BMC Microbiology

RESEARCH

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High throughput screening for human disease associated-pathogens and antimicrobial resistance genes in migratory birds at ten habitat sites in China

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Abstract

Background Migratory birds have been found to carry and spread pathogens, contaminating the environment and causing diseases in humans and other animals. To our knowledge, there hasn't been any systematic targeted screening for known pathogens in migratory birds. In the current study, customized real time PCR based TaqMan Array Cards (TAC) were used to detect 99 human disease related pathogens and 20 antimicrobial resistance (AMR) genes in migratory birds at 10 habitat sites in China.

Results The results showed that 30.5% (107/351) of migratory birds carried at least one of 14 pathogens. The most prevalent pathogens included *Aeromonas*, *Plasmodium*, *Cryptosporidium*, *Giardia lamblia*, enteropathogenic *Escherichia coli* (EPEC), *Campylobacter jejuni/coli*, and *Rickettsia*. Their distribution demonstrated certain host or region specificity. *Anseriformes* carried higher rate of pathogens (39.1%, 72/184) than *Charadriiformes* (23.2%, 33/142, $p < 0.05$). The overall pathogen detection rate was the highest in Hubei (87.1%, 27/31), possessing exclusively *Anser*. The pathogen quantities were estimated to be 10^3 to 2×10^8 gene copies per gram of feces. AMR genes associated with resistance to macrolides, quinolones, tetracyclines, and β-lactams were widely detected, with overall quantities ranging from 10^5 to 10^9 copies of interrogated genes for each drug class per gram of feces.

Conclusions Using such a multi-target detection and quantification platform, this study evaluated the potential role of migratory birds as reservoirs or vectors for a broad range of pathogens and AMR genes in the environment, indicating their capacity to transmit zoonotic diseases. These might provide evidence for implementation of targeted intervention with a one health approach.

Keywords Migratory birds, Pathogen, Antimicrobial resistance, TaqMan Array Card, Surveillance

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Background

Migratory birds carry a variety of microorganisms during migration, expanding the geographical and host range of pathogen transmission, which may be involved in the epidemiological chain of human diseases through environmental pollution and other pathways, posing potential public health risks [1]. Since the first large-scale outbreak of highly pathogenic avian influenza at Qinghai Lake in China in 2005, people have reinforced the investigation of migratory birds [2, 3]. Human intrusion into the natural habitats of wild birds, domestication of wild birds as pets or racing birds, and increased human consumption of poultry may promote pathogen transmission, leading to the emergence and spread of zoonoses [4]. More than 1,400 human pathogens have been described, of which about 62% are classified as zoonotic. Emerging infectious disease events were dominated by zoonoses (60.3%), most of which (71.8%) originated in wild animals [5]. Meanwhile, migratory birds have been found to be the disseminators of antimicrobial resistance across human-bird-environment interfaces [6–8], particularly over long distance to pristine environment like Antarctica [9]. Therefore, systematic surveillance of pathogens and AMR genes carried by migratory birds has been highlighted with a view to identifying their potential transmission risks and hazards [10], which can be essential for assessing the animal sources of infection [11].

Among the nine global migratory routes of birds, four pass through China, covering majority of the territory. The surveillance of avian flu has been widely implemented, while some studies have focused on individual bacteria or viruses [12–16]. Recent metagenomic sequencing approach has often aimed to discover novel microorganisms. However, there is a lack of molecular surveillance and epidemiological studies targeting multiple regions, representative migratory birds along migratory routes for a variety of diseases causing microorganisms. Many pathogens that cause pneumonia, diarrhea, meningitis, and febrile illness in humans, can like be spread through the human-animal-environment interface [17–19].

Nucleic acid based molecular methods have become a powerful tool for targeted pathogen detection because of their higher efficiency and accuracy compared to conventional methods such as bacterial culture and ELISA [20]. In particular, multiplex PCR increases the throughput and simplifies the procedure. Additionally, quantitative PCR (qPCR) enables the quantification of the pathogens. TaqMan Array Card (TAC) is a multiplex qPCR based microfluidic card that harbors 48 individual qPCR reactions that are physically separated for each of the 8 samples. This approach is greatly beneficial when a large number of pathogens need to be screened, including human diseases and animal or environmental surveillance [21–26].

We previously developed TAC cards for the detection of respiratory, enteric, and bloodstream pathogens as well as AMR genes, and utilized them widely for the surveillance of the relevant human diseases [22, 27–31]. In the current study, we applied these TACs to screen for 99 human disease related pathogens and 20 AMR genes in feces of migratory birds collected from ten regions along the migration routes in China to assess their potential transmission through bird droppings in the environment.

Methods

Sample collection

A total of 351 fresh fecal dropping samples from migratory birds were collected at ten bird wintering/stopover sites in China from September 2019 to November 2023, including Xingkai Lake in Heilongjiang, Cangzhou in Hebei, Longbao Nature Reserve in Qinghai, Grand View Pavilion and Dianchi Lake in Yunnan, Minjiang estuary in Fujian, Lingwu City in Ningxia, Wanghu Lake in Hubei, Tumuji National Nature Reserve in Inner Mongolia, Qingdao Zhanqiao in Shandong, and counties along the Yarlung Zangbo River in Xizang. The samples were preserved in the transport medium (0.9% NaCl, 0.2 g/L penicillin, 2 g/L streptomycin sulfate, 20% glycerol), transported with cold chain, and stored at − 80℃ until testing.

