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Research Article

Antibiotic resistance genes in *Bacteroides* isolated from faeces of Philippine ducks, *Anas luzonica* and *Anas platyrhynchos domesticus*

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Abstract
The problem of antimicrobial resistance (AMR) has severely afflicted the livestock industry because antibiotics are indiscriminately used for treating infectious diseases and for nontherapeutic purposes. Unfortunately, compared with human AMR research, livestock AMR research is lagging. Thus, this study aimed to contribute to the dearth of knowledge regarding livestock AMR by detecting 10 antibiotic resistance genes (ARGs) in *Bacteroides* isolated from duck faeces. The 10 ARGs were *tetQ, linA, bexA, msrSA, mefA, nim, cfiA, cepA, cfxA,* and *ermF*. In total, 32 isolates were grown, and their DNAs were extracted and subjected to polymerase chain reaction. All isolates were ARG-positive for 1–3 different genes. The ARG-positive genes were *linA* (21/32), *mefA* (20/32), and *bexA* (1/32). Of the 32 isolates, 25 (78%) contained 2–3 ARGs. Although all isolates were ARG-positive, AMR may not be that prevalent in the duck livestock industry because only a maximum of 3/10 ARGs were detected. This is possibly because the duck livestock industry is still a small-scale backyard industry; hence, the use of antibiotics in this industry is not that rampant. However, some reports have shown that *Bacteroides* exhibit extensive horizontal transfer of resistance and virulence genes. The prevalence of these genes may increase if the misuse of antibiotics in the duck industry is not addressed early.

Keywords: Antimicrobial resistance, Antibiotic resistance gene, *Bacteroides*, Ducks, Philippines

INTRODUCTION

Antibiotics have revolutionized the healthcare industry. With this discovery, common deadly diseases and fatal cuts were treated effectively, and dangers associated with surgery were considerably reduced. However, bacteria and other pathogens, just like all living things, have evolved such that they developed resistance due to overuse and/or misuse of antibiotics (Ventola, 2015). The effects of antimicrobial resistance (AMR) have increased globally both in the proportion and total number of pathogens that developed resistance (Roca *et al.,* 2015). AMR infections have claimed the lives of hundreds of thousands and have become one of the principal public health problems of the 21st century (Prestinaci *et al.,* 2015; Ventola, 2015). Antibiotic use or misuse during the COVID-19 pandemic further exacerbated the problem (Lucien *et al.,* 2021; Pelfrene *et al.,* 2021). The emergence, spread, and persistence of multidrug-resistant (MDR) bacteria in the animal–human–environment interface that interlinks the "sharing" of pathogens within the triad worsened the situation (Aslam *et al.,* 2019).

Because of the success of antibiotic use in humans, antibiotics have been used in animals but primarily for nontherapeutic purposes, such as growth promotion and prophylaxis, leading to even broader environmental exposure (Berglund, 2015). When antibiotic-resistant bacteria (ARB) in livestock are excreted, ARB-associated antibiotic-resistant genes (ARGs) propagate into the surrounding environment, thereby becoming environmental pollution. This increases the likelihood of transmission of these ARGs to humans, particularly livestock workers (Rysz and Alvarez, 2004; Li *et al.,*

2015). However, most publications are on ARB and ARGs in human clinical contexts, with a meager 10% of studies focusing on antibiotic resistance (AR) from livestock or animal husbandry (He *et al.,* 2020).

Ducks were selected as the research subjects in this study because, compared with swine and chicken, they have not generated considerable recognition in terms of AMR. Although the scale of the duck industry is smaller than that of the swine, chicken, cow, and fish aquaculture industry, ducks are known to act as carriers of bacterial pathogens (Delabouglise *et al.* 2018; Eid *et al.,* 2019; Pauly *et al.,* 2019). This problem of lack of sufficient information on ARB from ducks needs to be resolved because the duck market is increasing worldwide, as reported by the Food and Agriculture Organization of the United Nations (FAO-UN, 2021). In the Philippines alone, the duck market, although still quite small, has already contributed 11% of the total value of overall Philippine agricultural production (Chang *et al.,* 2003; Chang and Dagaas, 2004).

