 30 073 and then submitted to

the NCBI Sequence Read Archive (SRA) under the project number of PRJNA555790. The taxonomic classification was carried out using the Ribosomal Database Project (RDP) Classifier at the cutoff of 50 % with the taxon below 0.01 % being remove, and the diversity indexes were calculated by the RDP associated modules (Cole et al., 2014).

2.4. High-throughput quantitative PCR (HT-qPCR)

Only 14 samples covering different CKDu levels were further selected for the HT-qPCR analysis including 6 samples in high CKDu zone, 5 samples in mild CKDu zone, 2 samples in moderate CKDu zone and 1 sample in non-prevalent zone. The HT-qPCR array had 296 primer sets covering 6 human pathogens, 10 MRGs (heavy metal resistance genes), 28 MGEs (mobile genetic elements), 251 ARGs and 16S rRNA gene, which was determined based on previous studies (Stedtfeld et al., 2018; Zhu et al., 2013). The classification and resistance mechanisms were resorted through the Comprehensive Antibiotic Resistance Database (CARD; http://arpcard.mcmaster.ca). The detail information of the primers, target gene annotation was provided in Table S2. The HT-qPCR analysis was conducted in triplicate for each sample using the thermal cycling conditions on the Wafergen SmartChip Real-time PCR system (Wafergen Biosystems, USA), which had 5184 nano-wells for the qPCR reactions. The PCR mixtures (100 nL for each well) consisted of 1 × TB Green Premix Ex Taq (TaKaRa, Japan), 1 mg/mL bovine serum albumin, 250 nM of each primer and a DNA template of 2 ng/μL. The PCR mixtures were dispensed into the nano-wells using the SmartChip Multisample Nanodispenser (MSND) followed by real-time qPCR using SmartChip Cycler. A non-template control was included on each chip for each primer set. The thermal cycling of the qPCR amplification is as follows: (1) 95 °C, 10 min; (2) 95 °C, 30 s; (3) 60 °C, 30 s; (4) repeat (2) through (3) 39 more cycles; (5) melt-curve analysis automatically conducted by Wafergen software: 60 °C–97 °C, 0.4 °C/read. The wells with multiple melting peak were discarded, and a threshold cycle (C_T) of 31 was used as the detection limit. Only well data with single effective melting peak and the amplification efficiencies within 1.7–2.3 for three replicates were regarded as positive and retained for further analysis. The comparative C_T method was used to calculate the relative abundances of target genes compared to the 16S rRNA gene using the equations below, where C_T is the threshold cycle of target genes in the 296 primers.

$$\text{Gene copies} = 10^{((31-C_T)/(10/3))}$$

$$\text{Relative abundance} = \textit{Target gene copies}/16s\ \textit{rRNA gene copies}$$

2.5. Data analysis

Principal component analysis (PCA) and redundancy analysis (RDA) were performed using Canoco 5.0. The Kruskal-Wallis H-test with the Benjamini-Hochberg FDR correction was adopted to determine the OTUs with significant difference between groups ($p < 0.05$) using STAMP 2.1.3, and the identification of environment-associated biomarker ARGs and microbes was performed through the LEfSe analysis, an approach that calculated effect sizes using a linear discriminant analysis from relative abundances (Segata et al., 2011). Network analysis based on the spearman correlation ($p < 0.05$) was constructed through the Gephi platform to figure out the factors influencing specific ARGs (Bastian et al., 2009). The structural equation model (SEM) based on the correlation matrix were conducted through AMOS (SPSS Inc., Chicago, IL, USA) as previously described (Zhang et al., 2019a).

3. Results and discussion

3.1. Microbial community composition in groundwater

In agreement with previous studies (Liu et al., 2018; Hao et al., 2019; Xi et al., 2009), the dominant phylum in the groundwater samples of Sri Lanka was Proteobacteria, which accounted for 69.7 ± 17.9 %, followed by Bacteroidetes (5.8 ± 8.1 %) and Actinobacteria (5.0 ± 5.7 %) (Fig. 1A). The high abundance of unclassified species (7.4 ± 9.0 %) also indicated many new species in Sri Lankan groundwater that need further investigation. The abundance of Bacteroidetes in some samples like HAka3, MAT5 could reach up to 40.2 % and 46.2 %, respectively (Fig. S2), and the abundance of Actinobacteria in the LMW3 reached 31.3 %, while the presence of Cyanobacteria/Chloroplast in the HAka2, LAG3, LAP4 at 25.4 %, 13.0 % and 33.2 %, respectively. The abundant Cyanobacteria warranted the further investigations to detect algal toxins, which could contribute to the CKDu level and might provide some clues for the search of causative agents for CKDu etiology. The phylum like Firmicutes and Acidobacteria required significant proportions of nutrients for their growth and could not adapt to the oligotrophic micro-environments (Turnbaugh et al., 2006). Nevertheless, the higher diversity and adaptation of Proteobacteria contributed to its dominance in the groundwater (Lagier et al., 2016).

