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A Novel Tool for Specific Detection and Quantification of Chicken/Turkey Parvoviruses To Trace Poultry Fecal Contamination in the Environment

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Poultry farming may introduce pathogens into the environment and food chains. High concentrations of chicken/turkey parvoviruses were detected in chicken stools and slaughterhouse and downstream urban wastewaters by applying new PCR-based specific detection and quantification techniques. Our results confirm that chicken/turkey parvoviruses may be useful viral indicators of poultry fecal contamination.

Animal populations can serve as reservoirs for human pathogens and may facilitate transmission of those crossing the species barrier. Therefore, the origin of animal fecal contamination must be identified and tracked to monitor water quality, assess potential health risks, and determine optimal remediation strategies. In particular, poultry farming is an industry that produces a large volume of different by-products occasionally used as manure to fertilize crops, which can introduce pathogens into the surrounding environment and into the food chain (1, 5, 8). However, until recently, there had been little effort to develop suitable techniques to characterize the origin of avian fecal contamination (2, 13, 17).

Bacterial fecal indicators often fail to predict the presence of pathogenic microorganisms in water and food (7). Thus, viruses have emerged as a promising tool to increase water quality standards, due to their high host specificity and stability in different environments (9, 11, 14, 20, 21).

The high levels of prevalence of parvovirus in chickens (ChPV) and turkeys (TuPV) in different countries (3, 18, 19, 23, 24) and the high level of stability of animal parvovirus (15, 22) have been described. Here, the potential role of ChPV/TuPV as a new tool for microbial source tracking was evaluated by developing nested and also quantitative PCR-based assays for the detection and quantification of ChPV/TuPV in environmental samples.

All sequences available in GenBank for ChPV and TuPV were aligned, and two nested PCR (nPCR) assays, targeting the nonstructural and VP1/VP2 regions, and a quantitative PCR (qPCR), targeting the VP1/VP2 regions, were optimized (Table 1).

A total of 30 chicken fecal pools were collected from different farms in Catalonia (coastal Northeast Spain), the Basque Country (Northern Spain), Patras (Greece), and Budapest (Hungary) between February and December 2010. Three turkey, 2 partridge, and 7 hen pooled fecal samples collected from farms in Catalonia were also tested. All samples were collected from the ground and distributed into sterile 50-ml polyethylene containers that were kept at 4°C for less than 24 h prior to the analysis. Viral particles were concentrated from 250 mg of fecal material that was homogenized by vortexing with 2.5 ml of phosphate-buffered saline (PBS) during 2 min and centrifuged at 3,000 × *g* for 15 min, after

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TABLE 1 Oligonucleotide primers used for the detection and quantification of chicken/turkey parvoviruses

Primers	Genome region[a]	Position[b]	Amplification reaction	Amplicon size (bp)	Annealing temp (°C)	Sequence (5′–3′)
Par1	NS	661–682	First	412	53	GGTACAAGATATGCTAGATTTG
Par2		1053–1073				CGGATGGCTAAATTATCATCT
Par3		718–739	Nested	325	53	CCATCGCAGGAATTAACTCCAG
Par4		1022–1043				GTGTCAACATCTCCATGTATTG
VP-Par1	VP1/VP2	3119–3140	First	373	56	TGGAATTGTGATACTATATGGG
VP-Par2		3473–3492				TCYTGATCTGCAAATATTTG
VP-Par3		3173–3196	Nested	249	64	CATTGTGTCTGTCTWATGCGTGAC
VP-Par4		3405–3422				GTTTTCTGGATGACTTGCA
Q-PaV-F	VP1/VP2	3326–3345	qPCR	81	60	AGTCCACGAGATTGGCAACA
Q-PaV-R		3388–3407				GCAGGTTAAAGATTTTCACG
Q-PaV-Pr		3356–3378				6FAM-AATTATTCGAGATGGCGCCCACG-BHQ1

[a] NS, nonstructural protein 1; VP1, virion protein 1; VP2, virion protein 2.
[b] The sequence positions are with reference to accession number GU214706 from GenBank.

