

Reemergence of reticuloendotheliosis virus and Marek's disease virus co-infection in Yellow-Chickens in Southern China

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ABSTRACT The reticuloendotheliosis virus (REV) and the Marek's disease virus (MDV) cause reticuloendotheliosis (RE) and Marek's disease (MD) in poultry, respectively. According to epidemiological results obtained in our laboratory from 2010 to 2017, the positive rates of REV and MDV co-infection remained at low levels. In the present study, during the period of October 2018 to July 2020, 4 clinical cases with high morbidity (5%-20%) and mortality (2%-10%), caused by the co-infection of REV and vv+MDV-like strains, were diagnosed and analyzed by histopathological observation, cell cultures and detection with ELISA and IFA, and the PCR and by sequencing of the isolates' genes. Sequencing and the sequence analysis on the complete genomes of the REV strains and the *meq* genes of the MDV strains were performed. The results, based on the complete genome, LTR, *gag*, *pol*, and *env* genes' nucleotide

sequences of the REV strains, showed that the REV isolates and 68.0 % (17/25) of the reference strains were in a same branch, and all had a high sequence similarity (>99.0%). The similarities between the four isolates and a vv+MDV strain GX18NNM4 were very high, up to 99.3-99.8%. Also, the amino acid residuals at locations 71, 77, 80, 115, 139, 176, and 217 were all the same as A, E, Y, A, A, R, and A, respectively, in the *meq* gene of the four MDV isolates. In addition, the substitutes at P176R and P217A interrupted the stretches of the proline-rich repeat PPPP, indicating that these strains belonged to the vv+ MDV-like category. Our findings indicated that the more recent and frequent reemergence of REV and the subsequent co-infection with vv+ MDV-like strain has become one of the causes of the clinical outbreaks of tumors and is undoubtedly a threat to the poultry industry in southern China.

Key words: reticuloendotheliosis virus, marek's disease virus, reemergence, co-infection, yellow-chickens

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INTRODUCTION

Reticuloendotheliosis virus (REV) is an oncogenic and immunosuppressive retrovirus that causes reticuloendotheliosis (RE) (Xu et al., 2020). REV can cause neoplasia and immunosuppression (Sun et al., 2017b), as well as runting stunting syndrome (RSS) in many avian hosts, including chickens, turkeys, ducks, geese, pigeon, and many other wild birds (Zhai et al., 2016; Thontiravong et al., 2019; Alfaki et al., 2020; Caleiro et al., 2020; Liu et al., 2020). The initial REV

strain T was isolated from a turkey with visceral lymphomas in 1957 (Robinson and Twiehaus, 1974). In China, REV was first isolated in 1986 (Jiang et al., 2014), and spread rapidly throughout the country, causing great loss to the poultry industry (Yang et al., 2017). The full-length of the genome sequences of REV was 8.0-9.0 kb, including the LTR, *gag*, *pol*, and *env* genes (Jiang et al., 2012).

Marek's disease virus (MDV) is an oncogenic alpha-herpesvirus that causes Marek's disease (MD) (He et al., 2019). MDV induces lymphomas and immunosuppression in chickens (Faiz et al., 2018) and the clinical signs include paralysis, skin leukosis, depression and death (Gergen et al., 2019). According to the virulence of the virus, Species 1 MDV (MDV-1) can be clustered into mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) strains (Teng et al., 2011). The full-length of the genome sequences of MDV was 160-180 kb (He et al., 2019) and encodes a large number of genes.

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Among these genes the *meq* gene was one of the genes most associated with viral oncogenicity and pathogenicity (Shamblin et al., 2004).

Clinically, few cases were caused by REV infection alone as most of them were the co-infection of REV and MDV or other tumor viruses (Li et al., 2019b). A previous study showed that the co-infection of REV and MDV can increase illness severity and reduce MD vaccine efficacy (Sun et al., 2017b). According to epidemiological results obtained in our laboratory from 2010 to 2017, the positive rate of the co-infection of REV and MDV had remained at a low level (Li et al., 2019a). However, during a period from October 2018 to July 2020, 4 clinical cases of different breeds of Yellow-chickens with high morbidity and mortality were sent to our laboratory for diagnosis and the results showed that all these cases were the co-infection of REV and MDV.

