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Application of metagenomic sequencing toward rapid and sensitive diagnosis of goose avastrovirus infection in China

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Abstract

The gosling gout, a newly emerged disease, has widely broken out in China since 2017. Typical signs for the disease include diarrhea, anorexia, depression, dehydration, emaciation and paralysis. At autopsy, uratosis was the main pathological change which could be found at kidney, pericardium, air sac, muscle and leg joint. In this study, gosling gout was firstly diagnosed by metagenomic analysis. Samples of kidney, Fabricius bursa, spleen and jejunum were collected and submitted to next-generation DNA sequencing. Our results demonstrated that goose avastrovirus was highly related with this disease. We confirmed the sequencing results by reverse transcription polymerase chain reaction method and artificial infection experiment and got consistent results. In summary, metagenomic sequencing method combined with traditional molecular identification was applied toward diagnosis of a novel gosling gout disease in China and revealed that goose avastrovirus was highly related with this disease. It has been proved to be a powerful tool for rapid and sensitive diagnosis of animal diseases, especially for some exceptional pathogens. In addition, host range, variation, molecular pathogenesis and potential zoonotic infection of this novel goose astrovirus need to be further studied.

Introduction

Avian astroviruses are members of the genus Avastrovirus, one of the two genera in the family Astroviridae. As of 2011, the International Committee on Taxonomy of Viruses recognized three species of the genus Avastrovirus. These three species are 1) Avastrovirus 1, including turkey astrovirus 1, 2) Avastrovirus 2, including avian nephritis virus (ANV) 1 and 2 and 3) Avastrovirus 3, including turkey astrovirus 2 and duck astrovirus 1.[1] However, there are also a number of unassigned species in the genus Avastrovirus.[1] Here, we diagnosed a newly outbreak disease, which was caused by a novel Goose-origin astrovirus (GAV), by metagenomic sequencing method.

In year 2018, an acute infectious disease in goose, with typical signs of urate nephritis and no response to antibiotic therapy, commonly recognized as gosling gout, was outbroke in many urban and rural areas throughout China. Molecular method like polymerase chain reaction (PCR) or quantitative-PCR was performed toward the frequent pathogens including avian influenza virus (AIV), Newcastle disease virus (NDV), duck reovirus (DRV), duck hepatitis A virus (DHAV), duck Tembusu virus (DTMUV), duck circovirus (DuCV), Muscovy duck parvovirus (MDRV) and reticuloendotheliosis virus (REV); but all showed negative reaction. In order to find the infectious pathogens, metagenomic sequencing was adopted to detect the exceptional pathogens in the diseased geese.

Materials and Methods

Case Description. Since August 2018, 18 goose farms from Guangdong (five farms), Shandong (six farms), Sichuan (two farms), Hubei (three farms) and Chongqing (two farms) provinces or municipality were found being involved with gout disease outbreak. Typical signs for the disease included diarrhea, anorexia, depression, dehydration, emaciation and paralysis. At autopsy, uratosis

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was the main pathological change in the kidney, pericardium, air sac, muscle and leg joint. The dead or close to death geese (totally 138 samples) from 18 investigated farms (Table 1) were transported at low temperature to the Animal Diseases Rapid Diagnosis Center, Southwest University, Chongqing, China, conferred for the diagnosis of death causes. Varieties of diseased geese included Landaise goose, Huoyan goose, Taizhou goose, Wanxi White goose, Shitou goose, Ma goose, Magang goose and Sichuan White goose, and their age of clinical signs onset ranged from 8 to 37 days. According to the description of farm owners, mortality of the different farms ranged from 8.90% to 73.20%. The peak death was usually occurred at 5 to 8 days after diarrhea initiation.

Samples processing. The gosling samples including kidney, bursa of Fabricius, spleen and jejunum were collected by aseptic methods and triturated in sterile mortar with stainless steel wire. The processed samples were diluted 1:1 (w/v) with chilled sterile normal saline, aliquoted 5.00 mL per bottle and stored at – 80.00 ˚C for further study.