TABLE 2 Detection of chicken/turkey parvoviruses in avian feces and in environmental samples by nPCR of the NS region

Source of sample	No. of positive samples/total no. of samples	% positive
Chicken feces	22/30	73
Catalonia	8/10	80
Basque Country	2/7	29
Greece	6/7	86
Hungary	6/6	100
Hen feces	5/7	72
Turkey feces	3/3	100
Partridge feces	1/2	50
Duck feces	0/17	0
Seagull feces	0/25	0
Chicken slaughterhouse raw wastewater	5/5	100
Chicken slaughterhouse treated wastewater	4/5	40
Urban raw wastewater with poultry industry affluents	4/9	44
Urban raw wastewater without poultry industry affluents	0/11	0
Urban treated wastewater	0/5	0
Urban biosolids	3/4	7

which the supernatant was recovered and kept at −80°C until the nucleic acid extraction was performed.

The presence of ChPV/TuPV was also evaluated in chicken slaughterhouse raw and effluent wastewater samples (5 samples of each) and in raw and treated urban sewage (9 and 5 samples, respectively), as well as in biosolids (4 samples) from a sewage treatment plant (STP) located downstream from the slaughterhouse to prove a potential route of dissemination of these viruses into the environment. Also, 11 raw sewage samples from an STP from an area with no poultry industry were analyzed.

Viral particles were concentrated as described in previous studies (4). Nucleic acids from all viral concentrates were extracted by using the QIAmp viral RNA kit (QIAgen, Inc.) using the QIAcube automated platform.

nPCR assays based on the NS region were performed in 50-μl reaction mixtures containing 1× Gold buffer, 50 mM $MgCl_2$, 25 mM each deoxynucleoside triphosphate, 10 μM each primer (Table 1), 2 U of AmpliTaq Gold polymerase (Applied Biosystems, Inc.), and 10 μl of DNA sample. For the nested amplification, 49-μl reaction mixtures were prepared identically and 1 μl of the first-round PCR product was added. nPCR assays targeting the VP1/VP2 regions were prepared in the same way except that all primers were used at a concentration of 25 μM (Table 1).

qPCR amplifications were performed in a 25-μl reaction mixture containing 10 μl of DNA sample and 15 μl of TaqMan environmental PCR master mix (Applied Biosystems, Foster City, CA), 0.3 μM forward primer, 0.9 μM reverse primer, and 0.25 μM fluorogenic probe (Table 1). qPCR standards were generated and used as previously described (10, 12). In our assays, the average R^2 value was 0.996 ± 0.003, and the slope values ranged between −3.164 and −3.417 (mean value, −3.297). The estimated mean efficiency of the assay was 97.4%.

The specificity of the assays was studied by testing a wide selection of samples: 3 raw porcine and 3 bovine slaughterhouse sewage samples, 9 pooled duck fecal samples of *Anas platirhyncos* and 8 of *Cairina moschata*, 14 seagull samples of *Larus michahellis* and 11 of *Larus audouinii*, 2 feline parvovirus attenuated vaccines (Felocell 4 [Pfizer] and PureVax RcPch FelV), 1 canine parvovirus attenuated vaccine (Eurican CHPPI2-LR), and porcine parvovirus viral particles obtained by cell culturing. Raw hospital sewage samples containing exclusively human fecal/urine contamination and serum samples containing human parvovirus B19 were also tested. None of the samples tested provided amplification with any of the assays developed.

ChPV/TuPV were detected in 73% of pooled chicken stool samples from the different geographical areas tested, with a mean value of 9.07×10^8 genome copies (GC)/g. No differences in the percentages of positive samples attributable to the number of animals represented in the pooled samples or to the geographic origin were observed. The viruses were also detected in turkey and partridge feces. All chicken slaughterhouse raw wastewater samples and 80% of slaughterhouse treated wastewater tested positive. The mean concentration of the virus in raw wastewater obtained from the slaughterhouse was 4.63×10^5 GC/ml. Forty-four percent of downstream raw urban sewage samples and 75% of the biosolids produced in this STP tested positive, with mean values of 2.65×10^2 GC/ml and 1.29×10^5 GC/g, respectively. Interestingly, none of the samples collected in a STP in an area that was not identified as receiving effluent from the poultry industry tested positive by the assays developed here (Tables 2 and 3).

Nucleotide sequences were obtained from VP1/VP2 nPCR assay amplicons and compared to sequences already available in GenBank (6). Intrasample variability ranging from 96.4 to 100% was observed by cloning one of the amplicons obtained and studying the sequences of 9 clones.

Phylogenetic analysis showed that the sequence grouping could not be associated with geographical origin or sample type. All sequences studied were similar to previously reported sequences, with similarity values ranging between 85 and 100% (Table 4).

