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NOTE

Avian Pathology

Microbiological identification and analysis of waterfowl livers collected from backyard farms in southern China

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ABSTRACT. In total, 985 livers were collected from 275 backyard waterfowl farms distributed in seven provinces of southern China. The virus that was most commonly isolated was avian influenza virus, with a 12.1% positivity rate. Of the other positive samples, 10.6% tested positive for avian Tembusu virus, 6.8% for duck hepatitis A virus, 3.8% for duck plague virus, 3.4% for Muscovy duck parvovirus, 3.1% for goose parvovirus, 1.0% for mycoplasma and 0.9% for respiratory enteric orphan virus. The bacterium that was most commonly isolated was *Escherichia coli*, with a 47.1% positivity rate. This survey suggests that backyard waterfowl in southern China could be an important vector for the storage, variation, and transmission of various pathogens.

KEY WORDS: backyard farms, liver, microbiological identification, waterfowl

China is one of the world's largest producers of poultry products. In 2016, 18.88 million tons of meat and 30.95 million tons of eggs were produced according to the National Bureau of Statistics of China [24]. Infectious diseases have a significant economic impact on the poultry industry. In 2004, the total loss caused by the H5N1 avian influenza virus (AIV) was more than 10 billion dollars in China [22]. Since 2013, H7N9 AIV infections in birds have impacted human welfare and caused financial losses of more than 152 billion U.S. dollars [11, 34]. Domesticated waterfowl, an important division of poultry, are known to be natural reservoirs of some pathogens and are responsible for the evolution, maintenance, and spread of diseases such as AIV and Newcastle disease virus (NDV) [3, 8, 35]. Therefore, it is important to characterize and survey potentially pathogenic microbes in waterfowl flocks to protect public health.

Although commercial poultry farming has increased in China, there are still several backyard farms that are an important source of eggs and meat, especially in southern China. In a backyard poultry farm, standard hygienic practices and organized health management are not generally applied, and contact with wild birds, pets, and farm animals is frequent [7, 26]. Therefore, the health status of such flocks is generally poor. In 2002–2003, an outbreak of exotic Newcastle disease (ND) in a Californian backyard flock soon spread to commercial poultry farms as well as to backyard flocks in other countries [13]. In 2006–2007, H5N1 virus was detected in nine African countries and most of the reported cases occurred on backyard poultry farms [30]. Due to their contact with the outside environment, backyard birds play a key role in the transmission of infectious diseases. Previous studies have shown that backyard flocks carrying pathogens can also be indicators of zoonoses that circulate in wild birds and other domestic animals [26]. Nevertheless, there is very little information about potential disease infection of these flocks in China even though it is a country with many backyard farms.

The liver is a central organ that performs a wide range of essential functions, including digestion, absorption, synthesis, and storage. The liver is also an important barrier mechanism for organisms and is the target organ of various pathogenic microorganisms. Bacteria such as *Escherichia coli*, *Salmonella* spp., *Pasteurella multocida* and *Riemerella anatipestifer* [2, 9, 16, 20], and viruses such as duck hepatitis virus (DHV) [15] and duck plague virus (DPV) [17], can be isolated from avian livers. Additionally, hepatic tissue has a greater ability to regenerate than many other tissues, and up to 80% of hepatic function can be compromised before a disease process becomes clinically apparent [18]. Therefore, the liver also plays a key role in early diagnosis and monitoring the progress of various diseases.

The main objectives of the current study were to gather data to determine the prevalence of microbiological populations in the

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Table 1. The prevalence of individual virus isolated from 849 liver samples of backyard waterfowl in Southern China