RNA and DNA extractions. The frozen samples were filtered using 0.80 μm membrane filters to remove debris. Filtrates were treated with DNases and RNases (Takara, Otsu, Japan) and incubated for 2 hr at 37.00 ˚C to destroy free unprotected nucleic acids. Extraction of viral RNA and DNA were performed using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, USA) and TaKaRa Minibest Viral DNA Extraction kit (version 5.0; Takara) according to the manufacturers' instructions, respectively. The concentration and purity of extracted RNA and DNA were measured respectively using a spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, USA) and Qubit fluorimeter (Invitrogen, Carlsbad, USA) and the integrity was visualized by electrophoresis in a 1.20% of 3-(N-Morpholino) propanesulfonic acid (MOPS)/ formaldehyde agarose gel stained with GelRed (Solarbio Life Sciences, Beijing, China).

Detection of common pathogens. All the collected samples were conducted with detection for the commonly infectious pathogens including AIV, NDV, DRV, DHAV, DTMUV, DuCV, MDRV and REV. All these pathogens were screened by PCR method using previously described conditions.[2-8]

Metagenomic sequencing and data analysis. Sequencing of a gosling sample with typical symptoms was performed on the BGISEQ-500 (BGI, Shenzhen, China) for 300 cycles (150-bp paired ends). Raw sequence reads were trimmed based on the Phred quality scores after removal of adapters reads and goose genomic sequences as well as duplicate reads. Reads passing the quality check were taken into analysis and subjected to De Novo contig assembly using Trinity assembly Software (V2.5.1; Broad Institute, Cambridge, USA) with the criterion of a 90.00% minimum overlap identity. Contigs were classified by BLASTx search against the NCBI non-redundant protein database (e-value cutoff 10^{-10}). Contigs with significant BLASTx hits were retained and used for a second BLASTx search against the GenBank® non-redundant nucleotide database using an e-value of 10^{-4}.

Reverse transcription PCR (RT-PCR) identification. Based on the results of metagenomic sequencing, a pair of primers (F: 5'-GATTGGACCCGTTATGAT-3'; R: 5'-TTTGA CCCACATACCAAA-3') was designed to detect partial genome of GAV and synthesized commercially (Sangon Biotech Co., Ltd., Shanghai, China). All the reserved

Table 1. Information of the investigated goose samples and the detection results of suspected pathogens.

Sampling area	Farms	Sample age (Days)	Goose breed	Samples (n)	Mortality (%)	Detection results of suspected pathogens								
						AIV	NDV	DRV	DHAV	DuCV	DTMUV	REV	MDRV	GAV
Guangdong province	A	8	Landaise	11	73.20	-	-	-	-	-	-	-	-	+(11/11)
	B	14	Magang	7	35.80	-	-	-	-	-	-	-	-	+(7/7)
	C	26	Taizhou	12	10.50	-	-	-	-	-	-	-	-	+(12/12)
	D	14	Ma	8	55.10	-	-	-	-	-	-	-	-	+(8/8)
	E	8	Landaise	12	68.90	-	-	-	-	-	-	-	-	+(11/12)
Shandong province	F	31	Huoyan	5	30.50	-	-	-	-	-	-	-	-	+(5/5)
	G	37	Huoyan	7	27.20	-	-	-	-	-	-	-	-	+(7/7)
	H	28	Shitou	9	33.10	-	-	-	-	-	-	-	-	+(7/9)
	I	15	Ma	6	29.40	-	-	-	-	-	-	-	-	+(5/6)
	J	19	Shitou	8	26.40	-	-	-	-	-	-	-	-	+(8/8)
	K	14	Landaise	7	64.80	-	-	-	-	-	-	-	-	+(7/7)
Sichuan province	L	22	Sichuan White	8	16.80	-	-	-	-	-	-	-	-	+(7/8)
	M	26	Sichuan White	4	18.90	-	-	-	-	-	-	-	-	+(4/4)
Hubei province	N	34	Wanxi White	3	8.90	-	-	-	-	-	-	-	-	+(3/3)
	O	21	Magang	9	26.70	-	-	-	-	-	-	-	-	+(7/9)
	P	29	Wanxi White	11	43.20	-	-	-	-	-	-	-	-	+(7/11)
Chongqing municipality	Q	22	Sichuan White	7	34.50	-	-	-	-	-	-	-	-	+(7/7)
	R	17	Magang	4	45.60	-	-	-	-	-	-	-	-	+(4/4)

AIV: avian influenza virus, NDV: Newcastle disease virus, DRV: duck reovirus, DHAV: duck hepatitis A virus, DuCV: duck circovirus, DTMUV: duck Tembusu virus, REV: reticuloendotheliosis virus, MDRV: Muscovy duck parvovirus, and GAV: goose-origin astrovirus.