Total nucleic acid extraction

Nucleic acid extraction was performed using the cador Pathogen 96 QIAcube HT kit with the automated QIAcube HT system (Qiagen, Hilden, Germany). Briefly, 200 mg or 200 µL of fecal sample was mixed with 0.8 mL of InhibitEX buffer (Qiagen, Hilden, Germany) and glass beads of 212 ~ 300 µm (Sigma-Aldrich, St. Louis, USA). The mixture was homogenized for four times in the OMNI Bead Ruptor 24 Elite (Kennesaw GA, USA) at 8 m/s for 30 s with an interval of 20 s, followed by incubation at 95 °C for 5 min. 200 µL of supernatant were collected after centrifugation and loaded onto QIAcube HT system for automated nucleic acid extraction. Two external controls (Phocine herpesvirus for DNA targets and MS2 bacteriophage for RNA targets) were spiked into each sample to monitor extraction and amplification efficiency. Each batch of extraction included one extraction blank to rule out laboratory contamination.

Detection of pathogens and AMR genes with TaqMan Array Cards

Three customized TaqMan Array Cards targeting pathogens (supplemental Table S1) and AMR genes (supplemental Table S2) involved in enteric, respiratory, and bloodstream infections, respectively, were adapted

from previous studies [22, 27–31], and manufactured by Thermo Fisher (Carlsbad, CA, USA). All the qPCR assays have previously been extensively validated (supplemental Table S1). In order to improve the throughput, 5 samples were pooled with equal volume, i.e. 15 µL each, then mixed with 25 µL of TaqMan Fast Virus 1-Step Master Mix (4×) reagent (Thermo Fisher) and loaded into each port of the TAC card following the manufacturer's instruction. Real time PCR was performed with QuantStudio7 Real-time PCR instrument (Thermo Fisher) and analyzed using QuantStudio7 Real-time PCR Software. The qPCR cycling conditions were set as 50℃ for 10 min, 95℃ for 20 s, 40 cycles of 95℃ for 3 s, and 60℃ for 30 s. Quantification cycle (Cq) of 35 was used to determine the positivity. The positive results were valid only when the corresponding extraction blank was negative for the relevant targets. The negative results were valid only when the corresponding external controls amplified with the expected signal. Pathogen and AMR gene copy numbers were further derived from Cq values based on the standard curves generated with pooled positive controls, and normalized as copy numbers per gram of feces.

Confirmation of positive pathogen detections

When a pooled sample was positive for a certain pathogen, the 5 individual samples of the pool were tested with the cognate qPCR assay on 96-well plate to identify the positive sample. The 10-µL reaction contained 2.5 µL of TaqMan Fast Virus 1-Step Master Mix, 9 pmol of primers, 2.5 pmol of probe, and 2 µL of nucleic acid extract. The cycling condition was the same as those for TAC.

For samples that amplified for *Cryptosporidium* 18 S rRNA, qPCR targeting *COWP* gene and nested PCR for 18 S rRNA gene (supplemental Table S3) followed by amplicon sequencing (Tsingke, Qingdao, China) were performed to confirm *Cryptosporidium* detection [32, 33].

Identification of bird types

For bird species identification, the published assay targeting mitochondrial cytochrome oxidase sub gene I (COI) were modified to accommodate a few more bird species in the current study [34], with the forward primer 5'- CA CGAATAAACAACATAAGCTTCTG-3', reverse primers 5'- CAGGGTGTCCGAAGAATC-3' and 5'- CTGGGTG-GCCRAARAATC-3'. The 20 µL reaction contained 10 µL of Taq Plus Master Mix II (Vazyme, Nanjing, China), 4 pmol of each primer, 1 µL of nucleic acid extract, and nuclease-free water up to 20 µL. The PCR cycling conditions were set as initial denaturation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. The PCR products were examined with gel electrophoresis and sent for amplicon sequencing.

Statistical analysis

The prevalence of multiple pathogens in each region was expressed as the percentage of samples positive by qPCR. One-sample t-tests were used to determine if there were statistical differences between regions and bird types. Pathogen quantities were compared with Mann-Whitney U test. SPSS software, version 26.0, was used for the analysis. Two-tailed p values were calculated, and values of < 0.05 were considered statistically significant.

Results

Distribution of migratory bird types

Based on our preliminary data with an average detection rate of 1.5% for major human disease associated pathogens such as *Campylobacter* and *Cryptosporidium* in migratory birds, the sample size per site was estimated to be 23. A total of 351 samples were collected as shown in Table 1. During fecal sample collection, the bird types were observed and recorded at some sites, including *Anser indicus* at Qinghai and Xizang sites, *Anser albifrons* at Heilongjiang and Inner Mongolia, and *Chroicocephalus ridibundus* at Yunnan and Shandong. These were confirmed by the COI amplicon sequencing (supplemental Table S4). Several *Anser* species were observed at Hubei site, while COI analysis identified them as *Anser cygnoides*, *Anser albifrons*, *Anser fabalis*, and *Anser anser*. A variety of migratory birds group lived at Fujian, Ningxia, and Hebei sites, and most were identified by COI as shown in Table 1. Overall, the bird species were identified for 94.3% (331/351) of the samples by COI sequencing, involving 15 genera in 4 orders (Table 1), with *Anseriformes* (52.4%, 184/351) and *Charadriiformes* (40.5%, 142/351) being the two predominate types.

Pathogen detection by pooled TAC followed by individual confirmation

Through testing on pooled samples by TAC followed by confirmation on each individual samples with qPCR on plate, a total of 14 pathogens were detected positive, including 8 (8/49) bacteria, 1 (1/27) virus, and 5 (5/17) parasites (supplemental Figure S1). None of the 6 fungi interrogated was detected. Additionally, *Cryptosporidium* results was further confirmed by *COWP* qPCR and nested 18 S rRNA PCR followed by amplicon sequencing (supplemental Tables S3 and S4) because the *Cryptosporidium* qPCR assay on TAC may detect algae that are genetically close and commonly present in the wetland [35]. At least one pathogen was detected in 107 samples (30.5%) (Table 2). *Aeromonas* (9.1%), *Plasmodium* (7.4%), and *Cryptosporidium* (5.1%) were the three most prevalent pathogens (Table 2). HIV I (0.9%) was the only virus detected.