Pathogen isolated	Host (No. of samples)					
	Muscovy duck (n=364)	Mule duck (n=104)	Cherry Valley duck (n=80)	Sheldrake (n=225)	Goose (n=76)	Total (n=849)
AIV	51 (14.1%)	9 (8.4%)	4 (4.7%)	29 (12.8%)	10 (13.2%)	103 (12.1%)
ATV	5 (1.4%)	1 (1.2%)	9 (10.9%)	66 (29.4%)	9 (11.5%)	90 (10.6%)
DHAV	19 (5.2%)	16 (15.6%)	6 (7.8%)	14 (6.1%)	3 (3.3%)	58 (6.8%)
DPV	9 (2.4%)	8 (7.2%)	0 (0%)	16 (7.2%)	0 (0%)	33 (3.8%)
MDPV	15 (4.1%)	14 (13.5%)	0 (0%)	0 (0%)	0 (0%)	29 (3.4%)
GPV	2 (0.6%)	2 (1.9%)	11 (13.8%)	0 (0%)	11 (14.8%)	26 (3.1%)
AIV + ATV	0 (0%)	0 (0%)	0 (0%)	13 (5.6%)	0 (0%)	13 (1.5%)
REOV	4 (1.0%)	3 (2.4%)	1 (1.6%)	0 (0%)	0 (0%)	8 (0.9%)

livers of waterfowl carcasses in backyard flocks in southern China, to gain a better understanding of a particular epidemiological characteristics of backyard waterfowl flocks, and to provide a scientific basis for the clinical diagnosis and prevention of pathogens that have health implications for both waterfowl and humans.

Waterfowl carcasses were collected from 275 backyard waterfowl farms distributed throughout Jiangsu, Anhui, Zhejiang, Jiangxi, Fujian, Guangdong, and Guangxi Provinces, southern China, from January 2014 to December 2016. The majority of the backyard farms surveyed contained reared waterfowl for meat or egg-laying, with between 50 and 1,000 birds per farm. Most of the waterfowl backyard farms we surveyed were in a semi-open environment and were easily exposed to wild animals, birds, and people. Therefore, we analyzed the status of communicable waterfowl disease in order to attract the attention of relevant personnel. After a primary diagnosis of the prevalence of the situation, clinical symptoms, and previous disease history, 985 livers were collected. We divided the samples into sections for processing in bacteriology or virology laboratories. In total, 849 suspected cases comprising 364 Muscovy ducks (*Cairina moschata*), 104 mule ducks (a cross between *Cairina moschata* and Cherry Valley ducks), 80 Cherry Valley ducks, 225 sheldrakes (including Jinding ducks, Shaoxing ducks, and domestic ducks in Longyan city, Fujian Provinces), and 76 geese (*Anser domestica*), were examined for virus infection and isolation. Overall, 136 livers of suspected bacterial infection cases were collected under strict sterile manipulation and quantified by culture and bacteriological analysis.

In the laboratory, all virus isolation from the liver samples and PCR-based genetic identification of isolates were carried out according to previously reported protocols [1, 12, 19, 21, 25, 32, 37]. All isolates were obtained by specific pathogen-free embryonated duck egg inoculation, and were identified using specific primer pairs for AIV [19], avian Tembusu virus (ATV) [32], duck hepatitis A virus (DHAV) [1], DPV [12], Muscovy duck parvovirus (MDPV) [21], goose parvovirus (GPV) [21], mycoplasma and respiratory enteric orphan virus (REOV) [25, 37]. Bacteria culture and detection were performed following standard operating procedures outlined in the Manual of Clinical Microbiology, including setup, culture, identification, and typing. The samples suspected to have bacterial infection were plated on tryptic soy agar (Difco, Detroit, MI, U.S.A.) containing 10 μg/m*l* NAD (Sigma, St. Louis, MO, U.S.A.) and 5% bovine serum, MacConkey agar, and blood agar (5% fresh sheep blood). All plates were incubated at 37°C in air for a minimum of 48 hr [23, 29]. Minimum inhibitory concentration (MIC) values for all isolates were determined by using the agar dilution method and adhered to Clinical and Laboratory Standard Institute (CLSI) standards [6].

Clinical data were collected by retrospective analysis of the protocols of 985 domesticated waterfowl cases. The data were statistically analyzed using the SPSS version 19.0 software (SPSS Inc., Chicago, IL, U.S.A.) and GraphPad Prism5 (GraphPad Software Inc., San Diego, CA, U.S.A.). Mean and standard deviation (SD) were used as descriptive statistics. Student's *t*-test was used for normally distributed variables. One-way analysis of variance (ANOVA) was used to detect significant changes and the differences between diseased animals and normal animals. *P* values <0.05 were considered statistically significant.