MATERIALS AND METHODS

Case History

In the present study, during the period October 2018 to July 2020, diseased chickens of different breeds with emaciation, white combs and depression from clinical disease cases were sent to our laboratory for diagnosis. The ages of the diseased birds ranged from 78 to 120 day-old. The affected birds exhibited morbidities of 5% to 20% and mortalities of 2% to 10% (Supplementary Table S1) at the dates of delivery to the laboratory for diagnosis. All birds had been vaccinated with the commercial MD vaccine CVI988/Rispens at hatching.

Sample Collection and Processing

Tissue samples of significant tumor-like lesions were collected from all submitted birds, and the anticoagulant treated blood samples were collected also from the

same birds. One portion of each tissue was fixed in 10% buffered formalin and used for histopathological observations according to routine procedures (Wang et al., 2019). Another portion of the tissue was homogenized and made into a 1:3 (v/v) suspension with phosphate buffered saline, freeze-thawed three times at -80 °C and room temperature, and then filtrated through a 0.22 μm filter, and stored at -80 °C until used. Lymphocytes were separated from the blood samples by using the lymphocyte separation medium according to the routine procedure (Zhang et al., 2019).

Virus Isolation and Identification

DF-1 cells were used to isolate ALV as recently described (Wang et al., 2020). Briefly, the filtrated tissue suspension was inoculated into DF-1 cells for 2 hs, then replenished with DMEM containing 1% fetal calf serum (Life, Australia) and passed for three serial passages (7 days for each passage). Chicken embryo fibroblasts (CEFs) were used to isolate REV and MDV as recently described (Zhang et al., 2019). Briefly, the harvested lymphocytes were inoculated onto the primary CEFs grown in the 24-well plate (two wells per each sample at least) and the cultures were incubated at 37 °C in 5% CO₂ and passed for three serial passages (5 days per passage). The DF-1 cell cultures were used for the detection of ALV P27 antigen with the Avian Leukosis Virus Antigen Test Kit (Biochek, Holland) according to the routine procedure (Wang et al., 2020). The CEFs cultures, two wells from each sample, were used for the identification of REV and MDV by IFA using the monoclonal antibodies (mAbs) 11B118 against REV and BA4 against MDV, respectively (Cui et al., 2016). In order to detect the viral isolates' genes, DNA samples from the cell cultures were extracted by using a commercial DNA extraction kit (Tiangen, Beijing). The primers for the PCR detections are listed in Table 1, and the PCR procedure was performed as described previously (Li et al., 2019a).

Table 1. Primer sequences used for detection or amplification of REV and MDV isolates.

Name	Sequence (5'-3')	Expected size (bp)
MDV meq F	CCGTCTAGAAGGCGGGCACGGTAC	1113
MDV meq R	CGGAAGCTTAAACATGGGGCATAGACG	
MDV 132bpr F	TGCGATGAAAGTGCTATGGAGG	185, 317
MDV 132bpr R	GAGAATCCCTATGAGAAAAGCGC	449, 581
REV LTR F	CATACTGGAGCCAATGGT	291
REV LTR R	AATGTTGTAGCGAAGTACT	
REV-1-F	GCTATATAAGCCAGGTGC	1520
REV-1-R	ACTGGGCTTCTATTTCTCGCT	
REV-2-F	GTGGATTAGAGTTCGGAGCA	3300
REV-2-R	CCGTCCGGTTACCAGTTGA	
REV-3-F	TGATCTACAGAGAAAGGGG	1102
REV-3-R	CTTAACGAAGACGAGGTAC	
REV-4-F	GGTCTCAAGCCTCCTGTA	600
REV-4-R	CCACTGTTGTCTAAATCCC	
REV-5-F	CTCCTGACAACCAAGAAG	1825
REV-5-R	AGCTCCCTCCCACATTC	
REV-6-F	AATGTGGGAGGGAGCTCC	800
REV-6-R	GTAACAAACAAACACACAAACCAC	
REV-7-F	GTGCATACTGGCATCAATCG	1243
REV-7-R	CCCCCAAATGTTGTACCGAAAT	