Instead of testing the traditional faecal indicator bacteria (FIB) such as *Escherichia coli* or *Enterococcus* sp., this study focused on *Bacteroides*. This is because gut microbiota mostly comprises obligate anaerobes such as *Bacteroides*, not the traditional FIB (Wexler, 2007). Second, many studies have reported on AMR and FIB (*E. coli*), but those reporting *Bacteroides*-caused infection in humans are few and those reporting this infection in animals are even fewer. Also, several studies have reported that *Bacteroides* exhibit extensive horizontal gene transfer (HGT) of ARGs, thereby contributing to AMR (Shoemaker *et al.,* 2001; Husain *et al.,* 2017; Boto *et al.,* 2019; Kent *et al.,* 2020). Hence, this study aimed to contribute to the dearth of knowledge regarding ARG propagation from ducks.

MATERIALS AND METHODS

Bacteroides strains and culture media

The present study analyzed a fresh collection of 32 *Bacteroides* isolates (Table 1). In an earlier study, these were originally isolated from the faeces of Philippine ducks, *Anas luzonica* and *Anas platyrhynchos domesticus* (Dela Rosa and Rivera, 2021). All isolates were reactivated in *Bacteroides* Bile Esculin agar (Laboratories Conda, S.A., Spain) and incubated for 4 days at 37°C under an anaerobic condition using the Thermo Scientific™ Anaeropack™ anaerobic gas generator (ThermoFisher Scientific, USA). All reactivated isolates were stored in a refrigerator until further use.

DNA extraction and polymerase chain reaction (PCR)

DNAs from the *Bacteroides* isolates were extracted using boiling method (Garcia *et al.,* 2015). The resulting extracts were maintained at -20 °C until use. The DNA samples were analyzed for 10 target ARGs (Table 2). PCR was performed with 2x GoTaq Green Mastermix (Promega Corp., Madison, WI, USA) in a total reaction volume of 30 µL. The PCR conditions for each primer were based on previous study protocols (Eitel *et al.,* 2013). Following amplification, 4 µL of each PCR product was separated using 1.5% agarose gel. Moreover, the PCR products were electrophoresed for 20 min at 100V in 0.5x TBE buffer and visualized using the SYBR Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific, USA) under UV illumination.

Positive controls used for agarose gel electrophoresis came from isolates positive for the gene/s; these results were confirmed through amplicon sequencing at Macrogen, South Korea. All sequences were submitted to GenBank and assigned an accession number.

In silico analysis of *mefA* gene

mefA is the genetic determinant for conferring macrolide resistance, and its genetic homology was identified by analyzing data from the GenBank database. The obtained *mefA* gene sequence from *Bacteroides* sp. MZ229610 served as the positive control. It was used to search for homology with gene sequences found and downloaded from GenBank. The downloaded sequences were *B. fragilis* KJ816753*, Streptococcus sanguinis* FJ66795*, S. agalactiae* DQ445272*, S. mitis* DQ304773, *S. anginosus* MT345159, *S. salivarius* MT345165, *S. dysgalactiae* MT345166, *S. pyogenes* MT345164, *Gemella haemolysans* DQ304772, *Acidovorax* sp. MT345174, and *Clostridium perfringens* EU553549. All sequences were aligned using CLUSTALW with MEGA X (Version 10.0.4) using the Tamura 3-parameter model at the 50% cutoff value (Beric *et al.,* 2018)

RESULTS AND DISCUSSION

Most AR studies are associated with human health. Therefore, studies on AR from the animal husbandry industry are fewer but considerably more than those from the relatively small-scale animal industries such as duck livestock. Thus, the mode and pathway of AR transmission from animals to humans are poorly understood (Manaia, 2017; He *et al.,* 2020). This underscores the necessity for more research on antimicrobial usage, mechanisms of ARG release into the environment, and entry of these ARGs into human resistomes. Ironically, human resistomes have received considerable attention, but animal wastes from farms contain remarkably more ARGs than wastes from hospitals and municipal wastewater (Sim *et al.,* 2011; Ekpeghere *et al.,* 2017; Kivits *et al.,* 2018; Liu *et al.,* 2018; Gao *et al.,* 2020; Macedo *et al.,* 2020). These ARGs of animal livestock origin enter the environment through faecal discharge. They eventually contaminate water, soil, and crops, ultimately affecting native microbial communities