Table 1 Migratory bird types identified by COI PCR amplicon sequencing in this study

Bird Order	Anseriformes		Charadriiformes									Gruiformes		Lariformes	Other*	Total
Bird Genus	*Anser*	*Aythya*	*Calidris*	*Charadrius*	*Chlidonias*	*Chroicocephalus*	*Himantopus*	*Numenius*	*Philomachus*	*Pluvialis*	*Recurvirostra*	*Tringa*	*Fulica*	*Grus*	*Sterna*		
Fujian	16	-	8	7	-	1	-	5	-	-	-	-	-	-	-	6	43
Hebei	2	-	4	-	2	9	4	-	-	-	-	2	-	-	3	14	40
Heilongjiang	28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	28
Hubei	31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	31
InnerMongolia	25	2	-	-	-	2	-	-	-	-	-	-	-	1	-	-	30
Ningxia	9	-	-	-	-	3	3	-	1	3	8	-	1	-	-	-	28
Qinghai	36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	36
Shandong	-	-	-	-	-	28	-	-	-	-	-	-	-	-	-	-	28
Xizang	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35
Yunnan	-	-	-	-	-	52	-	-	-	-	-	-	-	-	-	-	52
Total	184		142										2		3	20	351

* Bird species were not identified with COI

Distribution of pathogens in migratory birds by region and bird type

At least one pathogen was detected at each of the 10 habitat sites interrogated in the current study (Table 2), with Hubei (87.1%, 27/31) and Ningxia (64.3%, 18/28) having the highest detection rates while Hebei (7.5%, 3/40) and Fujian (2.3%, 1/43) had the least ($p < 0.05$). From the perspective of geographical distribution, multiple pathogens were detected in multiple regions. *Cryptosporidium* had a wide geographic distribution across 7 of the 10 sites (Table 2). On the contrary, some pathogens showed specificity to a certain region or a certain bird species, such as *Rickettsia* in *Charadriiformes* (*Himantopus* and *Recurvirostra*) at the Ningxia site, and *Borrelia* in three bird species of Hebei (*Chlidonias leucopterus*, *Anser indicus*, and *Larus relictus*). Enteropathogenic *E. coli* (EPEC) was exclusively detected in *Chroicocephalus ridibundus* while typical EPEC (tEPEC, possessing the *eae* and *bfpA* genes) was found in Shandong only and atypical (aEPEC, possessing the *eae* gene but lacking the *bfpA* gene) in both Shandong and Yunnan.

Since majority of the samples were collected from *Anseriformes* and *Charadriiformes*, the pathogen distribution was further compared between these two orders (supplemental Figure S2). Overall, the pathogen detection rate was significantly higher in *Anseriformes* (39.1%, 72/184) than *Charadriiformes* (23.2%, 33/142, $p < 0.05$). The parasitic pathogens were more prevalent in *Anseriformes*, particularly with *Plasmodium* being exclusively detected in *Anseriformes* while the bacterial pathogens more prevalent in *Charadriiformes* (Fig. 1). Most of the water-borne pathogens, such as *Cryptosporidium*, *Giardia lamblia*, and *Aeromonas* were detected in *Anseriformes* (supplemental Figure S2). The foodborne pathogens, *Campylobacter* and enteropathogenic *E. coli*, including both tEPEC and aEPEC, were mostly detected in *Charadriiformes.* Mixed pathogens were detected in 25.0% (18/72) of the positive samples from *Anseriformes* and 21.2% (7/33) in *Charadriiformes* (supplemental Figure S2).

Distribution of pathogens detected in *Anser* by season

Among the 182 samples from *Anser*, most were collected from autumn/winter (68.1%, 124/182). The pathogen detection rate varied from 29.6% (8/27) in spring, 22.6% (7/31) for summer, 24.1% (14/58) for autumn, and 65.2% (43/66) for winter ($p < 0.05$). *Plasmodium*, *Aeromonas*, and *Entamoeba* were mostly detected in winter while *Cryptosporidium* distributed across summer, autumn, and winter. *Giardia lamblia* was mostly detected in spring (Fig. 2).

Table 2 The detection rate (%) of 14 pathogens identified in migratory birds at ten habitat sites in China

	Aeromonas	*Plasmodium*	*Cryptosporidium*	*Entamoeba*	*Giardia*	EPEC	*C. jejuni/coli*	*Rickettsia*	*Borrelia*	HIV	*Plesiomonas*	*V.cholerae*	*T.gondii*	*E. faecalis*	Total
Fujian	-	-	2.3	-	-	-	-	-	-	-	-	-	-	-	2.3
Hebei	-	-	-	-	-	-	-	-	7.5	-	-	-	-	-	7.5
Heilongjiang	-	10.7	14.3	-	-	-	-	-	-	-	-	-	-	-	17.9
Hubei	51.6	16.1	9.7	35.5	19.4	-	3.2	-	-	-	-	-	-	-	87.1
Inner Mongolia	3.3	-	-	-	20.0	-	-	-	-	-	-	-	3.3	-	23.3
Ningxia	42.9	-	3.6	-	-	-	-	21.4	-	-	3.6	7.1	-	-	64.3
Qinghai	-	8.3	11.1	-	-	-	-	-	-	5.6	-	-	-	-	25.0
Shandong	10.7	-	7.1	10.7	3.6	25.0	3.6	-	-	-	-	-	-	-	35.7
Xizang	-	42.9	8.6	-	-	-	-	-	-	-	-	-	-	2.9	45.7
Yunnan	-	-	-	-	-	3.9	9.6	-	-	1.9	1.9	-	-	-	15.4
Total	9.1	7.4	5.1	4.0	3.7	2.6	2.0	1.7	0.9	0.9	0.6	0.6	0.3	0.3	30.5