The dominant genus varied significantly between samples, and most of them belonged to the *Acinetobacter*, *Pseudomonas* and *Aeromonas* with the relative abundance of 12.9 ± 16.7 %, 10.4 ± 18.3 % and 3.2 ± 8.9 %, respectively (Fig. 1B). Unfortunately, the three genus are potential human pathogens, and they have been cultured and demonstrated worldwide in the drinking water systems; however all of them contained several non-pathogenic strains as well (Chen et al., 2016). When compared to other DSDs, it was also noted that the abundance of *Enterobacter* was higher in Anuradhapura Mihintale, which indicated faeces pollution of groundwater. The *Acinetobacter* was found in a wide range of ecological niches (Fournier et al., 2006). The medically relevant species belonged to the *Acinetobacter baumannii* complex, and they were designated as a "red alert" human pathogen, due to its extensive antibiotic resistance spectrum (Fournier et al., 2006). *A. baumannii* is an emerging opportunistic bacterial pathogen primarily associated with hospital-acquired infections, and the distinct ability to survive under desiccation and prolong endurance to the environment makes it persistent and dangerous pathogen. In addition, since several members in the genus *Acinetobacter* are pre-eminent as emerging multi-drug resistant bacteria, their presence in water may allow transfer genes to other bacteria making water a useful reservoir for pathogens.

The functions of the *Pseudomonas* was diverse, i.e., it promoted denitrification and micro-pollutants degradation, showed antibiotics & human pathogens resistance, and tolerated heavy metals toxicity (Colliver and Stephenson, 2000; Iyer et al., 2012; Mermod et al., 2010; Inglis et al., 2016). The *Pseudomonas* played a dominant role in the formation of biofilms that improved water quality (Douterelo et al., 2018; Lv et al., 2014). The *Pseudomonas* contained a clinically important human pathogen *P. aeruginosa*, which could cause a variety of opportunistic infections ranging from eye infections in contact lens wearers, burn and wound infections leading to septic shock and lung infections in cystic fibrosis patients (Bouskill et al., 2007).

The genus *Aeromonas* is Gram negative, rod-shaped and facultative anaerobes, which contain two main groups, the first is the non-motile psychrophilic aeromonads with optimal growth temperatures of 22–28 □ represented by *Aeromonas salmonicida*, and the second much larger group contains the mesophilic motile aeromonads that have optimal growth temperatures of 30–37 ℃ (Sayers et al., 2018). In humans, the mesophilic *Aeromonas* were mainly linked to gastroenteritis and wound infections with more than 85 % of human clinical cases. Generally, the *Aeromonas* infections were not an important public health problem so epidemiology was not very well known, and the main targets of *Aeromonas* were fish, which were exposed to these natural pathogens (Sayers et al., 2018).

The dominant non-human potential pathogens were *Novosphingobium*, *Acidovorax* and *Comamonas*. Both *Novosphingobium* and *Comamonas* were strictly aerobic and chemo-organotrophic, which also showed micro-pollutant degradation. The *Acidovorax* was a nitrate-reducer and Fe(II) oxidizer, which correlated positively with the iron