Table 1. *Bacteroides* sp. isolated from Philippine livestock ducks

Strain	Source (Duck species*)	GenBank accession number
Bacteroides sp. d16	*A. luzonica*	MN428890
Bacteroides sp. d17	*A. luzonica*	MN428891
Bacteroides sp. d18	*A. luzonica*	MN428892
Bacteroides sp. d19	*A. luzonica*	MN428893
Bacteroides sp. d20	*A. luzonica*	MN428894
Bacteroides sp. d21	*A. luzonica*	MN428895
Bacteroides ovatus d22	*A. luzonica*	MN428896
Bacteroides ovatus d23	*A. luzonica*	MN428897
Bacteroides *ovatus* d24	*A. luzonica*	MN428898
Bacteroides sp. d25	*A. luzonica*	MN428899
Bacteroides sp. d26	*A. luzonica*	MN428900
Bacteroides sp. d27	*A. luzonica*	MN428901
Bacteroides sp. d28	*A. luzonica*	MN428902
Bacteroides sp. d29	*A. luzonica*	MN428903
Bacteroides ovatus d30	*A. luzonica*	MN428904
Bacteroides ovatus d31	*A. luzonica*	MN428905
Bacteroides sp. d32	*A. luzonica*	MN428906
Bacteroides ovatus sp. d33	*A. luzonica*	MN428907
Bacteroides ovatus d34	*A. luzonica*	MN428908
Bacteroides sp. d35	*A. luzonica*	MN428909
Bacteroides sp. d36	*A. luzonica*	MN428910
Bacteroides sp. d37	*A. luzonica*	MN428911
Bacteroides sp. d38	*A. luzonica*	MN428912
Bacteroides ovatus d39	*A. platyrhynchos domesticus*	MN428913
Bacteroides sp. d40	*A. platyrhynchos domesticus*	MN428914
Bacteroides sp. d41	*A. platyrhynchos domesticus*	MN428915
Bacteroides sp. d43	*A. platyrhynchos domesticus*	MN428917
Bacteroides sp. d44	*A. platyrhynchos domesticus*	MN428918
Bacteroides sp. d45	*A. platyrhynchos domesticus*	MN428919
Bacteroides sp. d46	*A. platyrhynchos domesticus*	MN428920
Bacteroides sp. d47	*A. platyrhynchos domesticus*	MN428921
Bacteroides sp. d49	*A. platyrhynchos domesticus*	MN435123

**A. luzonica – Anas luzonica*; A. *platyrhynchos domesticus – Anas platyrhynchos domesticus*

Table 2. Primers used for the detection of 10 antibiotic-resistance genes in *Bacteroides* isolates.

Primer	Sequences (5′ → 3′)	Size (bp) [1]
tetQ	F[2]: CTGTCCCTAACGGTAAGG	658
	R[3]: TTATACTTCCTCCGGCATCGGT	
linA	F: CTGGGGAGTGGATGTCTTGT	230
	R: AGTTGGCTTGTTTGGAAGTG	
bexA	F: TAGTGGTTGCTGCGATTCTG	195
	R: TCAGCGTCTTGGTCTGTGTC	
msrSA	F: GGGAACTGAAAGATGGCAAA	165
	R: TACGAGCCTGTTTTCGCTTT	
mefA	F: ATACCCCAGCACTCAATTCG	186
	R: CAATCACAGCACCCAATACG	
nim	F: ATGTTCAGAGAAATGCGGC GTAAGTG	458
	R: GCTTCCTCGCCTGTCACGTGCTC	
cfiA	F: AATCGAAGGATGGGGTATGG	302
	R: CGGTCAGTGAATCGGTGAAT	
cepA	F: TTTCTGCTATGTCCTGCCT	786
	R: ATCTTTCACGAAGACGGC	
cfxA	F: TGACTGGCCCTGAATAATCT	301
	R: ACAAAAGATAGCGCAAATCC	
ermf	F: TAGATATTGGGGCAGGCAAG	178
	R: GGAAATTGCGGAACTGCAAA	

that acquire ARGs via HGT (Kivits *et al.,* 2018; Gao *et al.,* 2020).