EPEC included both typical EPEC and atypical EPEC

The pathogens in mixed infections were counted for each individual target

Estimation of pathogen quantities

The pathogen quantity was calculated based on the Cq values using the standard curves. The results showed that the quantities ranged from 10^3 to 2×10^8 gene copies per gram of feces (Fig. 3). The bacterial pathogens were mostly present at 10^4 to 10^7 gene copies per gram of feces. The pathogenic loads of *Aeromonas* and *Plasmodium* in Hubei were higher than those in Ningxia and Xizang ($p < 0.005$), respectively.

Detection and quantification of AMR genes

Of the 20 AMR genes associated with resistance to four classes of drugs including macrolides, quinolones, tetracyclines, and β-lactams, all were detected in 36 pooled feces from the migratory birds but two genes, i.e. *blaZ* and *ermA* (supplemental Table S2). Majority (30/36) of the pools were from *Anseriformes*, while six from *Charadriiformes*. Most of the pooled samples were positive for at least one AMR gene interrogated except 7 samples from *Anseriformes.* The quantities of each gene were calculated based on the Cq values using the standard curves, then averaged among the total number of samples tested, including the negatives, to estimate the overall exposure doses and compare between *Anseriformes* and *Charadriiformes.* Most of the genes were detected in both orders except *qnrA*, SHV, and TEM in only *Charadriiformes.* The averaged quantities were highly variable across genes even within the drug classes, from 10^3 to $>10^8$ gene copies/gram of feces (Fig. 4). When summing copies of genes belonging to the same class, the total quantities were estimated to be 10^5-10^9 copies/gram of feces.

Discussion

A multi-pathogen detection tool, TaqMan Array Card, was used in the current study to screen 99 pathogens and 20 AMR genes in the feces collected from a variety of migratory birds at different habitats of China. The interrogated pathogens were all related to human diseases and the qPCR assays used were previously developed, validated, and utilized for etiological and epidemiological analyses. The results showed that 30.5% of the samples were positive for at least one of the 14 pathogens identified. The distribution of these pathogens revealed region and bird type specificity, and certain seasonality. The most prevalent pathogens included water-borne *Aeromonas*, *Cryptosporidium*, and *Giardia lamblia*, food-borne *Campylobacter* and EPEC, and vector-borne *Plasmodium* and *Rickettsia*. This method greatly improved the detection efficiency and further increased the diversity of intestinal microorganisms and pathogens carried by migratory birds.

Per the recommendation from the World Health Organization (WHO), quantitative microbial risk assessment (QMRA) includes four steps, i.e. hazard identification,