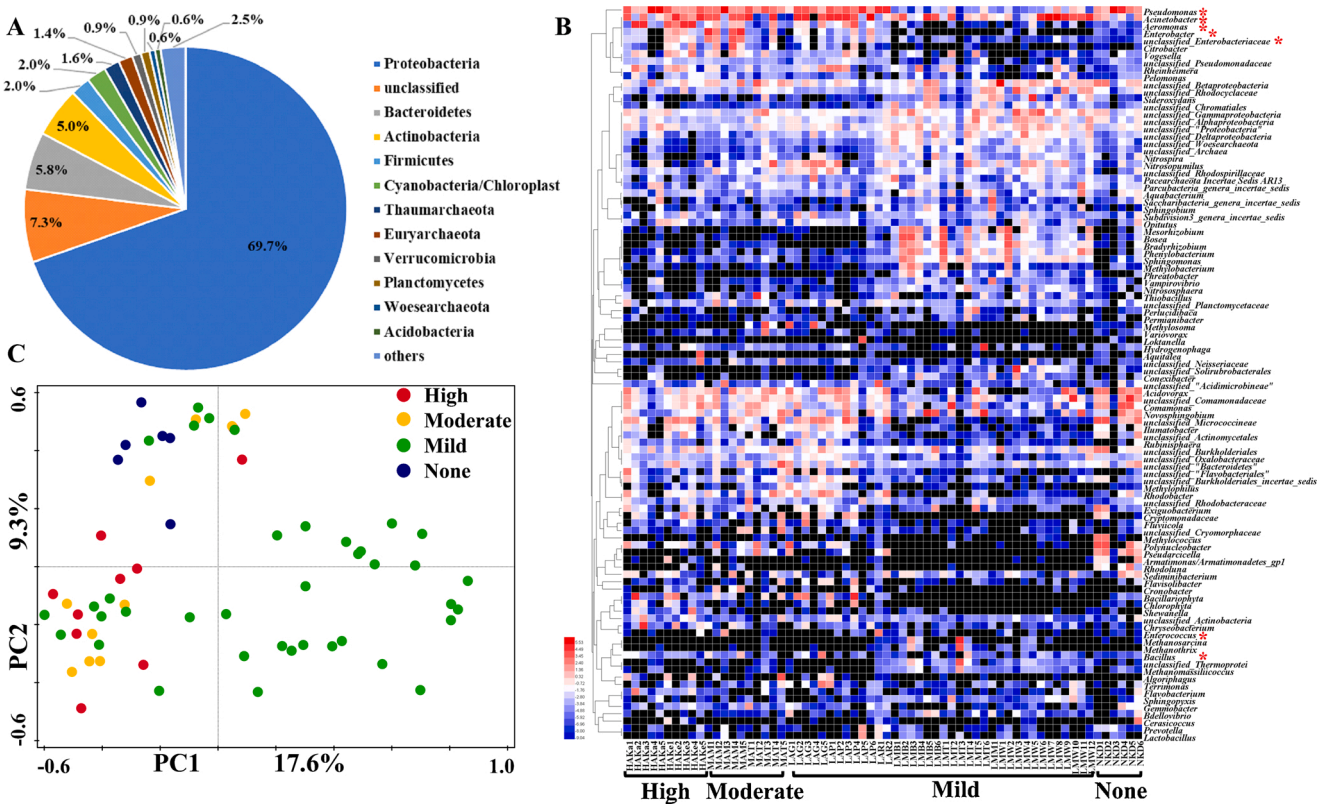

Fig. 1. The microbial community composition of the ground water of Sri Lank at phylum level (A), the heatmap showing the top 10 genus in each samples (B), and principal component analysis (PCA) of the microbial community based on the OTUs (C). The red asterisk represented the potential human pathogens indicated by the VFDB. The numerical colors indicated the values were log2 transformed based on the relative abundance.

(II) concentrations in well water (Navarro-noya et al., 2013). These genera further helped the improvement of the water quality of the drinking water. There were some genus that belonged to the specific samples like *Bacillariophyta* (24.1 %) and *Rheinheimera* (10.3 %) in HAKa2, which need further investigation to elucidate their specificity.

3.2. Prevalence of CKDu impacted the microbial community in ground water

The PCA analysis data indicated that the samples were generally clustered around based on the CKDu prevalence level, especially for most of samples from the High, mild and none CKDu zones (Fig. 1C). The one-way ANOSIM based on Bray-Curtis also showed that the variation of CKDu zones significantly affected the microbial community composition ($R = 0.1525$, $p = 0.0237$). The microbial community in the high CKDu zone varied significantly from the mild ($p < 0.05$) and none-prevalence ($p < 0.01$) CKDu zones, and the difference between mild and none was significant, while the difference with moderate zones were all not significant. These indicated that the demarcation of CKDu zones influenced the microbial community composition in groundwater in Sri Lanka.

LEfSe analysis showed the biomarker microbes in different CKDu zones, the high CKDu zone impacted Bacteroidetes and

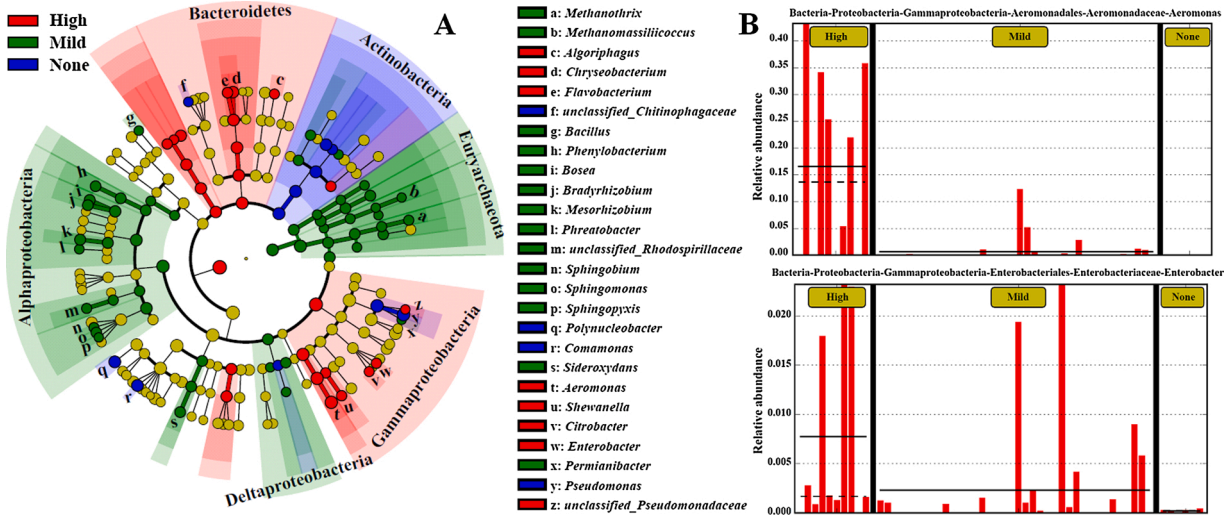

Fig. 2. LEfSe analysis showing the biomarkers in different CKDu affected area (A), and the biomarker of the high CKDu level (B).

Gamaproteobacteria, especially for the *Aeromonas* and *Enterobacter* which were both potential human pathogens (Fig. 2). The high CKDu zone might contribute to the enrichment of *Aeromonas* and *Enterobacter* in the groundwater, and the disease caused by the two genus should be paid more attention in high CKDu zones.

3.3. Antibiotic resistome in groundwater

The antibiotic resistome of water samples collected from different CKDu zones was determined through the HT-qPCR method. The distribution of water samples subjected for ARG was given below: high CKDu zone (6), CKDu moderate zone (2), CKDu mild zone (1), CKDu non-prevalent zone (1). Totally, 212 kinds of ARGs were detected in the water samples with an average of 147. The distribution of ARGs varied from CKDu zones markedly. The highest number of ARGs was detected from CKDu high risk zone (161) followed in order as high CKDu zone >> moderate CKDu zone > CKDu mild zone > CKDu non-prevalent zone. The abundance of ARGs showed a noteworthy relationship with the prevalence of CKDu (Fig. S3).