The findings of the present study address the dearth of information on AR in ducks. All the 32 *Bacteroides* isolates were successfully reactivated and subjected to ARG detection (Table 3). The positive controls of *linA, bexA,* and *mefA* were collected from the samples themselves. Strain d16 was the positive control for *linA*, strain d43 was for *bexA*, and strain d18 was for *mefA*. The PCR products of the positive controls were sequenced and confirmed to contain the ARG primer sequences. The sequences were deposited in GenBank under accession numbers MZ209269, MZ144741, and MZ229610 for *linA, bexA,* and *mefA*, respectively.

Of the 32 *Bacteroides* isolates, 31 were positive for at least 1 ARG. To be exact, of the 31 isolates, 19 contained just 1 ARG. In those 19 isolates, the most common ARG was *linA* (10 isolates). On the other hand, 11 isolates contained 2 ARGs (*linaA* + *mefA*). At last, only 1 isolate contained 3 ARGs. The study results also showed that, of the 32 isolates, 12 (37.5%) contained multiple ARGs (11 isolates with 2 ARGs and 1 isolate with 3 ARGs). Regarding ARG frequency, *linA* was the most common ARG detected (22/32 or 65.6%) (Figs. 1–3). The ARGs *tetQ, msrSA, nim, cfiA, cepA, cfxA,* and *ermF* were not detected in any of the *Bacteroides* isolates.

So far, this is the extent of AR in ducks in the Philippines — the first documentation in the country. All the duck faeces collected in this study came from the small-scale backyard farms. No large-scale duck farming industry exists in the Philippines yet. However, as observed in the present study, 12 isolates already contained multiple ARGs. It is both fortunate and unfortunate that the Philippine duck industry is also growing fast. Hence, the problem of ARB and ARGs associated

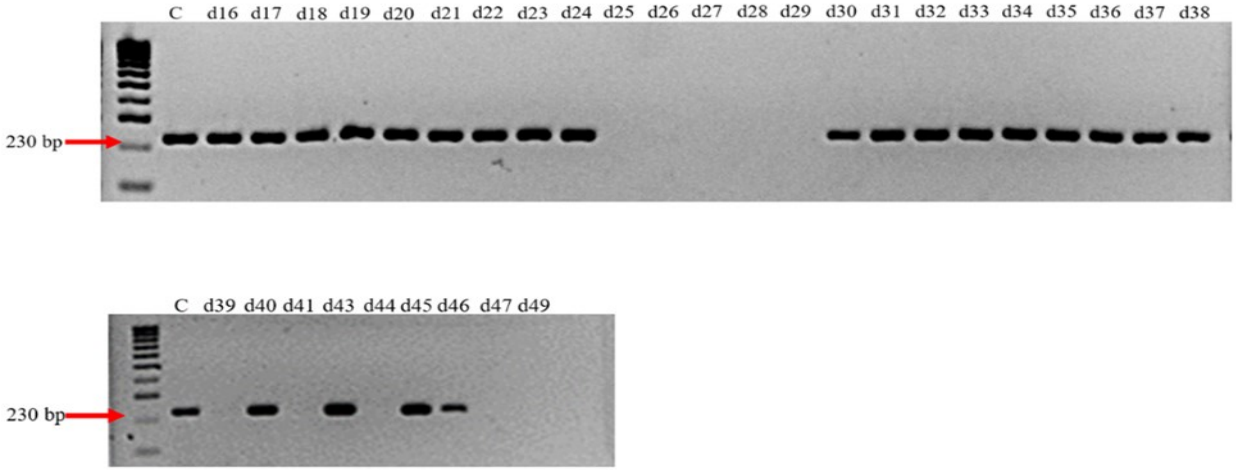

Fig. 1. *Agarose gel electrophoresis of the PCR-based detection of antibiotic-resistant gene linA with an amplicon size of 230 bp. C (2nd lane), control from Bacteroides isolate d16, and all succeeding lanes are the different Bacteroides isolates tested for linA ARG*