Sequencing and Analysis of the Isolates' Gene and Genome

Seven primer-sets for the amplification of the full genome of REV and one primer-set for the amplification of the MDV *meq* gene were designed by our team with Primer Premier software version 5, listed in Table 1. The conditions for PCR were as follows: 95 °C, 5 min; 95 °C 15 s; 50-60°C, 15 s; 72 °C, 15s/kb (30 cycles), 72 °C, 10 min. The PCR products were purified using a Universal DNA Purification Kit (TIANGEN, Beijing, China), and then cloned into the vector pMD18-T (TaKaRa, Dalian, China), respectively. Three independent clones of each of the strains were sequenced by Beijing Genomics Institute (BGI, Guangzhou, China).

The nucleotide (nt) sequences were retrieved, edited using the EditSeq modules of DNASTAR (ver. 7.1.0). All the nt and amino acid (aa) sequences (including the isolates and reference strains) were analyzed using the ClustalW software program with DNASTARR (ver. 7.1.0). Phylogenetic analysis of the REV complete genome and MDV *meq* sequences were performed with the maximum-likelihood (ML) method using MEGA X (ver.10.1.7).

Ethics Statement

All the experimental animals of this study were cared for and maintained throughout the experiments strictly following the ethics and biosecurity guidelines approved by the Institutional Animal Care and Use Committee of Guangxi University (NO.2018-GXU-168).

RESULTS

Gross and Histological Observations

Postmortem examinations were performed on 4 to 6 diseased birds per case. The birds showed stunted growth, and prominent sternums (Figure 1A). Birds in case 1 had white nodules in the liver, spleen and kidney and some birds had marked hepatosplenomegaly (Figure 1B, 1C, 1D); Birds in case 2 had white nodules in the heart, proventriculus and cecum (Figure 1E, 1F, 1G); Birds in case 3 had white nodules in the chest wall (Figure 1H); Birds in case 4 had hyperplasia in the lung (detailed information is shown in Table 2). The histopathological observations of the tissue sections indicated that a large area of neoplastic lymphocytes was observed in the liver, spleen, kidney, heart, proventriculus, and cecum (Table 2, Figure 1I–1N). Specifically, the spleen, kidney, and proventriculus had been infiltrated by both neoplastic lymphocytes and primitive reticular cells (Table 2, Figure 1J, 1K, 1M).

Detection and Identification of the Isolates

The results of ELISA applied on the DF-1 cultures showed that all samples were negative for ALV (data not shown). The IFA applied on the CEF cultures using

the MDV-specific and REV-specific mAbs, respectively, showed that specific immunofluorescence signals were both detected in two wells of cultures from every sample (Figure 2A–2D, 2E–2H). The PCR detection results using the cell cultures also showed every well from each sample was positive for both MDV and REV (Figure 2K).

Sequencing Results of the Provirus Genome of REV and the MegGene of MDV Isolates

For the samples of each case, one REV isolate and one MDV isolate were used for the sequencing. Four REV provirus genomes (GX18NNR1, MW046297; GX19YLR1, MW046296; GX19NNR1, MW046295; GD20R1, MW046294) and four MDV *meq* genes (GX18NNM8, MW046300; GX19YLM5, MW046293; GX19NNM3, MW046292; GD20M1, MW046291) were successfully sequenced and submitted to GenBank. The complete genomes of the four REV isolates all had a length of 8284 bp with the typical replicative structure of γ -retroviruses. The nt-sequence similarities of the complete genome, LTR, *gag*, *pol* and *env* genes among the four isolates were 99.6–99.8%, 99.4–99.8%, 99.7–99.8%, and 99.7–99.9%, respectively (Supplementary Table S2). The *meq* genes of the four MDV isolate all had a length of 1020 bp and show 99.3–100% similarities among the four isolates (Supplementary Table S3).