The dominant ARGs could be categorized as multidrug resistance genes (45.2 %), beta-lactamase resistance genes (31.3 %) and Aminoglycoside resistance genes (10.3 %), respectively (Fig. 3A). Nonetheless, the dominant antibiotic resistance mechanisms could be identified as antibiotic efflux (47.0 %), antibiotic inactivation (44.4 %) and antibiotic target alteration (5.1 %) (Fig. 3B). The dominance of antibiotic inactivation was a double-edged sword; in one way, the antibiotic inactivation availed the degradation of target antibiotics, alternatively the antibiotic inactivation genes had horizontal gene transfer potential to clinical pathogens. The multidrug resistance genes dominated in all the samples, but the abundance of beta-lactamase and aminoglycoside resistance genes (especially the beta-lactamase resistance genes) in the CKDu affected areas were notably higher than the none-prevalence zone (Fig. 4A). Vancomycin resistance genes were not detected in the none-prevalence zone, but it was generally detected in the CKDu affected zones. Both beta-lactamase aminoglycoside and vancomycin antibiotics were widely used for the CKDu treatment, and therefore our observations were in compliance with drug usage in respective CKDu zones.

The main mobile genetic elements (MGEs) were plasmid followed by integrase, while transposase dominated in LAG3 and LAP4 samples (Fig. 4B). In the CKDu zones, the relative abundance of plasmid and integrase were generally higher than the none-prevalence zone. A significant correlation between MGEs and ARGs also existed ($p < 0.01$) based on the spearman correlation analysis, which indicated the role of MGEs on the proliferating ARGs into ground water. The heavy metal resistance genes could reflect the selective pressure from heavy metals in the drinking water. The copper resistance gene, *copA*, generally dominated in all samples, and the mercury resistance gene, *merA*, dominated in LG1 and LG3 (Fig. 4C). The qPCR results of virulence factor reflected the occurrence of human pathogens in the groundwater, especially the *Acinetobacter. baumannii*, which was comparable with the microbial community where the *Acinetobacter* generally dominated in the samples (Fig. 4D). Although the results of high-throughput sequencing were not reliable at the species level, the identical dominance and changing trend of *A. baumannii* were well mutual corroboration between high throughput sequencing and HT-qPCR. These indicated that the disease caused by *A. baumannii* should be paid attention in Sri Lanka.

As shown in Fig. 5A, in non-prevalence zone, the dominant ARG was *mexF* belonging to the multidrug resistance genes, and the dominant MGEs belonged to the pNI05map-F and *intI1*. Nonetheless, the ARGs distribution in the CKDu affected zones was quite different. The *aac*, *cphA-01*, *cphA-02*, *vanTc-02*, *vanC-03* were enriched significantly when compared to the none-prevalence zone. Interestingly, the dominant MGEs changed little (Fig. 5A). Through the one-way ANNOSIM analysis, the degree of CKDu prevalence significantly affected the ARGs distribution ($R = 0.321$, $p = 0.0188$), and the difference between high and mild CKDu zones was significant ($R = 0.321$, $p = 0.0189$). Further, a potent biomarker for the high and mild CKDu zones could be found through the LEfSe analysis (Fig. 5B). Our results showed that the beta-lactamase, *cphA-01*, *cphA-02* could well represent the influence of high CKDu prevalence on the ARGs distribution. The dominance of *mexF* could be considered as a biomarker for the mild CKDu zones. The dominance of *mexF* might reflect the intrinsic composition of antibiotic resistome in Sri Lankan groundwater, while the enrichment of beta-lactamase, especially the *cphA-01* and *cphA-02*, indicated the influence caused by the CKDu.

3.4. Potential mechanisms of the antibiotic resistome affected by the CKDu

Many factors influenced the occurrence of antibiotic resistome in groundwater. A significant correlation existed between antibiotic resistome with microbial community ($R = 0.546$, $p = 0.0002$), co-selection from heavy metals reflected by MRGs ($R = 0.3535$, $p = 0.0091$) and horizontal gene transfer reflected by MGEs ($R = 0.5271$, $p = 0.0001$). However, no relationships were noted with the human pathogens and environmental factors for the antibiotic resistome. The role of these factors on the fate of antibiotic resistome has been widely elucidated (Bengtsson-Palme et al., 2019; Pallares-Vega et al., 2019; Yu et al., 2020; Ju et al., 2016). Procrustes analysis also indicated that the MGEs and microbial community could explain the difference of antibiotic resistome between samples by 44.4 % and 36.6 %, respectively. Nonetheless, which factor dominated the influence on the antibiotic resistome as a whole required further clarification.