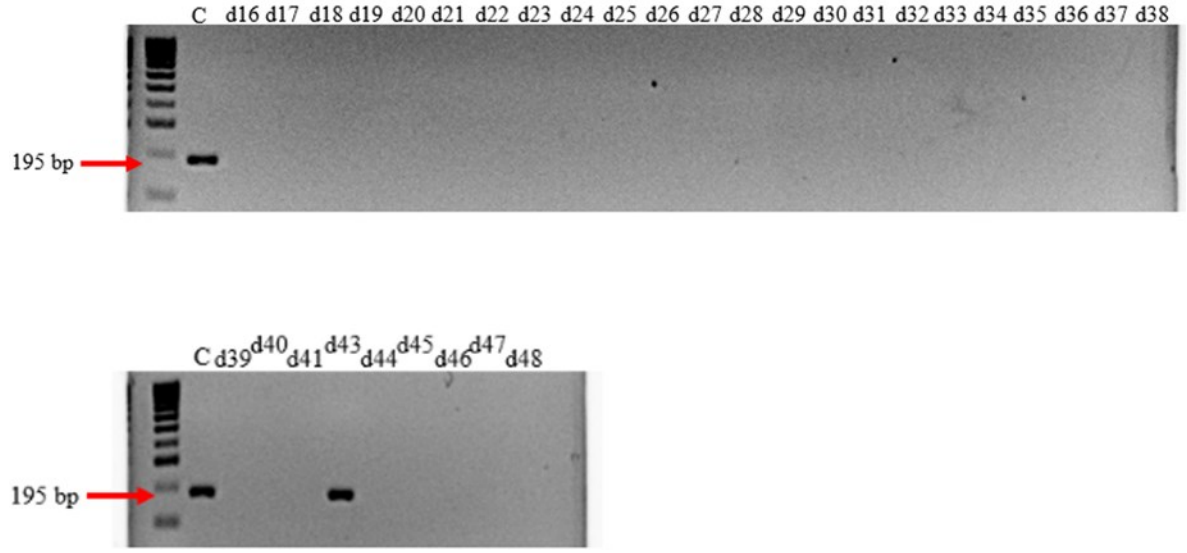

Fig. 2. *Agarose gel electrophoresis of the PCR-based detection of antibiotic-resistant gene bexA with an amplicon size of 195 bp. C (2nd lane), control from Bacteroides isolate d43, and all succeeding lanes are the different Bacteroides isolates tested for bexA ARG*