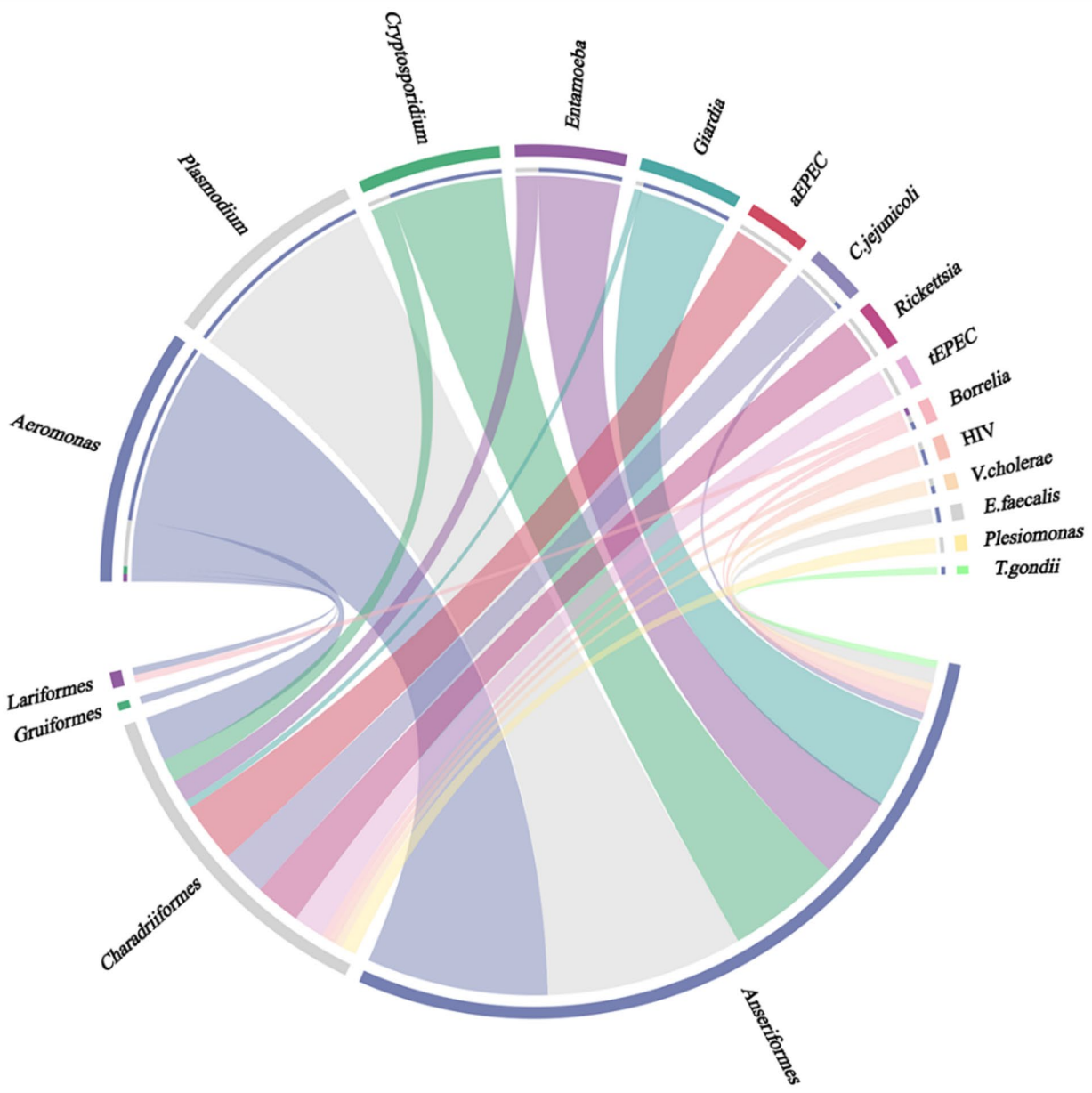

Fig. 1 Distribution of the detected pathogens in the different migratory bird orders

exposure assessment, dose-response modeling, and risk characterization [36]. qPCR based TAC platform enabled both detection and quantification of the pathogens. In the current study, the pathogen loads of bacteria ranged mostly between 20 and 1000 copies per mg of bird dropping. It is known that infection doses for some of the pathogens were low, such as 500–800 bacteria for *Campylobacter* [37]. Extensive estimation of the exposure doses of different pathogens in various hosts may be informative for more accurate risk assessment.

As expected, most of the pathogens were diarrhea associated enteropathogens. *Aeromonas* was the most common pathogen detected (9.1%, 32/351), predominantly in *Anseriformes*. About half of the migratory birds, mostly *Anser*, in Hubei and Ningxia carried *Aeromonas*. *Aeromonas* has previously been isolated from a variety of environmental sources, such as contaminated and drinking water, as well as tissues and body fluids of cold-blooded and warm-blooded animals [38–40]. It is commonly associated with sepsis in various aquatic organisms and gastrointestinal or parenteral diseases in humans [41]. The migration of waterfowl across national and intercontinental borders can provide a mechanism for the global spread of bacterial species. Halpern et al. identified three *Aeromonas* species in 19 birds of three different species with different abundance, suggesting that waterfowl had