samples were confirmed by RT-PCR. Briefly, RNA was extracted (as described before) from each sample and used for reverse transcription. A total reaction volume of 20.00 µL containing 4.00 µL of 5X reverse transcriptase buffer (50.00 mM Tris-HCl, 8.00 mM $MgCl_2$, 30.00 mM KCl and 1.00 mM dithiothreitol; pH: 8.30), 0.50 mM each deoxynucleotide triphosphate (dNTP), 1.00 µM antisense primers of GAV, respectively, 20.00 U of RNase inhibitor, 2.00 µL of the prepared RNA and 5.00 U of avian myeloblastosis virus reverse trans-criptase (Takara). After incubation for 60 min at 42.00 ˚C, the mixture was heated for 3 min at 95.00 ˚C to denature the products. The mixture was then chilled on ice. The total PCR reaction volume of 25.00 µL contained 2.00 µL of cDNA, 0.50 µM of each primer, 2.50 µL of 10X PCR buffer (100 mM Tris-HCl; pH: 8.80, 500 mM KCl and 0.80% NP-40), 2.00 mM $MgCl_2$, 0.20 mM each dNTP, 1.25 U of Taq polymerase (Sangon Biotech Co., Ltd.) and 17.25 µL distilled water. The PCR reaction was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, USA) using the following procedure: Initial denaturation at 94.00 ˚C for 5 min; 30 cycles of denaturation at 94.00 ˚C for 1 min, annealing at 48.00 ˚C for 1 min and extension at 72.00 ˚C for 1 min; then, final extension at 72.00 ˚C for 10 min. The PCR products were analyzed by electrophoresis in 1.00% agarose, followed by ethidium bromide staining and visualization under ultraviolet light. Positive PCR products were purified by Mini BEST Agarose Gel DNA Extraction Kit (TaKaRa) and sequenced in Sangon Biotech Co., Ltd. Genetic homology analysis was done for individual samples as well as for each GAV strains detected by metagenomic sequencing using GenBank® function in MegAlign Software (version 5.0; Dnastar Inc., Madison, USA).

Experimental infection of goslings. The frozen astrovirus-positive tissue samples were homogenized and spinned by 12,000 *g* for 10 min at 4.00 ˚C. After freezing and thawing for three times, the suspensions were filtered through 0.45 µm and 0.22 µm filters orderly (Ultrafree-MC; Millipore, Bedford, USA) to get rid of other potential bacterial contaminations. Then, the filtered suspensions were used for experimental infection. In order to test Koch's postulates, 40 healthy one-day-old Landaise geese were selected and purchased from the Laboratory Animal Center of Chongqing Medical University, Chongqing, China, and divided into two groups (one infection group and one negative control group) with 20 goslings in each group. Each bird in the infection group was orally inoculated with 1.00 mL (dosage based on a pre-infection experiment) astrovirus-positive tissue suspensions; while, 1.00 mL normal saline solution was administered to each animal of negative control group as equal. The geese were monitored daily for signs of disease. Following death of the goslings, necropsy examinations were performed and tissues including liver, spleen and kidney were collected and processed regarding molecular detection as described above. All animals used in this study were handled in strict accordance with the guidelines of the Southwest University Animal Care and Use Committee, Chongqing, China (Approval Number: SWU-AUC-2018-36).

Results

Clinical symptoms of the diseased geese. Diseased goslings displayed symptoms including diarrhea, anorexia, depression, dehydration, emaciation and paralysis. Anatomical investigation showed that uratosis was the main pathological change which could be found at kidney (Fig. 1A), pericardium (Fig. 1B), air sac and muscle as well as leg joint. Soft content like embolus was presented in the jejunum in rare cases (approximately 5.00%; Fig. 1C). Usually, excess urates were observed in kidneys and ureters in the early stage of the disease (about 2-4 days after diarrhea onset); while, visceral, intra-muscular and articular uratosis was appeared in the middle stage (about 5-8 days after diarrhea onset). Swollen kindeys were found in most cases during the agonal stage (about 8-13 days after diarrhea onset) and uratosis could also be found in other organs. In the present study, 90.60% (125/138), 70.30% (97/138), 31.90% (44/138), 22.50%(31/138), 20.30% (28/138) and 12.30% (17/138) of cases were found with uratosis in pericardium, liver, intestines, air sac, muscle and leg joint, respectively.