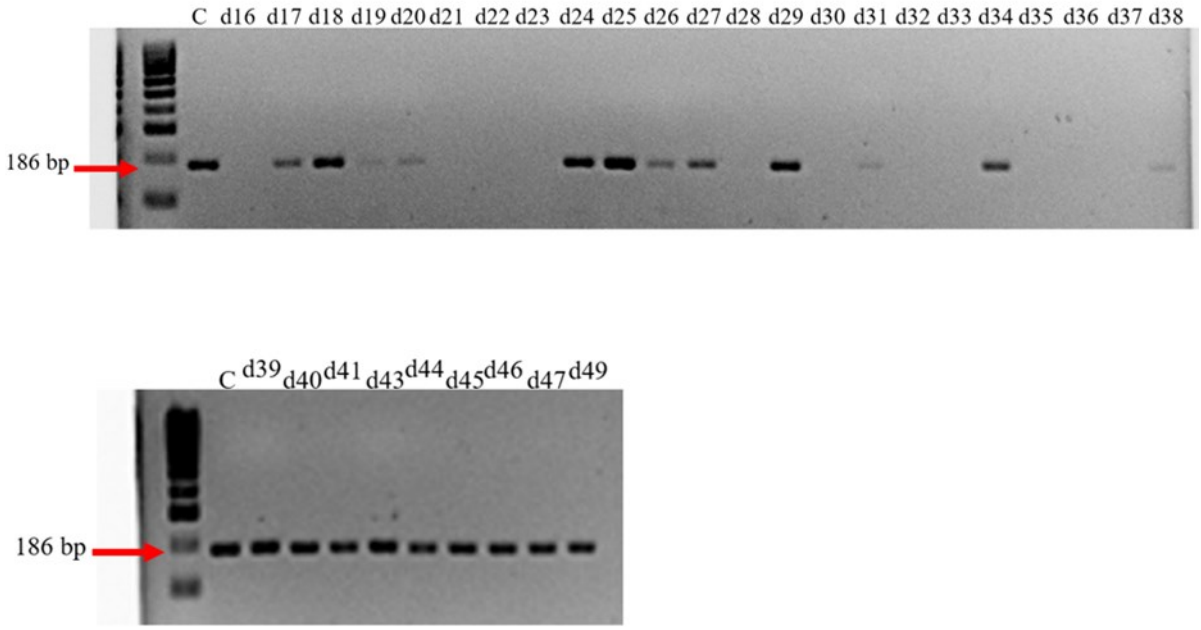

Fig. 3. *Agarose gel electrophoresis of the PCR-based detection of antibiotic-resistant gene mefA with an amplicon size of 186 bp. C (2nd lane), control from Bacteroides isolate d18, and all succeeding lanes are the different Bacteroides isolates tested for mefA ARG*

with those large-scale animal livestock industries may eventually be noted in the fast-growing duck industry. Therefore, the issue of antimicrobial usage in the duck industry must be recognized and addressed while the situation is still more manageable.

This present study did not use FIB such as *E. coli* and faecal coliforms because they do not best represent the gut microbiome, which is in reality only present in low numbers in the gut (Wexler, 2007; Cabral, 2010). The most abundant gut microflora are anaerobes, with the main anaerobic genera being *Bacteroides* and Bifidobacteria. Moreover, *Bacteroides* spp. are the most dominant and abundant in faeces (Hong *et al.,* 2008; Cabral, 2010; Teixeira *et al.,* 2020)**.** They are deemed clinically important anaerobes because of their high prevalence in both gut and faecal microbiota (Hong *et al.,* 2008). *Bacteroides* spp. are the most common opportunistic pathogenic group of anaerobes that cause intraabdominal, pelvic, gynaecological, skin, soft tissue, and bloodstream infections in humans (Wexler, 2007; Wang *et al.,* 2020; Rong *et al.,* 2021)**.**

Vast information is available on *Bacteroides* because of the regular resistance surveys conducted for humans (Niestepski *et al.,* 2019; Ogane *et al.,* 2020; Wang *et al.,* 2020; Rong *et al.,* 2021). Accordingly, *Bacteroides* in humans are generally known to be almost completely resistant to β-lactams and tetracyclines and moderately resistant to cefoxitin, amoxicillin, clavulanic acid, clindamycin, and moxifloxacin, and exhibit low resistance for carbapenems, piperacillin/tazobactam, metronidazole, and tigecycline (Szekely *et al.,* 2015)**.** However, this general picture in humans is not well-established in animals and less so, if not, is nonexistent in ducks. The results of the present study provide a picture concerning ARGs in duck-associated *Bacteroides.* Moreover, this study could serve as a starting point for further investigations regarding ARGs' prevalence, incidence, and transmission from duck intestinal microbiota.

The *linA* gene codes for the lincomycin resistance protein LinA and is carried by the transposon NBU2. This protein is responsible for lincosamide resistance (Wang *et al.,* 2000)**.** Lincomycin is usually used to treat avian mycoplasmosis, which causes high morbidity in ducklings**.** Therefore, lincomycin has already been given in ducklings as prophylaxis (Stipkovits and Szathmary, 2012). However, interestingly, none of the duck breeders from where the duck faeces were collected admitted that they had ever used any lincosamide antibiotics. Nevertheless, a possible explanation for the many *linA*-positive *Bacteroides* is that *Bacteroides linA* is a homolog of the *linA* gene in *Staphylococcus aureus* (Spížek and Rezanza, 2017). Because the *linA* gene is carried by a transposon, HGT between the two microorganisms belonging to different genera may have occurred, resulting in the high number of *linA*-positive strains in this study.