Phylogenetic Analysis of the REV and MDV Isolates

The complete genome sequences of the four REV isolates and 25 other reference strains (isolated from chickens, geese, ducks, and wild birds in different countries and regions) from GenBank were aligned, and the identities of different regions of the genes (LTR, *gag*, *pol*, *env*) were assessed (Supplementary Table S2). The results showed that the sequence similarities between the four REV isolates complete genome and the 25 reference strains were 91.0–99.9%, and those of LTR, *gag*, *pol*, and *env* genes between the four REV isolates and all the reference strains were 91.0% to 100.0%, 95.7% to 99.9%, 95.9% to 100%, and 94.5% to 100%, respectively (Supplementary Table S2). All of the phylogenetic trees, which were based on the complete genome, LTR, *gag*, *pol*, and *env* genes, showed that 68.9% (20/29, including the four isolates in the study) of the REV strains were in the same branch, and almost all showed high similarities of more than 99.00% (Figure. 3A–3E, and Supplementary Table S2).

The comparisons of *meq* on the nt and aa sequences between the four MDV isolates and 26 other reference strains were assessed (Supplementary Table S3) and the similarities were 98.3–99.8% (Supplementary Table S2). It is noteworthy that the similarities between the four isolates and GX18NNM4, a new vv+MDV strain just identified by our group in 2020 (Shi et al., 2020), were