The virus that was most commonly isolated was AIV, with 103 (12.1%) positive samples. The subtypes of AIV isolates were also determined and has co-published with other cooperative units in previous works [4]. Other positive samples included 90 ATV (10.6%), 58 DHAV (6.8%), 33 DPV (3.8%), 29 MDPV (3.4%), 26 GPV (3.1%) and 8 REOV (0.9%), while 13 samples (1.5%) were positive for both AIV and ATV (Table 1).

AIV, ATV, and DHAV have a wide range of hosts and were isolated from Muscovy duck, mule duck, Cherry Valley duck, sheldrake, and goose. ATV was found in 66 out of 225 sheldrakes examined (29.5%), which was significantly higher than the rate in other hosts (P<0.05). The typical clinical symptoms of sheldrakes infected with ATV are a decrease in egg laying but with a low mortality [28]. However, we found that 13 out of the 225 the sheldrakes examined (5.6%) were co-infected with AIV and ATV, which increased mortality according to clinical investigations. In addition, we found that the lesions of some Muscovy duck (n=14, 24.2%) or mule duck (n=9, 15.5%) infected with DHAV were obviously altered, which was characterized by hemorrhagic or yellowed pancreatitis, but congestion and hemorrhage of the liver were not present (Table 2) (Fig. 1). We tentatively entitled the disease "pancreatitis" and determined the molecular characteristics [10], but the pathogenic mechanisms need to be studied further.

Duck plague (DP) was once an important disease of ducks in china, but the occurrence of the disease has been quite rare since the introduction of the DPV vaccine. Recently in China, DP has been occasionally reported in some duck flocks following DP vaccinations and has resulted in tremendous economic losses to the duck industry [33]. In our research, DPV was isolated from Muscovy duck, mule duck, and sheldrake, with an isolation rate of 3.8%. Therefore, the cause of this recent disease breakout needs

Table 2. Gross pathology caused by duck hepatitis a virus in backyard waterfowl

Host	No. of samples	Hepatitis [a)]	Pancreatitis [b)]
Muscovy duck	19	5 (8.6%)	14 (24.1%)
Mule duck	16	7 (12.1%)	9 (15.5%)
Cherry Valley duck	6	6 (10.3%)	0 (0%)
Sheldrake	14	14 (24.1%)	0 (0%)
Goose	3	3 (5.2%)	0 (0%)
Total	58	35 (60.3%)	23 (39.7%)

a) Liver enlargement or bleeding, b) Pancreatic yellowing or hemorrhage.

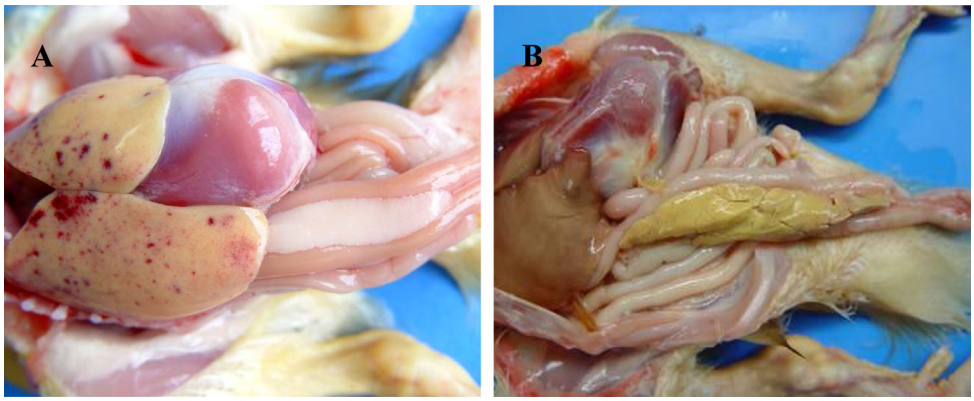

Fig. 1. Different gross lesions in ducklings infected with duck hepatitis A virus. A) Liver enlargement and internal bleeding (age: 10 days); B) Pancreatic yellowing or hemorrhaging (age: 18 days).

to be elucidated further.