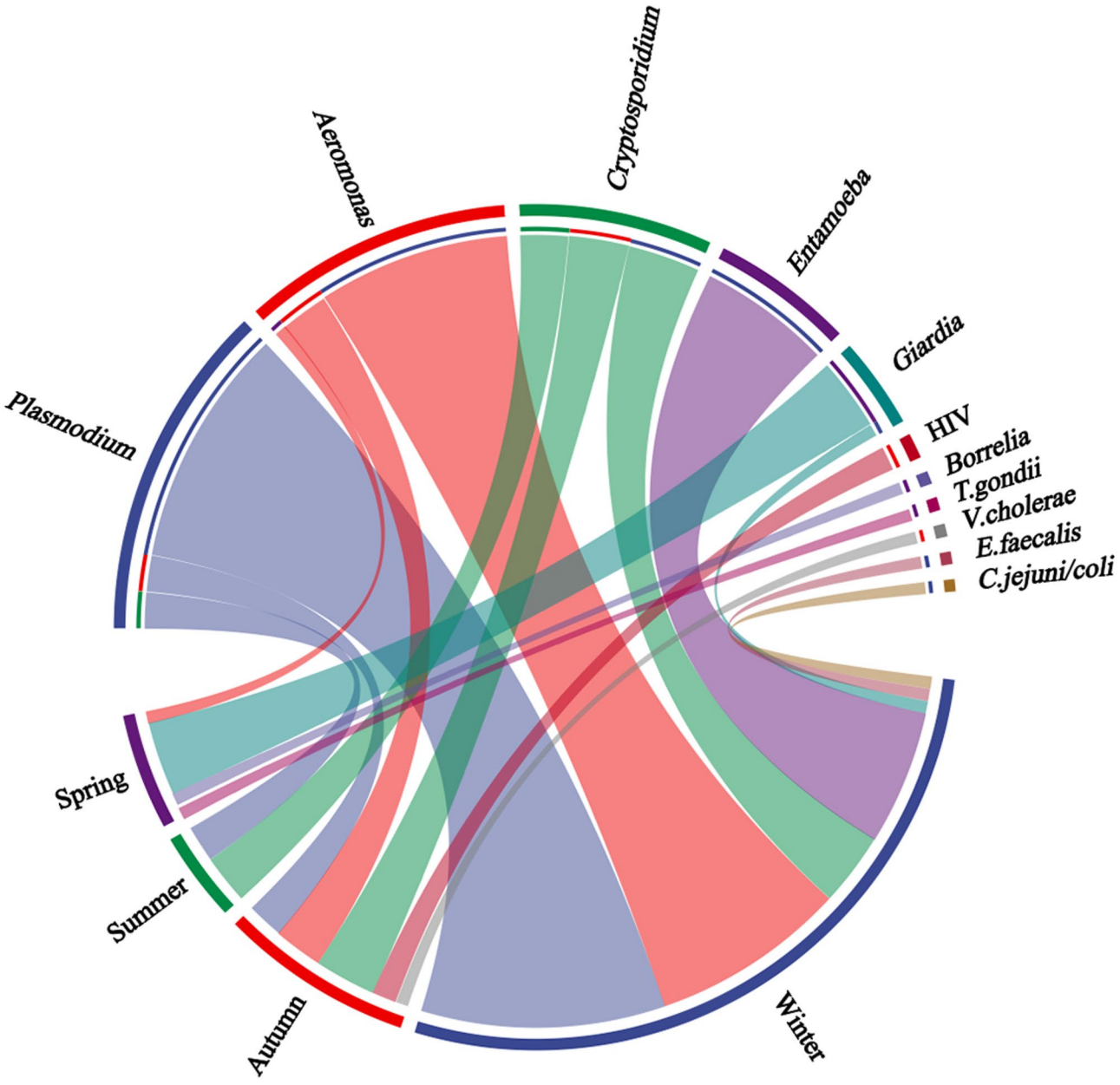

Fig. 2 Distribution of pathogens detected in *Anser* by season

the potential to transmit *Aeromonas* across continents, thereby establishing new endemic areas far from the source of infection [42]. Liang et al. analyzed the drug resistance, virulence, and genetic diversity of *Aeromonas* in migratory birds from Guangxi, Guangdong, Ningxia, Jiangxi and Inner Mongolia in China, and the results showed that migratory birds carried high virulence and multidrug-resistant *Aeromonas* [43]. The current study revealed its geographical, seasonal, and host distribution, then the next step would be to delve deeper into its molecular characteristics to assess the potential threat to public health.

Notably, the most prevalent enteric parasite was *Cryptosporidium* (5.1%, 18/351), which is consistent with the findings from previously studies. *Cryptosporidium* has a broad host range, including humans, domestic animals, birds, fish, etc., and it is mainly transmitted by fecal-oral route [44]. As one of the leading causative agents of mortality due to diarrhea in children under 5, it is a ubiquitous water contaminant and widely transmitted [45–48]. A large-scale epidemiological survey of *Cryptosporidium* in chickens and ducks in Henan, China, by Wang et al. showed that infection rates varied from 3.4% in broilers to 16.3% in Pekin ducks [49], while the detection rate of *Cryptosporidium* was only 1.7% among migrating

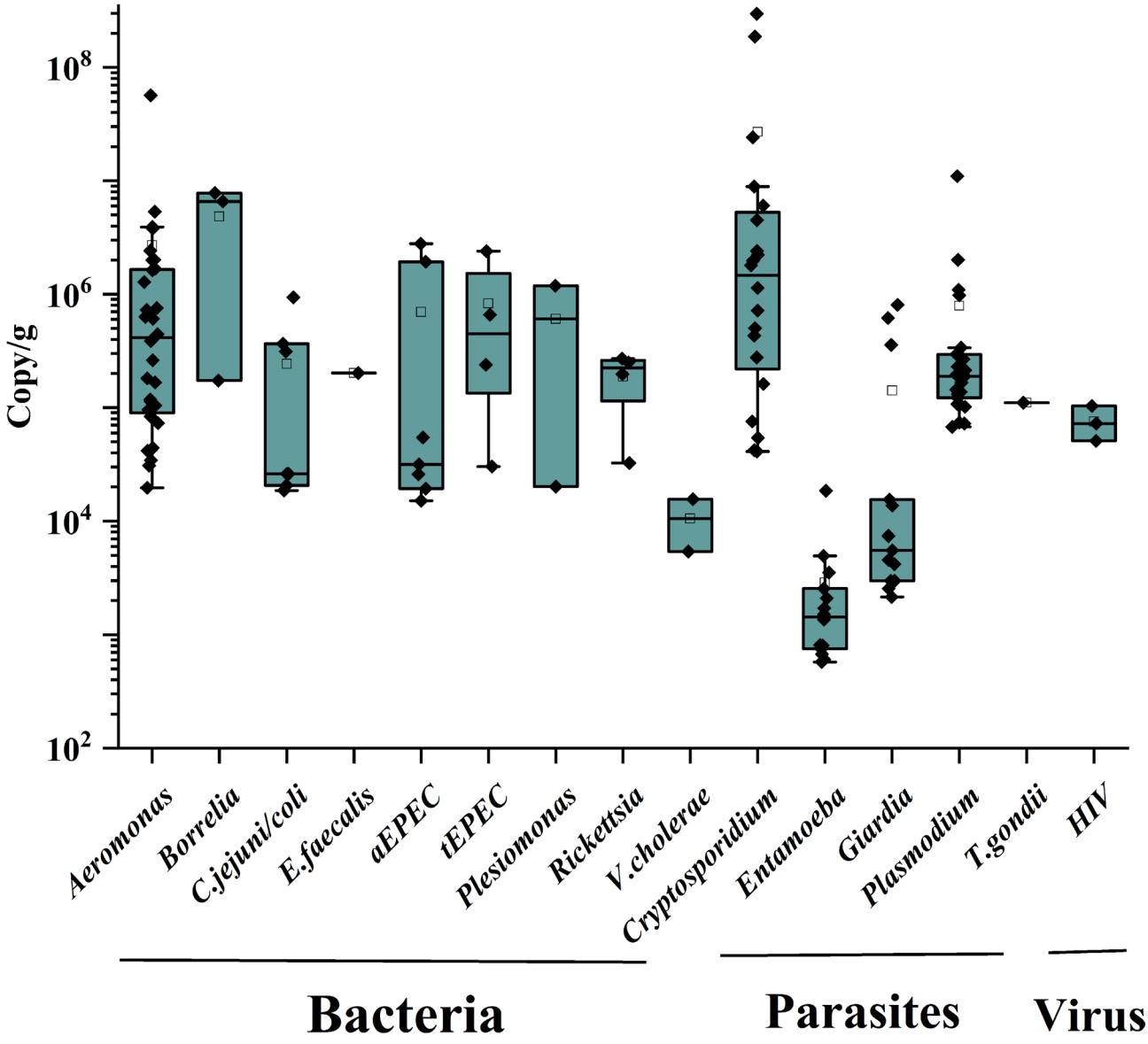

Fig. 3 Pathogen quantities in the fecal samples of migratory birds determined by qPCR. The quantity was expressed as the copy number of gene per gram of feces