Detection results of some frequent pathogens. As shown in Table 1, all samples showed negative reactions with AIV, NDV, DRV, DHAV, DuCV, DTMUV, REV and MDRV. These findings indicated that some exceptional pathogens should be existed in the diseased geese.

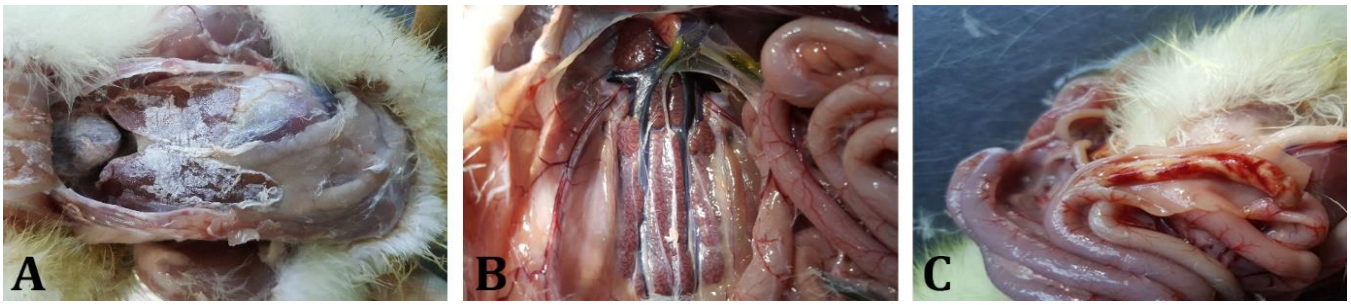

Fig. 1. Clinical symptoms of the diseased geese. **A)** uratosis at pericardium; **B)** uratosis at kidney; and **C)** soft content in the jejunum.

Metagenomic sequencing results. Metagenomic sequencing analysis demonstrated that nine species of bacteria including *Propionibacterium acnes*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Ralstonia pickettii*, *Cupriavidus metallidurans*, *Streptococcus salivarius*, *Stenotrophomonas maltophilia*, *Pseudomonas sp TKP* and *Pseudomonas stutzeri* and one kind of virus (GAV) had been detected in the diseased goose samples (Table 2). Although the top two sequencing reads were referred to *Propionibacterium acnes* and *Pseudomonas putida*, the causative pathogen could not be bacteria according to the epidemiological investigation and the progress of disease. Based on the sequencing results, GAV was the only virus associated with this disease (RNA targeted rate of 6.90%). Given the symptoms above, GAV is likely to be the causative pathogen in this disease.

Re-identification results by RT-PCR. Goose astrovirus infection in all clinical samples (138) collected from the 18 goose farms were confirmed by RT-PCR. In summary, 127 samples were astrovirus-positive (Table 1) and the positive ratio was approximately 92.00% (127/138). Homology analysis done based on the sequencing results of the positive products showed that the samples shared 95.70 % to 99.20% similarity with GAV strains registered in GenBank®. Phylogenetic analysis of existing GAV strains revealed that the queried sequence clustered with all astrovirus strains that had been isolated from geese (Fig. 2); while, it displayed relative far relatedness with chicken-origin ANV. These findings confirmed that the virus belonged to GAV.

Artificial infection experiment results. In the infection group, 3, 7, 5 and 5 goslings died at days 3, 8, 10 and 14 after infection, respectively. Clinical symptoms including diarrhea, anorexia and paralysis were found; consistent with those of naturally infected animals. At necropsy, urates were clearly observed in the kidneys, ureters and pericardiums in the later dead geese (8, 10 and 14 days after infection). However, no obvious uratosis was found in the animals died at day 3. Hyperemia and hemorrhage in the jejunum were the major pathological changes in those birds. All animals in the negative control survived without obvious clinical symptoms during the experiment. According to the PCR and sequencing identification results, GAV was detected in all infection samples.

Table 2. Sequencing and database analysis of the diseased geese samples.