Structural equation model (SEM) has been commonly applied to

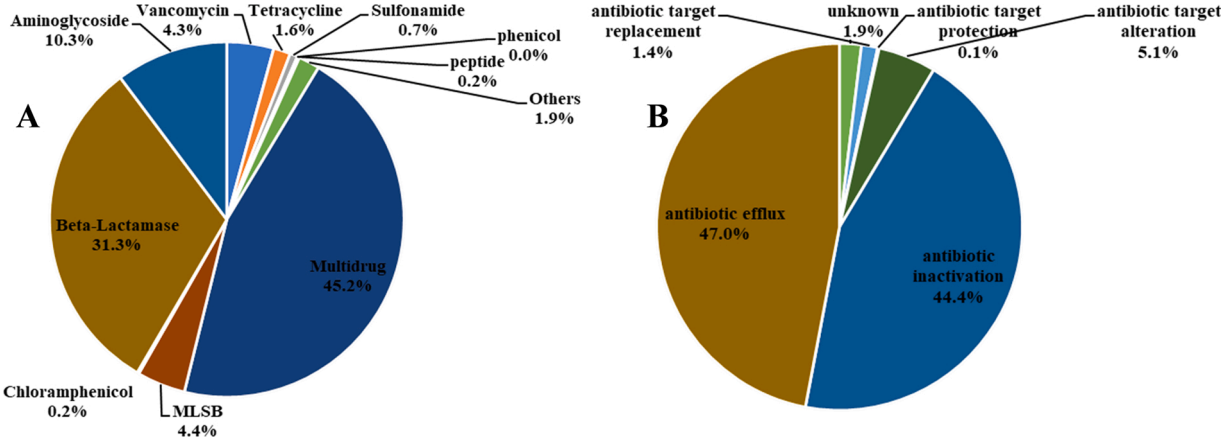

Fig. 3. Characterization of the antibiotic resistome in the ground water of Sri Lanka at the type level (A) and resistance mechanisms (B).

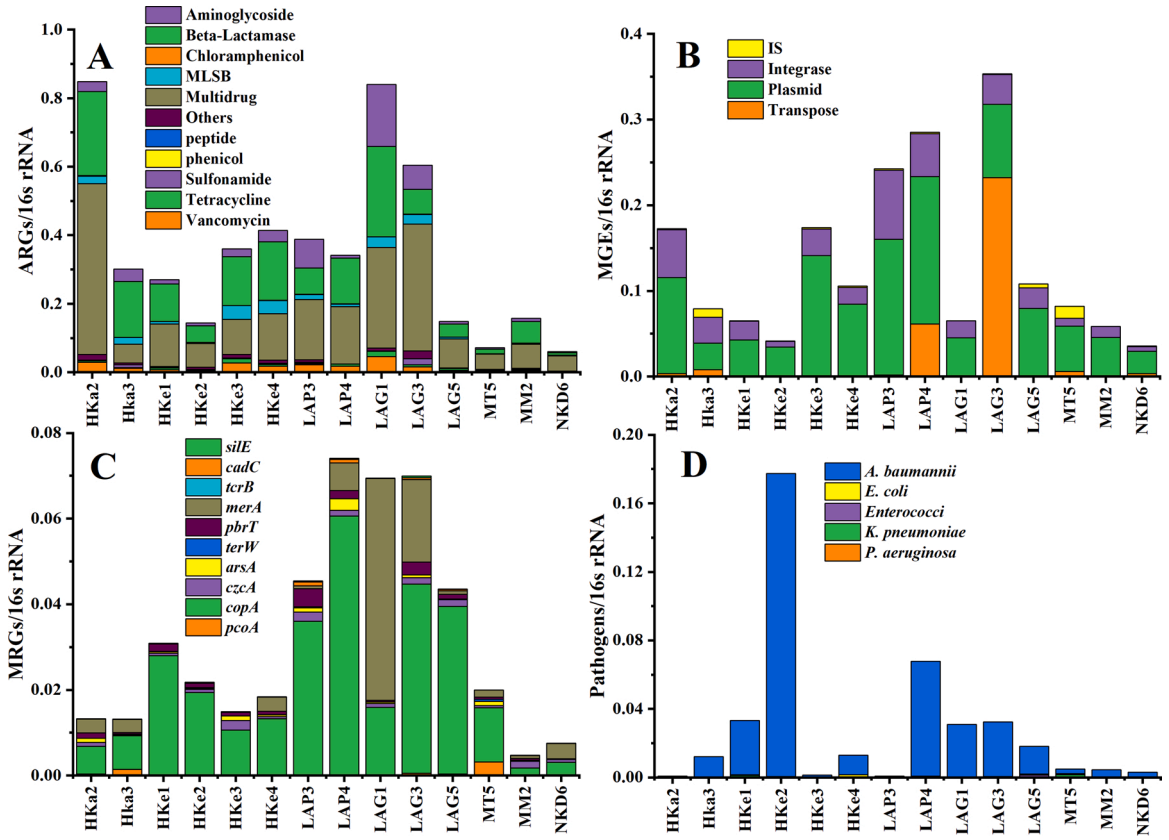

Fig. 4. Occurrence of the antibiotic resistome (A), mobile genetic elements (MGEs, B), heavy metal resistance genes (MRGs, C) and human pathogens (D) in the ground water of Sri Lanka.

develop relationships in complex eco-environments from observational data (Chen et al., 2019; Zhang et al., 2018). In this study, the SEM was well established to explore the direct, indirect and total effects of microbial community, MGEs, MRGs, environmental factors and human pathogens on the ARGs patterns in the groundwater (Fig. 6A). Generally, the factors influencing the ARGs patterns in terms of standardized total effect followed the order as microbial community ($\lambda = 0.535$) > MGEs ($\lambda = 0.501$) > EV ($\lambda = 0.299$) > MRGs ($\lambda = 0.197$) > human pathogens ($\lambda = 0.06$), which indicated that the microbial community and MGEs generally determined the ARGs patterns (Fig. 6B). Further, the effects from microbial community and MGEs on CKDu prevalence were direct and significant ($p < 0.05$). Our data signified the impacts of CKDu prevalence on the fate of antibiotic resistome through the changes on the microbial community and MGEs, and ARGs profiles in the groundwater were more closely associated with their inherently molecular microbiological mechanisms.