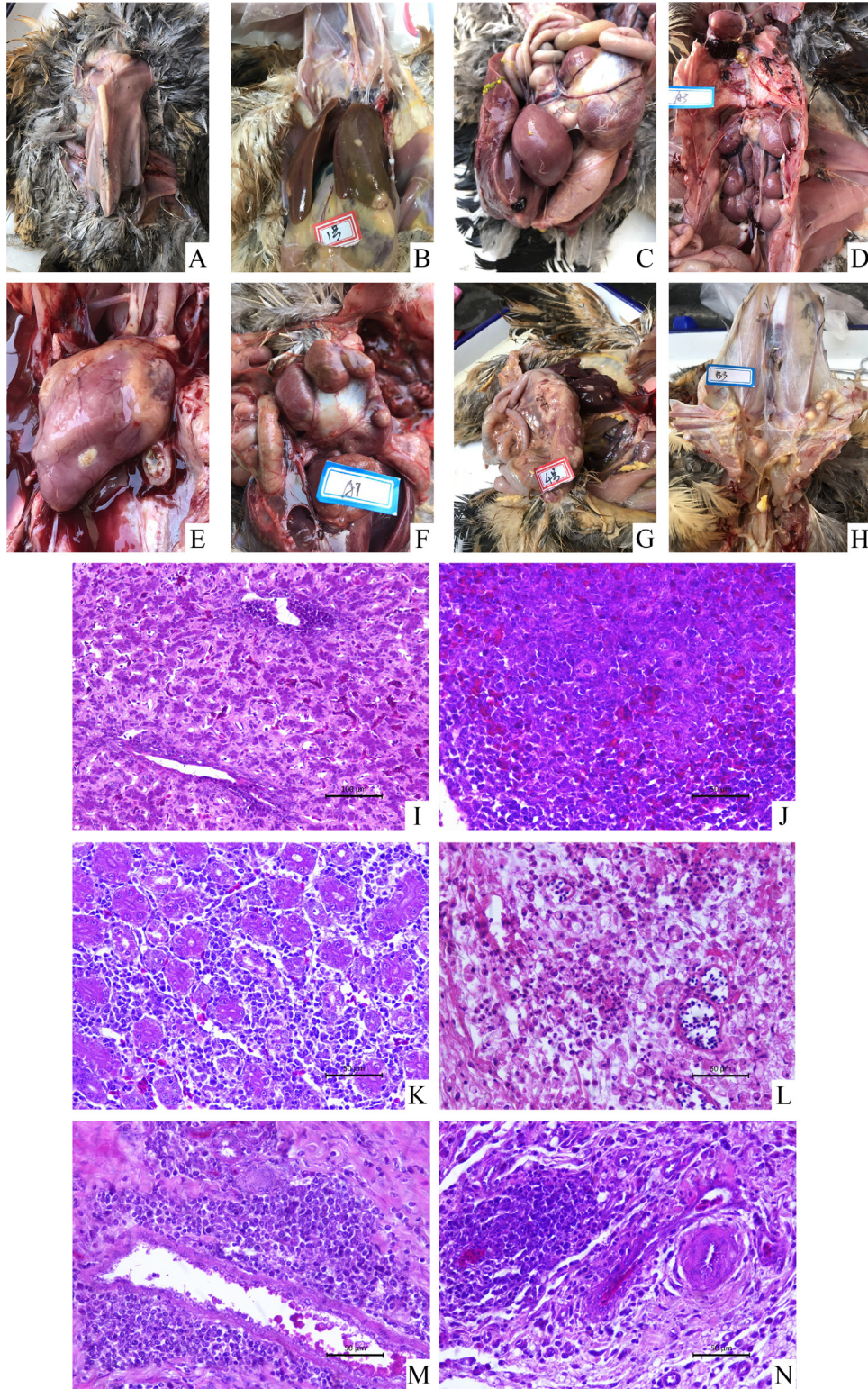


Figure 1. Gross and histopathological changes. The birds showed stunted growth, and prominent sternums (A). Birds in case 1 had white nodules in the liver, spleen and kidney (B–D). Birds in case 2 had white nodules in the heart, proventriculus and cecum (E–G). Birds in case 3 had white nodules in the chest wall (H). A large area of neoplastic lymphocytes was observed in the liver, spleen, kidney, heart, proventriculus, and cecum (I–N).

high, up to 99.3% to 99.8%. The phylogenetic tree showed that the four MDV isolates and 11 of the reference strains (including the new vv+MDV strain GX18NNM4) were in a same branch (Figure 3F). In addition, the aa residuals of the four MDV isolates at positions 71, 77, 80, 115, 139, 176, and 217 were all the

same as A, E, Y, A, A, R, and A, respectively (Table 3), consistent with the characteristics of the vv+ MDV strains like GX18NNM4. Also, the substitutes of P176R and P217A interrupted the stretches of the proline-rich repeat (PRR) PPPP, which might result in an increase in virulence (Mescolini et al., 2019).

Table 2. Histopathologic findings in the 4 clinical cases.

Case	Breed	Age (day)	Postmortem examination (4-6 chickens)	Microscopic examination
1	Native Chicken	120	Liver: hepatomegaly or tumor nodule Spleen: splenomegaly or tumor nodule Kidney: none or tumor nodule	Neoplastic lymphocytes, and primitive reticular cells Neoplastic lymphocytes, and primitive reticular cells Neoplastic lymphocytes
2	Three-Yellow chicken	95	Heart: none or tumor nodule Liver: hepatomegaly or tumor nodule Spleen: splenomegaly or tumor nodule Kidney: none or tumor nodule Proventriculus: swollen proventriculor or tumor nodule Cecum: none or tumor nodule	None or neoplastic lymphocytes Neoplastic lymphocytes, and primitive reticular cells Neoplastic lymphocytes, and primitive reticular cells Neoplastic lymphocytes Neoplastic lymphocytes
3	Native Chicken	100	Heart: none or pericardial effusion or tumor nodule Liver: hepatomegaly or tumor nodule Chest wall: none or tumor nodule Spleen: none or tumor nodule Kidney: none or tumor nodule	Neoplastic lymphocytes Neoplastic lymphocytes, and primitive reticular cells Neoplastic lymphocytes Neoplastic lymphocytes, and primitive reticular cells Neoplastic lymphocytes
4	Qingyuan-Ma Chicken	78	Heart: none or pericardial effusion or tumor nodule Liver: bleeding point or hepatomegaly or tumor nodule Spleen: none or splenomegaly or tumor nodule Lung: none or hyperplasia Proventriculus: swollen proventriculor or tumor nodule	Neoplastic lymphocytes Neoplastic lymphocytes, and primitive reticular cells Neoplastic lymphocytes, and primitive reticular cells None or Neoplastic lymphocytes Neoplastic lymphocytes

DISCUSSION

REV can be transmitted vertically in eggs, horizontally by contact with infected birds (Xu et al., 2020),

and also could disperse through the contamination of attenuated virus live vaccines (Schat and Erb, 2014). Previous studies have shown that some attenuated live vaccines against IBDV, MDV, HTV, and FPV are