Pathogens	DNA		RNA	
	Reads	Targeted rate (%)	Reads	Targeted rate (%)
Propionibacterium acnes	1269	14.70	12783	20.60
Pseudomonas putida	1081	12.50	7362	11.90
Goose astrovirus	145	1.70	4294	6.90
Pseudomonas fluorescens	537	6.20	3125	5.00
Ralstonia pickettii	50	0.60	2427	3.90
Cupriavidus metallidurans	49	0.60	1963	3.20
Streptococcus salivarius	20	0.20	1952	3.20
Stenotrophomonas maltophilia	86	1.00	1404	2.30
Pseudomonas sp TKP	240	2.80	1239	2.00
Pseudomonas stutzeri	68	0.80	724	1.20

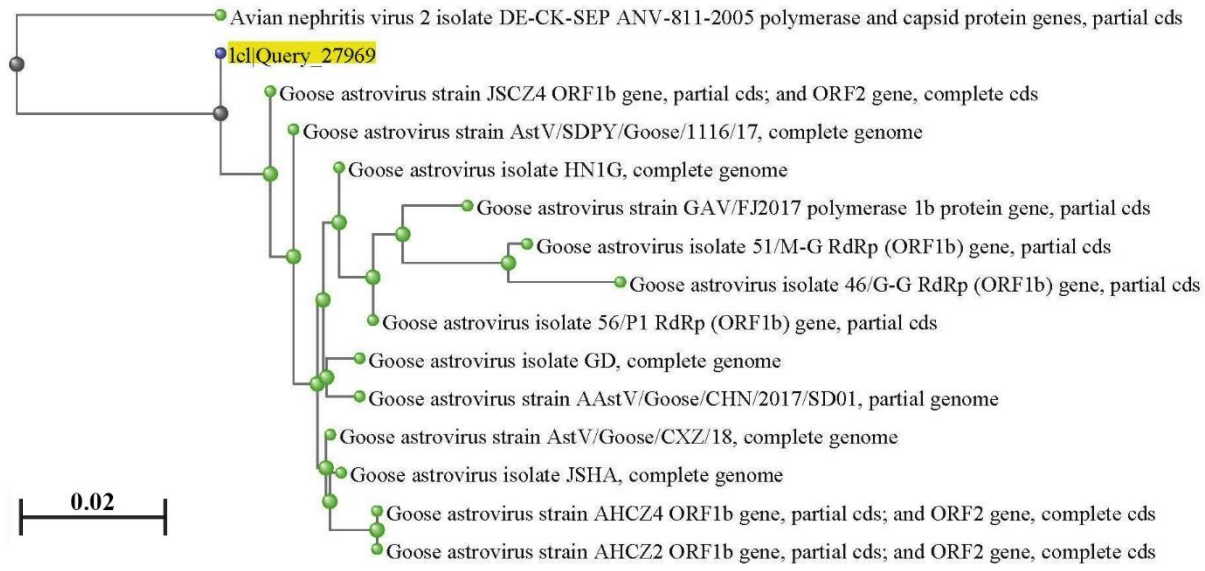

Fig. 2. Phylogenetic tree of the isolate and goose astrovirus based on partial sequence of the ORF1b gene. Lcl Query_27969 was the isolate in this study.

Discussion

Traditional pathogen detection methods in infectious disease diagnosis rely upon the identification of agents that are already known to be associated with a particular clinical syndrome.[9] Whereas in the present study, a newly emerged disease with typical signs of gout was rarely reported before. Preliminary diagnosis was performed for the frequent pathogens including AIV, NDV, DRV, DHAV, DTMUV, DuCV, MDRV and REV. However, results indicated that they were all negative. Therefore, metagenomic sequencing method was applied because it has been proved to be highly sensitive in detecting the exceptional pathogens in the recent years.[10,11] This method had the potential to revolutionize pathogen detection by allowing the simultaneous detection of all microorganisms in a clinical sample, without a priori knowledge of their identities, especially to detect rare and novel pathogens. As next-generation sequencing technologies improved and costs continued to drop, the metagenomic approaches to infectious disease investigations will become increasingly common in public laboratories.

According to our survey, the mortality rates of geese in different farms might be related to the onset age and breed of the infected geese. We could find that younger goslings often exhibited a relative higher mortality rate in these cases. Another interesting finding was that Landaise goose displayed higher mortality rate than other seven domestic goose breeds. Since it is a new disease outbreak, few studies have focused on this novel virus and no literature has addressed this issue. Therefore, more studies on its host range, age, variety and molecular pathogenesis are still needed.