whooper swans in the same area [50]. The total prevalence rates of *Cryptosporidium* and *Giardia* reported by Jian et al. in wild birds in Qinghai Lake and the surrounding areas of the Tibetan Plateau in China were 8.98% and 3.39%, respectively [51]. The overall prevalence of *Cryptosporidium* in animal inhabiting drinking water catchments in three states across Australia was 18.3% [52]. Cautions have to be taken to select the proper PCR assays for *Cryptosporidium* detection due to high homology of ribosomal RNA gene, as the most often used target, between *Cryptosporidium* and algae, which is often present in environment samples and interfere the assay specificity. We further performed amplicon sequencing to validate the *Cryptosporidium* detection, confirmed its broadest distribution at seven of the ten sites, mostly from *Anser*.

Furthermore, *Cryptosporidium* and *Giardia* have been considered as important indicators for water quality due to the high stability and resistance of their oocysts in the environment [53–55]. In our study, *Cryptosporidium* or *Giardia lamblia* or both were detected in migratory birds, mostly from *Anseriformes*, inhabiting at lakes, swamps, and other wetlands, so their droppings likely affect the water quality. When humans, poultry, or other animals come into contact with these waters, it increases the risk and spread of relevant waterborne infectious diseases [56]. Therefore, monitoring pathogens, including other waterborne such as *Aeromonas*, *V. cholerae*, carried

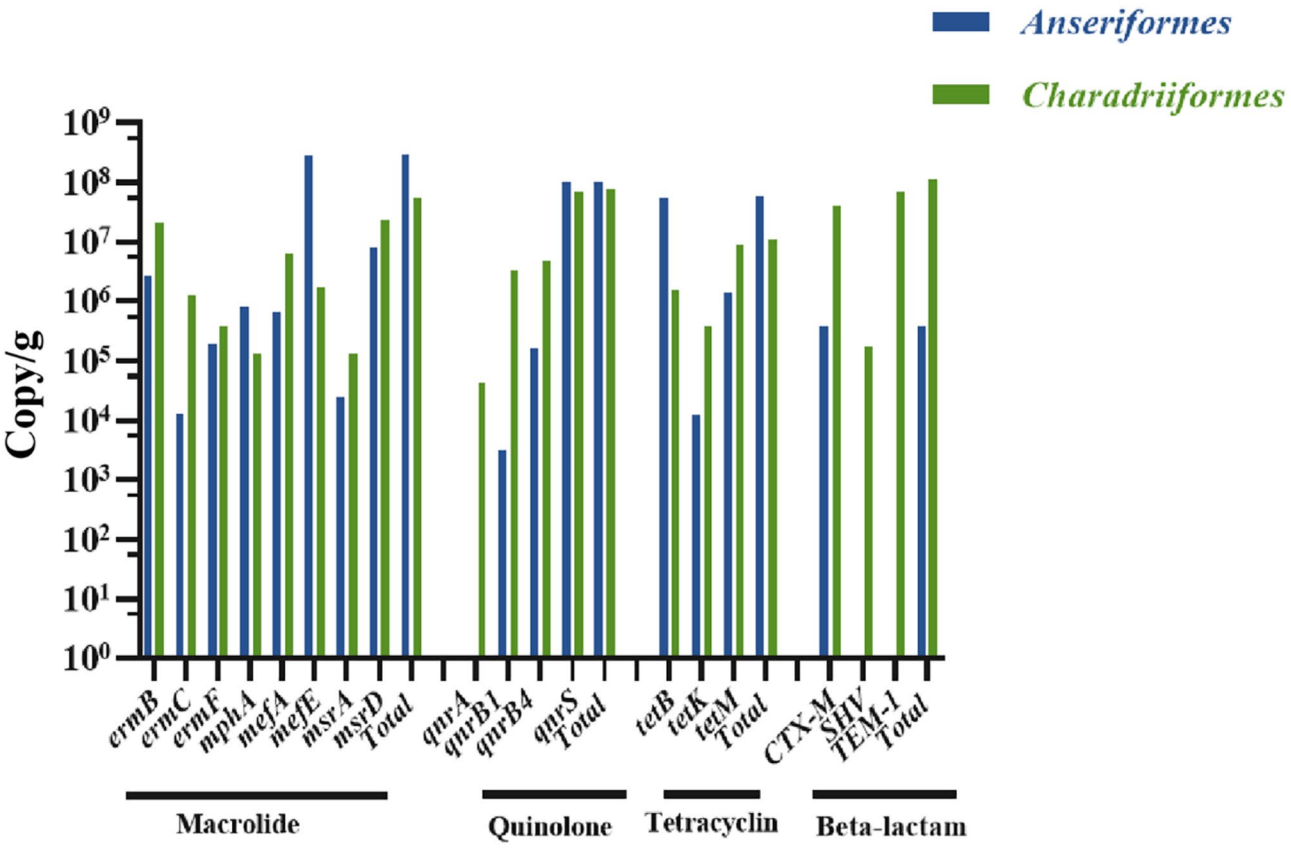

Fig. 4 Averaged quantities of AMR genes detected in *Anseriformes* and *Charadriifornes*. The quantity of each gene was averaged among all the samples tested regardless of positivity for the relevant gene. The total quantity indicates the sum of the averaged quantities of the genes belonging to the same drug class

by migratory birds is essential for early warning of water pollution and it is also necessary to strengthen the eco-epidemiological research on the transmission dynamics in endemic areas and reduce the impact on human health.