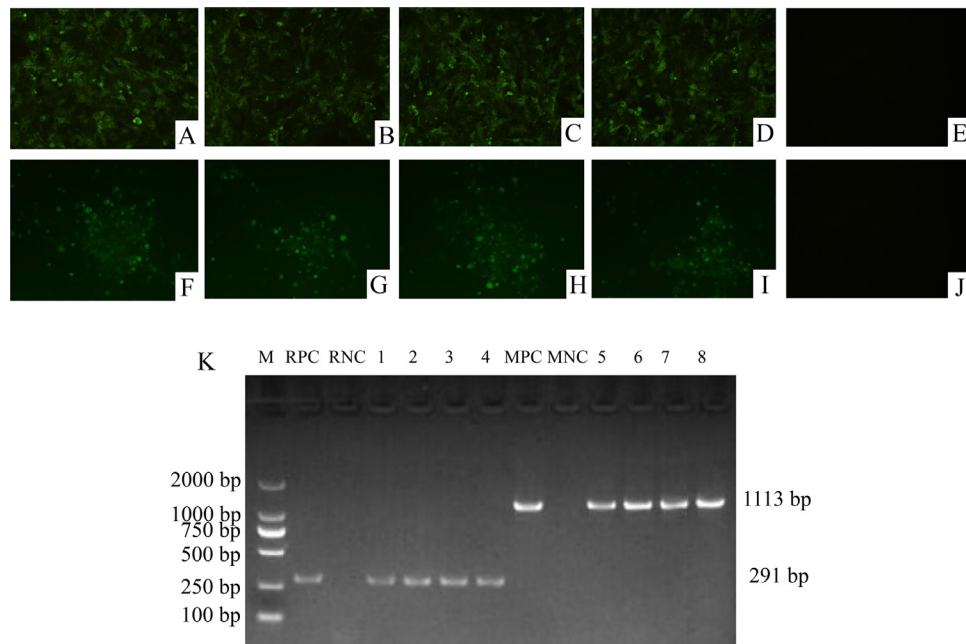


Figure 2. IFA and PCR results of CEF cell cultures infected with the samples. Positive fluorescence was observed in the GX18NNR1, GX19YLR1, GX19NNR1, and GD20R1 infected cells by the IFA with REV-specific monoclonal antibody 11B118 (A–D), while no specific fluorescence was observed in the non-infected cells (E). Positive fluorescence was observed in the GX18NNM7, GX19YLM5, GX19NNM3 and GD20M1 infected cells by the IFA with MDV-1 *gB*-specific monoclonal antibody BA4 (F–I). No specific fluorescence was observed in the non-infected cells (J). (K) PCR was used to identify the REV and MDV positive samples. M, Marker, DL2000; RPC, CEF cells infected with GD1210 as positive control; RNC, uninfected CEF cells used as a negative control; 1-4, positive PCR products obtained using the primers specific for REV LTR of GX18NNR1, GX19YLR1, GX19NNR1 and GD20R1, respectively; MPC, CEF cells infected with GX18NNM4 as positive control; MNC, uninfected CEF cells used as a negative control; 5-8, positive PCR products obtained using the primers specific for MDV meq of GX18NNM7, GX19YLM5, GX19NNM3 and GD20M1, respectively.