The SEM and mantel test data identified the dominant factors influencing the antibiotic resistome on CKDu prevalence as a whole, but the fate of specific ARGs required to be further determined. Network analysis based on the spearman correlation provided a plausible approach to determine the factors responsible for fate determination of specific ARGs (Zhang et al., 2019b, 2020). The outline of the network analysis could be treated analogues to different parts of a human body. Accordingly, as shown in Fig. 6C, the human body like image was divided into three parts as head, body and feet. In the head part, the ARGs significantly affected by the CKDu level were shown. and particularly the *cphA-01* and *cphA-02* were considered as the biomarkers of the high CKDu zones. The contributing factors in this part were potential human pathogens including *Aeromonas*, *Enterobacter*, *Actinetobacter*. *K. pneumoniae*, *P. aeruginosa* and *E.coli*. Interestingly, the *Aeromonas* and *Enterobacter* were also considered as biomarkers of high level CKDu zones from the perspective of microbial community. These results indicated that the antibiotic resistome affected by the CKDu level might be due to the microbial community especially the *Aeromonas* carrying the *cphA-01* and *cphA-02*. We also found out the important role of the water hardness on the *cphA-01* and *cphA-02*, and our previous studies also indicated the importance of hardness in groundwater of the CKDu affected areas (Cooray et al., 2019a, b). It was hypothesized that higher water hardness might contribute to the higher CKDu level, which lead to the usage of amounts of β-lactams for the CKDu patients, and the beta-lactam resistance gene *cphA* was further enriched in the groundwater of higher CKDu prevalence zones. But further investigation was needed to elucidate the hypothesis. The hardness and human potential pathogens especially the *Aeromonas* and *Enterobacter* should be paid more attention in the high level CKDu zones, which contributed the most to the changes of antibiotic resistome in the ground water.

In the body part (Fig. 6C), most of the ARGs belong to the multidrug resistance genes e.g., *mexF*, *oprJ*, *oprD* which were present in all samples. Therefore, they were considered as intrinsic ARGs in the groundwater. In this area, the dominant MGEs were pNI105map-F and *intI1*, which indicated that the MGEs might contribute mostly to the distribution of intrinsic ARGs. Particularly, the *mexF* was considered as the biomarker of the mild or none-prevalence CKDu zones. Besides, the role of MRGs in the antibiotic resistome mainly happened in the body part, and there existed a significant correlation between MRGs and ARGs. In the feet part (Fig. 6C), the abundance of aforementioned ARGs was generally low, and it was also the less abundant MGEs contributing to the distribution.

In summary, the degree of the CKDu prevalence significantly affected ARGs especially *cphA* which were mainly determined by the changes of the microbial community especially the human potential pathogens of *Aeromonas*, *Enterobacter*, while most of the ARGs including *mexF* constituted the background antibiotic resistome present in the ground water, and its distribution was mainly associated with the MGEs of pNI105map-F and *intI1*.