Another frequently isolated virus was waterfowl parvovirus which can be divided into two groups, MDPV and GPV [27]. Our study shows that MDPV was only found in Muscovy duck and mule duck, while GPV was isolated in four species of waterfowl: Muscovy duck, mule duck, Cherry Valley duck, and goose. Moreover, according to our clinical diagnosis, we found that both MDPV and GPV may cause the newly emerged "Dwarf Syndrome" in ducks. This may be due to new recombinant strains of both MDPV and GPV; similar results have been documented in previous studies [5, 14, 31, 36]. Further statistical analysis found that MDPV merely causes duck "dwarfism" and "short beak" (n=20, 70.0%) in Muscovy or mule duck, while GPV could cause dwarfism, short beak, and protruding tongue in Cherry Valley duck (Table 3) (Fig. 2), but the difference of biological characteristics, pathogenicity, and pathogenesis of these two recombinant strains also needs to be elucidated further. In the same flock, ducks with a lower body weight than normal ducks ($P<0.05$) were diagnosed with "dwarfism"; ducks with the ratio of beak length to weight lower than that of the normal duck ($P<0.05$) were diagnosed as "short beak"; ducks with a longer tongue than beak ($P<0.05$) were diagnosed as "protruding tongue"; ducks were classified as normal in the absence of any positive diagnosis among the ducks tested by RT-PCR for the above pathogens.

The identities and frequency of each bacterium isolated and the antimicrobial susceptibility tests are shown in our study. The bacterium that was most commonly isolated was *E. coli*, with 64 positive samples (47.1%). In addition, 21 samples (15.4%) were positive for *R. anatipestifer*, 10 samples (7.4%) were positive for *P. multocida*, six samples (4.4%) were positive for *Salmonella* spp., and five samples (3.7%) were positive for both *E. coli* and *Salmonella* spp. In addition, we detected Mycoplasma in nine of 985 liver (1.0%) samples. Antimicrobial susceptibility tests showed that all isolates had multiple antimicrobial resistance and no antimicrobials were sensitive to all strains. Nineteen out of 64 *E. coli* strains (29.7%) were susceptible to florfenicol, while 22 strains (34.4%) were resistant to all antimicrobials, which was the highest resistance among all isolates. In 21 strains of *R. anatipestifer*, sensitivity to cefazolin (71.4%), doxycycline (52.4%), florfenicol (85.7%), and actinospectacin (66.7%) was more than 50%, while two strains (9.5%) were resistant. In 10 strains of *P. multocida*, the antimicrobial sensitivity was above 50% for cefazolin (60.0%) and florfenicol (60.0%). In six strains of *Salmonella* spp., sensitivity to cefazolin (83.0%), enrofloxacin (83.0%), florfenicol (83.0%) and pediatric compound sulfamethoxazole tablets (66.7%) was more than 50%. In addition, 29.7% of *E. coli*, 85.7% of *R. anatipestifer*, 60.0% of *P. multocida*, and 83.0% of *Salmonella* spp. isolates were found to be more sensitive to florfenicol than to other antimicrobials. The above results will provide a reference for medicinal requirements in clinics.

In summary, this survey provides detailed information on the prevalence of microbiological populations in backyard waterfowls of southern China. Our results indicate that backyard waterfowl could be an important vector for the storage, variation and transmission of various pathogens, and should not be neglected. Therefore, further etiology and serological studies in backyard waterfowls are urgently required in China to monitor waterfowl infections.