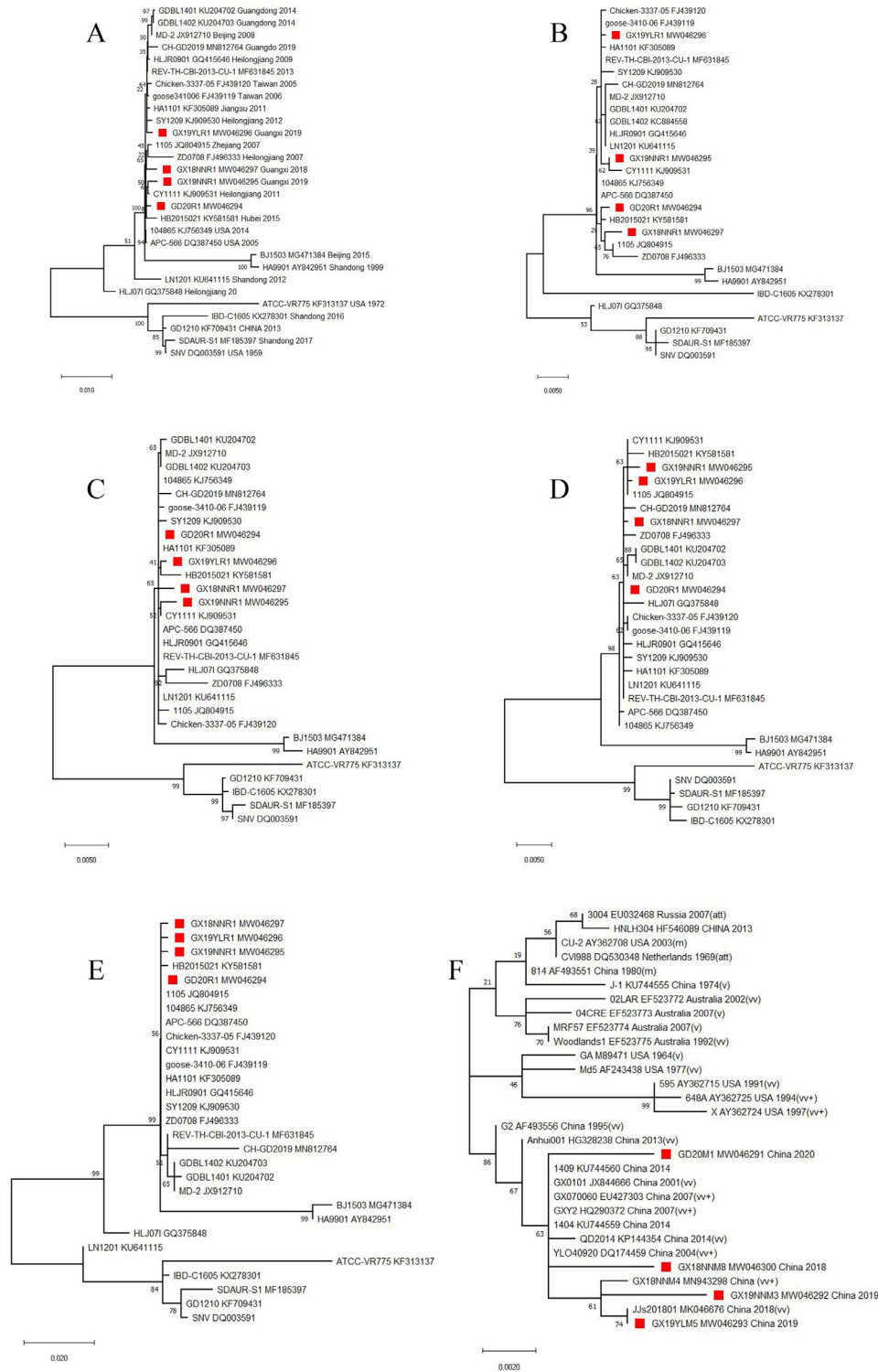


Figure 3. Phylogenetic trees based on the sequences of the complete-genome (A), LTR (B), *gag* (C), *pol* (D) and *env* (E) of REV strains, and *meq* gene of MDV strains (F). The phylogenetic trees were constructed by using the Maximum Likelihood method based on nucleotide alignment with the GTR Substitution Model with 1000 bootstrap replicates using the software MEGA X 10.1.7.

contaminated by REV (Fadly et al., 1996; Li et al., 2013; Li et al., 2015; Li et al., 2016), and we believe that this is one of the most common reasons for the high positive rate of REV infection in commercial chickens. A previous study showed that during the years 2005-2015, the seroprevalence in individual chickens in China ranged from 7.49% to 22.83% (Yang et al., 2017), indicating REV infection was common in China in this period.

Since the first description of MD in 1907, MD has spread rapidly all over the world (Eschke et al., 2018). At present, dramatic success has been achieved through the extensive use of the CVI988/Rispens and 814 vaccines (Ralapanawe et al., 2016; Reddy et al., 2017). Nevertheless, MD outbreaks have been continually reported in southern China in recent years (Zhang et al., 2015; Sun et al., 2017a; Li et al., 2019a), which is likely due to

Table 3. Amino acid substitutions in the MEQ proteins of the MDV strains.