Twenty-one isolates were positive for the *mefA* gene (Table 3). Moreover, the *Bacteroides* sp. d18 isolate was sequenced and used as the positive control in this study. Further, the sequence of this isolate was used as a reference to search for other bacteria with the *mefA* gene deposited in GenBank. The sequence data obtained from the GenBank database revealed that the *mefA* gene was well distributed within the genus *Streptococcus* (Fig. 4).

Table 3. Occurrence of 10 antibiotic-resistance genes in *Bacteroides* spp. isolated from duck faeces.

***Bacteroides* isolate**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Number of genes detected per strain**
d16	−	+	−	−	−	−	−	−	−	−	1
d17	−	+	−	−	+	−	−	−	−	−	2
d18	−	+	−	−	+	−	−	−	−	−	2
d19	−	+	−	−	+	−	−	−	−	−	2
d20	−	+	−	−	+	−	−	−	−	−	2
d21	−	+	−	−	−	−	−	−	−	−	1
d22	−	+	−	−	−	−	−	−	−	−	1
d23	−	+	−	−	−	−	−	−	−	−	1
d24	−	+	−	−	+	−	−	−	−	−	2
d25	−	−	−	−	+	−	−	−	−	−	1
d26	−	−	−	−	+	−	−	−	−	−	1
d27	−	−	−	−	+	−	−	−	−	−	1
d28	−	−	−	−	−	−	−	−	−	−	0
d29	−	−	−	−	+	−	−	−	−	−	1
d30	−	+	−	−	−	−	−	−	−	−	1
d31	−	+	−	−	+	−	−	−	−	−	2
d32	−	+	−	−	−	−	−	−	−	−	1
d33	−	+	−	−	−	−	−	−	−	−	1
d34	−	+	−	−	+	−	−	−	−	−	2
d35	−	+	−	−	−	−	−	−	−	−	1
d36	−	+	−	−	−	−	−	−	−	−	1
d37	−	+	−	−	−	−	−	−	−	−	1
d38	−	+	−	−	+	−	−	−	−	−	2
d39	−	−	−	−	+	−	−	−	−	−	1
d40	−	+	−	−	+	−	−	−	−	−	2
d41	−	−	−	−	+	−	−	−	−	−	1
d43	−	+	+	−	+	−	−	−	−	−	3
d44	−	−	−	−	+	−	−	−	−	−	1
d45	−	+	−	−	+	−	−	−	−	−	2
d46	−	+	−	−	+	−	−	−	−	−	2
d47	−	−	−	−	+	−	−	−	−	−	1
d49	−	−	−	−	+	−	−	−	−	−	1
Frequency	0/32	21/32	1/32	0/32	20/32	0/32	0/32	0/32	0/32	0/32	

1: *tetQ* gene; 2: *linA* gene; 3: *bexA* gene; 4: *msrSA* gene; 5: *mefA* gene; 6: *nim* gene; 7: *cfiA* gene; 8: *cepA* gene; 9: *cfxA* gene; 10: *ermF* gene; (+): ARG detected; (−): ARG not detected.

Moreover, it showed that the *mefA* gene was also well distributed in other anaerobic genera such as *Gemella, Acidovorax, Clostridium*, and other *Bacteroides,* which also includes *Bacteroides* sp. d18 that came from this study. The pairwise distances between the rooted *S. pyogenes* and the two *Bacteroides* sp. unveiled that their *mefA* genes were highly similar to each other (Supplementary Table S1). The pairwise distance values were 0.01007 between *S. pyogenes* and *Bacteroides* sp. d18 and 0.19855 between *S. pyogenes* and *B. fragilis*. The *mefA* gene codes for the macrolide efflux protein A and is carried by a conjugative transposon, CTnGERM1 (Wang *et al.,* 2003). This gene confers macrolide resistance and is a homolog of the *mefA* gene from *S. pyogenes* (Clancy *et al.,* 1996)**.** Because *mefA* is carried by a transposon, HGT may occur between susceptible bacteria with different Gram reactions. Thus, genetic exchanges between different groups can occur when the groups come into contact in the environment in the presence of appropriate permissible conditions. Fig. 4 presents the distribution of the *mefA* gene in other anaerobic bacteria in humans such as *Gemella* and *Clostridium perfringens*; these findings are based on *in silico* data. Moreover, studies on different species with the *mefA* gene have demonstrated that the gene transfer occurred due to mobile elements (Cai *et al.,* 2007; Soge et al., 2009; Tazumi *et al.,* 2009) which is consistent with other studies reporting *mefA* gene transfer through a transposon or a mobile genetic element. Combining high bootstrap values and low pairwise distance values between species can determine the strong relatedness of mefA genes of these species. This also suggests the possibility of HGT of macrolide resistance elements to other anaerobes or facultative anaerobes. Consequently, these anaerobes/ facultative anaerobes can now serve as reservoirs for