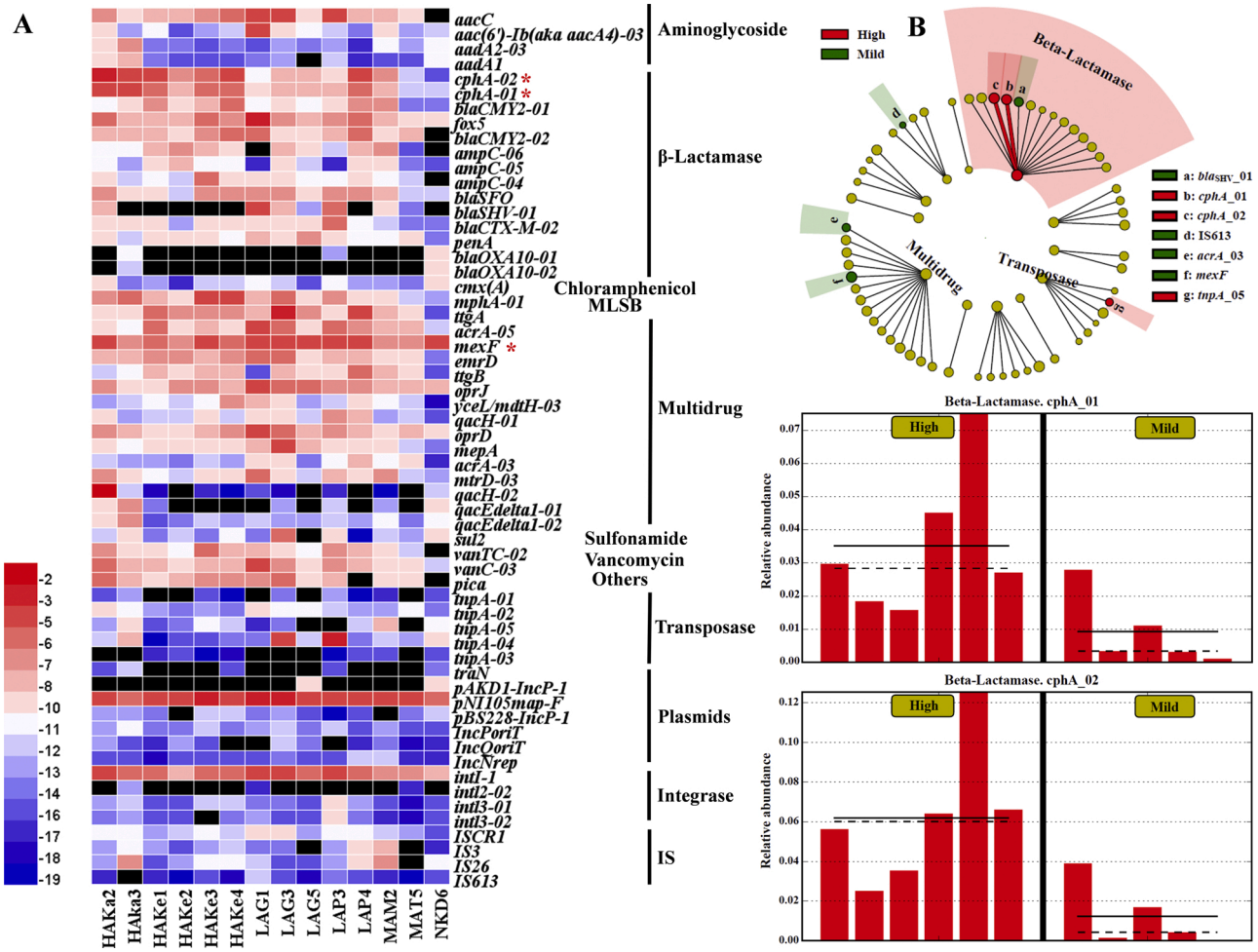

Fig. 5. Heatmap (A) showing the occurrence of the top 10 ARG subtypes and mobile genetic elements (MGEs), the numerical colors indicated the values were log2 transformed based on the relative abundance; LEfSe analysis (B) indicating the ARGs significantly affected by the CKDu.

3.5. Discussion and implications

The *mexF* was a multidrug inner membrane transporter of the MexEF-OprN complex, and it encoded the resistance-nodulation-cell division (RND) antibiotic efflux pump that exported intracellular antibiotics like phenicol, diaminopyrimidine and fluoroquinolone out of cells (Alcock et al., 2019). The *mexF* gene has previously been observed in many environments, such as soil, sediments, river, and drinking waters. It was also prevalent in pigs not exposed to antibiotics, but interestingly, was not found in pigs undergone antibiotic treatment (Götz et al., 1996). Both *mexF* and *oprD* genes also occurred in natural waters (Wang et al., 2016). Therefore, it could be concluded that the *mexF* was an intrinsic ARG in the groundwater of Sri Lanka as for the non-endemic CKDu zones.

The pNI105 was described as a small plasmid (5 kb in length), and conferred high-level resistance to kanamycin and neomycin, while ARGs associated with resistance to these antibiotics were not detected (Wang et al., 2016). Interestingly, the small plasmid pNI105 was non-transmissible but showed a conjugation activity when coexisting with the conjugative plasmids like pAKD1 and pBS228 that resulted in long-term persistence in the microbes (San Millan et al., 2014). A significant correlation between abundance of the dominant ARG, *mexF* and levels of the dominant MGEs, PNI105map-F and *intI1* suggested that the dissemination of ARGs could be attributed to the horizontal gene transfer in the drinking water. Although PNI105map-F and *intI1* could not mobilize by themselves, they could be mobilized by a trans-acting conjugative element present in the same host (Amos et al., 2018). The PNI105map-F helped the persistence of the *mexF* in the microbes, while *intI1* carrying the *mexF* could be transferred through the insertion into other conjugative elements.

The most abundant group of ARG among *Aeromonas* species in natural water were beta-lactam ARG, and a chromosomally encoded β-lactamase *cphA* has been found in *A. hydrophila* isolated from estuarine water, as it has already been observed in *Aeromonas* strains from wastewater (Piotrowska and Popowska, 2014). *Aeromonas* species have been also detected in sources of treated urban water for human consumption, such as water treatment plants, wells, tap water or even drinking mineral water. The *cphA* was an ambler class B MBL; subclass B2 originally isolated from *A. hydrophilia*. This enzyme had specific activity against carbapenems and was active as a mono-zinc protein. The *cphA* gene was intrinsic in the environmental isolates of *A. hydrophila* and *A. jandaei*, which well explained the observed significant correlation between *cphA* and *Aeromonas*, and it further elucidated our hypothesis that the degree of CKDu prevalence significantly affected the presence of ARGs, especially the *cphA*, which was mainly determined by the changes of the microbial community, especially the human potential pathogen of *Aeromonas*.

Previous studies generally focused on identifying causative for the CKDu etiology, while how the CKDu prevalence patterns affecting the environment was neglected. This study systematically elucidated the impacts on the antibiotic resistome and microbial community in the groundwater by the CKDu prevailing areas in Sri Lanka. The human activity to the CKDu treatment like the special antibiotic usage for the CKDu patients had significant impacts on the antibiotic resistome in the