Table 3. Frequency of different symptoms caused by Muscovy duck parvovirus (MDPV) or goose parvovirus (GPV) in waterfowl

Host	MDPV (n=29)				GPV (n=33)			
	Classical symptoms [a)]	Clinical symptoms			Classical symptoms [b)]	Clinical symptoms		
		Dwarfism [c)]	Short beak [d)]	Protruding tongue [e)]		Dwarfism [c)]	Short beak [d)]	Protruding tongue [e)]
Muscovy duck	9 (31.0%)	6 (20.7%)	6 (20.7%)	0 (0%)	2 (6.1%)	0 (0%)	0 (0%)	0 (0%)
Mule duck	0 (0%)	14 (48.3%)	14 (48.3%)	0 (0%)	2 (6.1%)	0 (0%)	0 (0%)	0 (0%)
Cherry Valley duck	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (6.1%)	9 (27.3%)	9 (27.3%)	9 (27.3%)
Goose	0 (0%)	0 (0%)	0 (0%)	0 (0%)	11 (33.3%)	0 (0%)	0 (0%)	0 (0%)

a) The classical symptoms in ducklings infected with MDPV include panting, anorexia, diarrhea, dehydration, and rapid emaciation. b) Ducklings infected with GPV have highly contagious gastrointestinal disease. c) Weight of the diseased duck is lower than that of the normal duck ($P<0.05$). d) Ratio of beak length to weight of the diseased duck is lower than that of the normal duck ($P<0.05$). e) Tongue is longer than the beak ($P<0.05$).

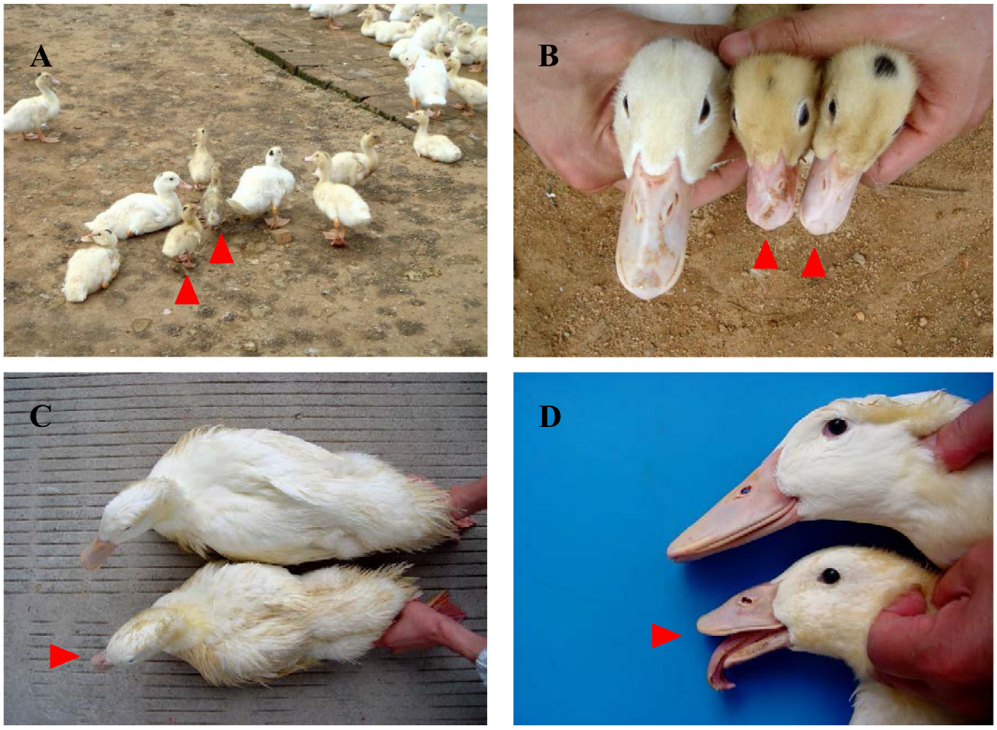

Fig. 2. Ducks infected with Muscovy duck parvovirus (MDPV) or goose parvovirus (GPV) causing the newly emerged "Dwarf Syndrome". A) Mule duck infection with MDPV results in dwarfism (age: 37 days); B) Mule duck infection with MDPV results in short beak (age: 37 days); C) Cherry Valley duck infection with GPV results in dwarfism (age: 41 days); D) Cherry Valley duck infected with GPV results in short beak and protruding tongue (age: 41 days). All the "Dwarf Syndrome" ducks are indicated by "▲".

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