The detection of other enteric pathogens showed geographical relevance. For example, *V. cholerae* was detected in Ningxia. *V. cholerae* O1 and O139 have been considered to be the only serotypes that cause diarrhea. However, recent studies demonstrated non-toxigenic non-O1/non-O139 *Vibrio cholerae* are also associated with diarrheal disease globally [57], indicating the importance of further characterization of these *V. cholerae* positives. *C. jejuni/coli* and EPEC were mostly detected in *Chroicocephalus ridibundus* in Shandong and Yunnan. Interestingly, aEPEC were present in birds from both regions while tEPEC only from Shandong, which likely reflected the difference in ecological environments.

In our study, some vector-borne pathogens were also detected in feces. *Plasmodium* was detected exclusively from *Anser* across four sites. Previous studies have suggested that migratory bird species might carry blood parasites with high diversity and prevalence. In addition, *Rickettsia* and *Borrelia*, as tick-borne pathogens, were detected in Ningxia and Hebei, respectively. Migratory birds, as hosts for ticks, may facilitate the spread of pathogens to new geographic areas through migration. Studies have shown that migratory birds may increase the risk of human exposure to ticks and their infections, especially during seasons of high tick activity [58–60]. Parker et al. in their analysis of tick and bird hosts showed that infection intensity was the greatest in birds captured during autumn migration [61]. In this study, six *Rickettsia*-positive samples were all collected from birds of *Anseriformes* in autumn in Ningxia while three *Borrelia* samples from different birds in Hebei. Hebei may serve as an important stopover site for birds along multiple migration routes, where a large number of birds congregate, potentially facilitating host exchange among birds. The reasons or mechanisms that such bloodstream pathogens were present in feces require further investigation.

As a demonstration of proof-of-concept, 20 AMR genes related to four important drug classes were tested along with the pathogens. Most were detected positive with the exposure dose mostly between 10^7 and 10^8 copies/gram of feces. Metagenomic sequencing has revealed

a broad range of AMR genes in a variety of migratory birds [10]. By comparison (supplemental Table S2), our qPCR results revealed the presence of several AMR genes that were not identified by metagenomic sequencing, including *mphA*, *msrA*, and *msrD* for macrolide resistances, *qnrA*, *qnrB1*, *qnrB4*, and *qnrS* for quinolone resistances, CTX-M for β-lactam resistance. These discrepancies are worth further clarification because of the importance of these resistances in human health and poultry industry. Admittedly, only a small subset of the AMR genes was interrogated in the current study. Extensive and systematic targeted screening with such high throughput approach may provide more comprehensive understanding of the drug resistances carried and spread by migratory birds.

There are a few limitations in this study. Due to the sampling accessibility, temporal variation at a certain region was not captured, and the sample sizes for some migratory bird types were small. The individual specimens from a few pooled samples didn't amplify for the relevant targets, leading to a potential underestimate of the detection. Alternative methods may be required to confirm these results. The presence of the pathogens was determined only by qPCR, without the confirmation of the viability or pathogenicity. While most of the targets were tested at the species level, several were at genus level, such as *Plasmodium*, *Rickettsia*, *Aeromonas*, etc. (supplemental Table S1). Currently targeted and metagenomic sequencing is ongoing to further characterize the genetic features, virulence, diversity, and host specificity of the detected pathogens, which can be novel species or strains as described previously [62]. The coverage of AMR gens was limited in the current study. More broad screening is needed for a comprehensive profile of drug resistance that can be spread by the migratory birds. Furthermore, similar high throughput strategy is being implemented to screen the environmental samples and those from poultry farms to explore the link and the potential transmission between the wild and domestic birds.

Conclusions

In conclusion, TaqMan Array Card enabled high throughput screening of a broad range of pathogens and AMR genes in migratory birds to evaluate the potential hazardous effect to the environment. The advantage of this platform is modular, with the flexibility to accommodate additional targets including virulence genes, drug resistance genes, and any emerging agents, e.g. arboviruses such as Usutu virus. Further combined with a one health approach, future studies using such a tool would be of great value to estimate the spread risks from wildlife to domestic animals and humans. Rapid and long-term surveillance may provide guidance to implement targeted interventions to reduce the risk of pathogen transmission and zoonotic outbreaks.

Abbreviations

TAC	TaqMan Array Card
qPCR	Quantitative polymerase chain reaction
AMR	Antimicrobial resistance
EPEC	Enteropathogenic *Escherichia coli;*
tEPEC	Typical EPEC
aEPEC	Atypical EPEC
COI	Cytochrome oxidase sub gene I

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04059-4.

Supplementary Material 1

Author contributions

J.Liu. conceptualized the study. R.J. and Y.W. collected and processed the samples. L.W., R.M., J.Li, S.W, Y.F., and D.Z. performed the pathogen/AMR detection. L.W., G.Z., and J.Liu interpretated and analyzed the data. L.W. and J.L. wrote the original manuscript and L.W. prepared all the figures and tables. All authors reviewed the manuscript.

Funding

National Key Research and Development Program of China (2021YFC0863400, To J.Liu); State Key Laboratory of Veterinary Public Health and Safety (2024SKLVPHS06, To J.Liu); National Natural Science Foundation of China (no. 32070530 to G.Zhang).

Data availability

The sequences generated during the current study are deposited and available at NCBI, with the accession numbers PQ047601, PQ047618-PQ047627, PV124875-PV124878, PV124901-PV124936.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Medical College of Qingdao University (QDU-AEC-2024388).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 9 February 2025 / Accepted: 19 May 2025
Published online: 06 June 2025

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