Strains	71	77	80	115	139	176	217	Number of PPPP repeats
CVI988 (att)	S	E	D	V	T	P	P	8
CU-2 (m)	S	E	D	v	T	P	P	7
814 (m)	S	E	D	a	T	P	P	4
GA (v)	A	K	D	V	T	P	P	5
J-1 (v)	A	K	D	V	T	P	P	5
Md5 (vv)	A	K	D	V	T	P	P	4
G2 (vv)	A	E	Y	A	T	P	A	4
QD2014 (vv)	A	E	Y	A	A	R	A	3
GXY2 (vv+)	A	E	Y	A	A	R	A	3
YLO40920 (vv+)	A	E	Y	A	A	R	A	3
GX070060 (vv+)	A	E	Y	A	A	R	A	3
GX18NNM4	A	E	Y	A	A	R	A	3
GX18NNM8	A	E	Y	A	A	R	A	3
GX19YLM5	A	E	Y	A	A	R	A	3
GX19NNM3	A	E	Y	A	A	R	A	3
GX20M1	A	E	Y	A	A	R	A	3

Boldface represents the virus strains isolated in the study.

MDV evolution to increased virulence (Shi et al., 2020) and the co-infection with other viruses (Cui et al., 2016; Li et al., 2019b). According to our previous study, MDV and ALV-J co-infection was about 15% responsible for the clinical tumor outbreaks during 2010 to 2016 and jumped to 57.14% (8/14) in 2017 (Li et al., 2019a). Interestingly, there were few reports of co-infection of REV and MDV in clinical cases during January 2016 to September 2018 in southern China

In the present study, four clinical cases with high morbidity and mortality due to tumors were diagnosed by histopathological observation, cell cultures and the detection with ELISA and IFA, and with PCR and the sequencing of the viral isolate gene and/or genome. The results showed that all the cases exhibited co-infection of REV and MDV. To our knowledge, this is the re-isolation of the REV in clinical cases since 2015 from Yellow-chickens in southern China. Previous study showed that compared with the mono-infection of REV, MDV or ALV, co-infection with different tumor viruses (REV and/or MDV and/or ALV) has more potent pathogenicity: the co-infection with MDV and REV could increase the severity of the diseases and reduce MD vaccine efficacy (Sun et al., 2017b); the co-infection of ALV-J and REV could cause more serious synergistic pathogenic effects (Dong et al., 2015); the ALV and MDV co-infection could cause greater pathogenic effects than the MDV single infections (Wang et al., 2020).

In order to further characterize the pathogens isolated from the diseased birds, the complete genome of the four REV strains and the oncogenic *meq* gene of four MDV strains were sequenced and analyzed. Based on the phylogenetic tree, 68.9% (20/29) REV strains included the four REV isolates and 16 other reference strains were in the same branch and shared a high similarity (>99.00%), indicating that they may have the same origin. However, we cannot identify the exact source of the four REV strains here, since there are two transmission routes, vertical or horizontal, of REV to the chickens including through the contaminated live vaccine

(Schat and Erb, 2014; Li et al., 2016; Xu et al., 2020). The sequence analysis based on *meq* gene indicated that these four MDV strains belong to vv+ MDV-like strains. A previous study has shown that the vvMDV strain can break through the protection of the vaccine (Eschke et al., 2018) and a recent study by our group also showed that the vv+MDV strain can cause severe immunosuppression to the challenged chickens and break through the protections provided by the current used commercial vaccines CVI988/Rispens and 814 (Shi et al., 2020).

In the present study, four clinical cases with high morbidity and mortality caused by the co-infection of REV and vvMDV in Yellow-chickens in southern China were diagnosed and the isolated pathogens were further characterized as to gene/genome. Our finding indicated that the frequent reemergence of REV and the subsequent co-infection with vv+MDV have become one of the causes of the clinical outbreaks of tumors and undoubtedly become a threat to the poultry industry in southern China. Since the pathogenicity of the co-infection cases is much higher than that of the mono-infection cases, eradication programs, elimination of the contamination of the live vaccines, strict biosecurity practices, and vaccinations plus the resistance breeding measures should be universally implemented in the effective prevention and control programs against these clinical tumor diseases.

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findings of this study are available within the article [and/or] its supplementary.

DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi: <https://doi.org/10.1016/j.psj.2021.101099>.

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