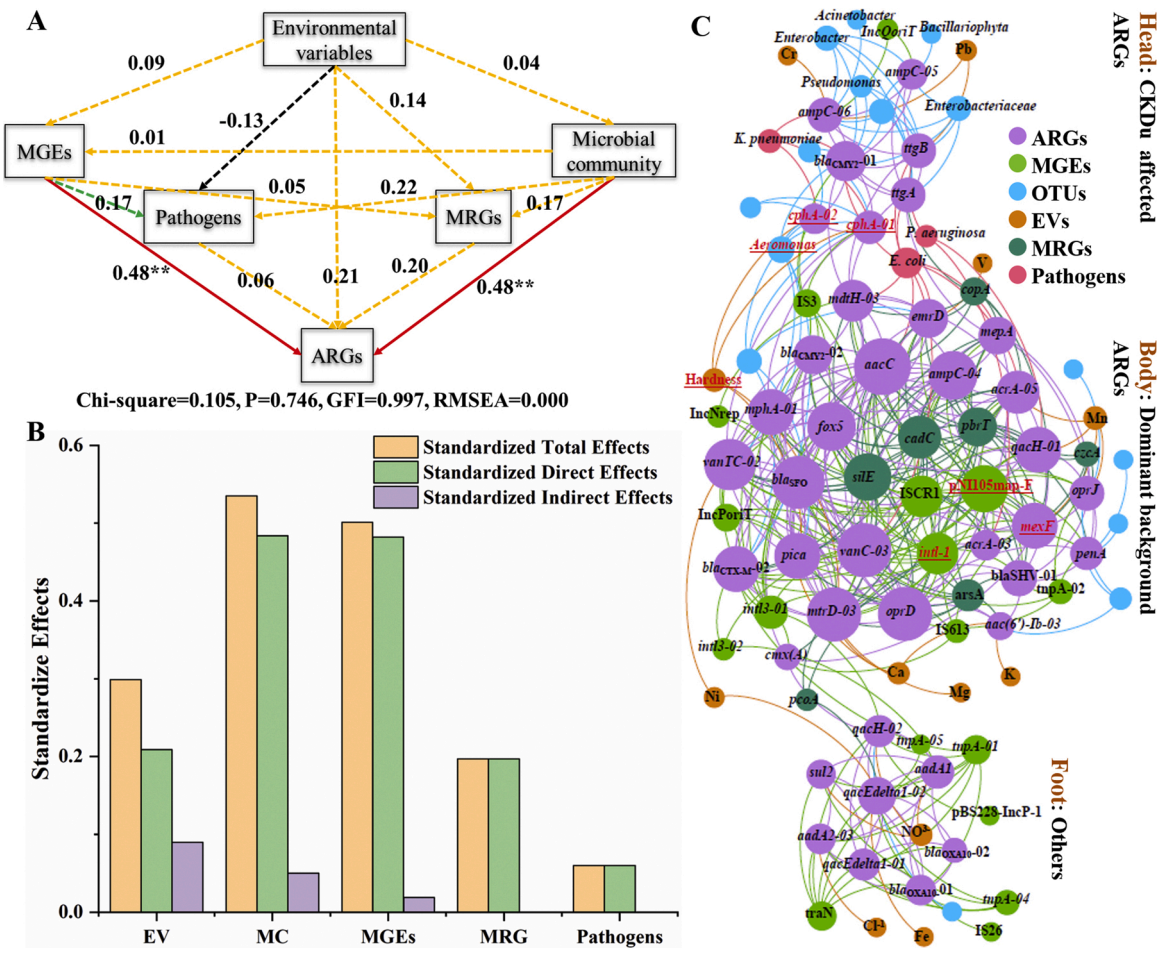

Fig. 6. Structural equation models (SEM) constructed for the relationship between concerned factors and antibiotic resistome (A), red solid line and double asterisks indicated the significance of the path coefficient; The standardized effects of concerned factors on the antibiotic resistome (B); Network analysis showing the factors that have significantly positive correlation with specific ARGs (C). The top 10 genus and ARGs were selected, and the line color indicated the source of the significant correlation with ARGs.

groundwater, which would in return deteriorated the CKDu prevalence. The monitor and control of the ARGs, especially the *cphA* in the groundwater of the CKDu affected areas, were emphasized. Besides, the diseases such as gastroenteritis and wound infections caused by potential human pathogen of *Aeromonas* in the CKDu affected areas should also be paid attention when recommending the groundwater for drinking.

4. Conclusions

For the first time, this study clarified the effects of CKDu prevalence on groundwater microbial environment in Sri Lanka, and the following conclusions could be drawn through the comprehensive analysis of the antibiotic resistome and microbial community of the ground water in the CKDu affected areas of Sri Lanka.

1) The prevalence of CKDu significantly affected the presence of microbial community in the groundwater of Sri Lanka. *Aeromonas* could be considered as a biomarker of the high CKDu zones. Identification of the role of potential human pathogens like *Acinetobacter*, *Pseudomonas* and *Aeromonas* in the groundwater for the disease prevalence should be paid more attention.
2) In terms of antibiotic resistome, *cphA* was considered as a biomarker of the high CKDu zones. The multidrug resistance gene *mexF* dominating in all samples could be treated as a background ARG in the groundwater of Sri Lanka.
3) Microbial community followed by the MGEs contributed to the changes of antibiotic resistome affected by the CKDu prevalence as a whole. The specific enrichment of the biomarker ARG of *cphA* was attributed to the increase of the *Aeromonas* in the microbial community. Nonetheless, the distribution of the dominant *mexF* was significantly associated with the MGEs like pNI105map-F and *intI1*.

CRediT authorship contribution statement

Titus Cooray: Conceptualization, Investigation, Writing - original draft, Funding acquisition. **Junya Zhang:** Conceptualization, Software, Writing - original draft, Writing - review & editing, Supervision. **Hui Zhong:** Software, Writing - review & editing. **Libing Zheng:** Software, Validation, Writing - review & editing. **Yuansong Wei:** Supervision, Funding acquisition, Writing - review & editing. **Sujithra K. Weragoda:** Validation, Writing - review & editing. **K.B.S.N Jinadasa:** Validation, Writing - review & editing. **Rohan Weerasooriya:** Validation, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.123816.

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