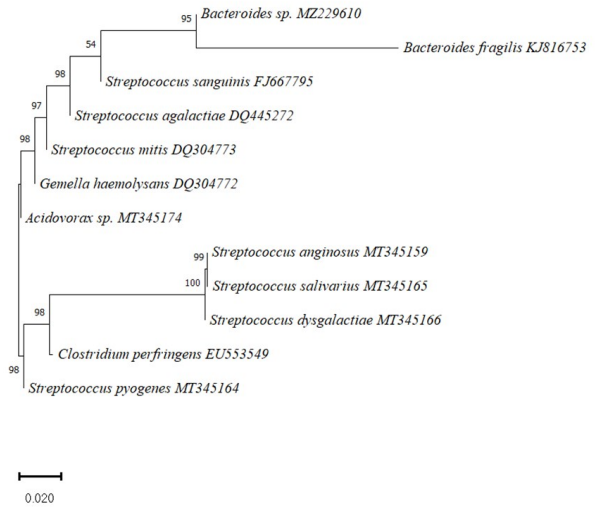

Fig. 4. *Phylogenetic relationships of bacterial species based on their mefA sequences. Branching patterns, rooted using Streptococcus pyogenes mefA gene sequence, were constructed using Neighbor-Joining tree based on the Tamura-3 parameter model with 1000 replicates*

ARGs in the environment.

Finally, the plasmid-acquired *bexA* gene (BexA protein) is a member of the multidrug and toxic compound extrusion class and is responsible for fluoroquinolone and moxifloxacin resistance**.** The low frequency of the *bexA* gene is similar to the low frequency noted in *Bacteroides* from humans (Eitel *et al.,* 2013). This low frequency of *bexA*-positive strains indicates that duck-associated *Bacteroides* are still susceptible to fluoroquinolones, unlike *Bacteroides* in humans that have already developed resistance to third- and fourth-generation fluoroquinolones.

This new knowledge on duck faeces is a public health concern because, in a backyard industry setting such as in the Philippines, ducks primarily scavenge for their feeds around their areas and shed their faecal droppings within that same area. The ARGs from ARB from the duck faeces can be transferred to other microbial communities through HGT or the ARB can also find their way into the food chain, eventually affecting humans.

Conclusion

The present study investigated the ARGs of duck-associated *Bacteroides* previously isolated from duck faeces. The results revealed that multiple ARGs are already present in ducks. The Philippine agricultural sector may not be fully aware of them because the focus is more on swine, chicken, and cow husbandry. Although all strains were positive to at least 1 ARG and the most is only 3 out of 10 ARGs, extensive AR is not yet prevalent in *Bacteroides*. However, with the continuous nontherapeutic use of antibiotics and/or because of contact with farm workers possibly harboring ARB strains, ARGs may be inexplicably introduced in the duck farm. When that happens, HGT may occur. As mentioned, most AMR research focuses on the human clinical context. Therefore, further studies are warranted to fill the gaps in information, such as data on the abundance and prevalence of ARGs from livestock. A more collaborative livestock study is required to discern the interrelationship between livestock antibiotic usage and the pattern and transmission of ARGs into the receiving environments or in a clinical setting.

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Supplementary information

The authors are responsible for the content of the supplementary information. Any queries regarding the same should be directed to the corresponding author. The data of pairwise distances between the rooted *S. pyogenes* and the two *Bacteroides* sp.are shown in Supplementary Table S1. The supplementary information is downloadable from the article's webpage and will not be printed in the print copy.

Conflict of interest

The author declare that they have no conflict of interest.

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