In this study, artificial infection experiment was performed with tissue sample suspensions because the virus isolation was failed. We had attempted to isolate the virus with specific pathogen free chicken and duck embryos. However, the virus could not reproduce in those hosts. Our findings were in line with what Yuan *et al.,* reported.[12] Recently, some reports showed that GAV could be isolated by goose embryos and LMH cells.[12,13] Based on the findings, we had successfully isolated three strains of GAV by goose embryos; however, the data were not presented in this paper.

Although metagenomic analysis indicated that GAV was the only viral pathogen in this case and even pathogens including AIV, NDV, DRV, DHAV, DuCV, DTMUV, REV and MDRV were not able to be detected in the diseased goslings, we could not exclude the potential co-infections with other pathogens because of the small quantity of tested samples. What's more, whether the high protein level in the feed promotes the occurrence of gout, it also needs to be further investigated.

The mechanism for the gout disease induced by GAV in this study needs to be further elucidated. Previous study has indicated that astroviruses could increase the permeability of the epithelial cells and the increased permeability of the kidney epithelial cells might contribute to the gout disease.[14] In addition, due to arginase lack in poultry, ammonia cannot be processed into urea instead of purine, hypoxanthine and xanthine; then, it oxidized to uric acid, forming sodium urate and calcium urate being excreted through kidney finally. If the rate of urate formation is greater than the excretory capacity of the urinary organs, gout can be caused with the urate deposits on visceral surfaces.[13] In the present study, plenty of uratosis found at kidney, pericardium, air sac, muscle and leg joint in the diseased geese should be partially elucidated by the above explanations.

Acknowledgments

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Conflicts of interest

The Authors declare that there is no conflict of interest.

References

1. Zhang Y, Wang F, Liu N, et al. Complete genome sequence of a novel avastrovirus in goose. Arch Virol 2017; 162(7): 2135-2139.
2. Li K, Gao H, Gao L, et al. Development of TaqMan real-time PCR assay for detection and quantitation of reticuloendotheliosis virus. J Virol Methods 2012; 179(2): 402-408.
3. Miller PJ, Torchetti MK. Newcastle disease virus detection and differentiation from avian influenza. Methods Mol Biol 2014; 1161: 235-239.
4. Spackman E. Avian influenza virus detection and quantitation by real-time RT-PCR. Methods Mol Biol 2014; 1161: 105-118.
5. Wan C, Huang Y, Cheng L, et al. The development of a rapid SYBR Green I-based quantitative PCR for detection of Duck circovirus. Virol J 2011; 8: 465. doi: 10.1186/1743-422X-8-465.
6. Wan C, Shi S, Chen C, et al. Development of a PCR assay for detection and differentiation of Muscovy duck and goose parvoviruses based on NS gene characterization. J Vet Med Sci 2018; 80(12): 1861-1866.
7. Wang Y, Zhu S, Hong W, et al. A multiplex PCR for detection of six viruses in ducks. J Virol Methods 2017; 248: 172-176.
8. Zhang YF, Xie ZX, Xie LJ, et al. GeXP analyzer-based

multiplex reverse-transcription PCR assay for the simultaneous detection and differentiation of eleven duck viruses. BMC Microbiol 2015; 15: 247. doi: 10.1186/s12866-015-0590-6.
9. Miller RR, Montoya V, Gardy JL, et al. Metagenomics for pathogen detection in public health. Genome Med 2013; 5(9): 81. doi: 10.1186/gm485.
10. Bibby K. Metagenomic identification of viral pathogens. Trends Biotechnol 2013; 31(5): 275-279.
11. Brown JR, Bharucha T, Breuer J. Encephalitis diagnosis using metagenomics: application of next generation sequencing for undiagnosed cases. J Infect 2018; 76(3): 225-240.
12. Yuan X, Meng K, Zhang Y, et al. Genome analysis of newly emerging goose-origin nephrotic astrovirus in China reveals it belongs to a novel genetically distinct astrovirus. Infect Genet Evol 2019; 67: 1-6. doi: 10.1016/j.meegid.2018.10.014.13.
13. Zhang X, Ren D, Li T, et al. An emerging novel goose astrovirus associated with gosling gout disease, China. Emerg Microbes Infec 2018; 7: 152. doi: 10.1038/s41426-018-0153-7.
14. Bulbule NR, Mandakhalikar KD, Kapgate SS, et al. Role of chicken astrovirus as a causative agent of gout in commercial broilers in India. Avian Pathol 2013; 42(5): 464-473.