The assays designed here have proved to be useful for the specific detection and quantification of poultry fecal contamination, for evaluating their dissemination within the environment, and for discriminating poultry pollution from many other sources of fecal contamination potentially present in urban wastewater. Further studies for determining the presence of ChPV/TuPV in environmental samples susceptible of receiving poultry contamination via polluted water or as a consequence of the application of polluted biosolids may be conducted by applying the tools developed here.

TABLE 3 Quantification of chicken/turkey parvoviruses in different types of environmental samples

Type of sample	No. of samples	% positive	Mean value	Range
Chicken feces	21	81	9.07×10^8 GC/g	1.97×10^2–1.07×10^{10} GC/g
Slaughterhouse raw wastewater	3	100	4.63×10^5 GC/ml	1.90×10^5–8.14×10^5 GC/ml
Urban raw wastewater	2	50	2.65×10^2 GC/ml	2.65×10^2 GC/ml
Urban biosolids	2	100	1.29×10^5 GC/g	1.07×10^5–1.51×10^5 GC/g

TABLE 4 Typification and diversity of the chicken/turkey parvovirus strains identified by sequencing the amplicons obtained from the analyzed samples

Sample (GenBank accession no.)	Type of sample	Geographic origin	Genomic region[a]	% similarity to indicated virus[b] (geographic origin)
CT-Par1 (JX434399)	Turkey feces	Catalonia	NS	97, TuPV 1078 (USA)
CT-Par2 (JX434400)	Chicken feces	Catalonia	NS	95, TuPV 260 (USA)
CT-Par3 (JX434401)	Chicken feces	Catalonia	NS	95, TuPV 260 (USA)
CT-Par4 (JX434402)	Chicken feces	Catalonia	NS	95, TuPV 260 (USA)
CT-Par5 (JX434403)	Slaughterhouse sewage	Catalonia	NS	97, TuPV 260 (USA); 96, ChPV ABU-P1 (Hungary)
CT-Par6 (JX434404)	Slaughterhouse sewage	Catalonia	NS	96, TuPV 260 (USA); 96, ChPV ABU-P1 (Hungary)
CT-Par7 (JX434405)	Slaughterhouse sewage	Catalonia	NS	98, ChPVABU-P1 (Hungary); 97, TuPV 260 (USA)
CT-Par8 (JX434406)	Slaughterhouse sewage	Catalonia	NS	94, TuPV 260 (USA); 93, ChPV ABU-P1 (Hungary)
CT-Par9 (JX434407)	Urban sewage	Catalonia	NS	97, ChPVABU-P1 (Hungary); 97, TuPV 260 (USA)
CT-Par10 (JX434408)	Urban sewage	Catalonia	NS	98, ChPVABU-P1 (Hungary); 97, TuPV 260 (USA)
CT-Par11 (JX434409)	Urban sewage	Catalonia	NS	98, ChPVABU-P1 (Hungary); 97, TuPV 260 (USA)
CT-Par12 (JX434410)	Chicken feces	Hungary	NS	94, TuPV 260 (USA); 94, ChPV ABU-P1 (Hungary)
CT-Par13 (JX434411)	Chicken feces	Hungary	NS	93, TuPV 260 (USA); 92, ChPV ABU-P1 (Hungary)
CT-Par14 (JX434412)	Chicken feces	Hungary	NS	94, TuPV 260 (USA); 93, ChPV ABU-P1 (Hungary)
CT-Par1 (JX434386)	Turkey feces	Catalonia	V1/VP2	98, TuPV 1078 (USA)
CT-Par7 (JX434387)	Chicken feces	Catalonia	VP1/VP2	97, TuPV 260 (USA); 97, ChPV ABU-P1 (Hungary)
CT-Par8 (JX434388)	Chicken feces	Catalonia	VP1/VP2	95, TuPV 260 (USA); 95, ChPV ABU-P1 (Hungary)
CT-Par9 (JX434389)	Chicken feces	Catalonia	VP1/VP2	94, TuPV 260 (USA); 94, ChPV ABU-P1 (Hungary)
CT-Par10 (JX434390)	Chicken feces	Catalonia	VP1/VP2	95, TuPV 260 (USA); 95, ChPV ABU-P1 (Hungary)
CT-Par11 (JX434391)	Chicken feces	Catalonia	VP1/VP2	95, TuPV 260 (USA); 95, ChPV ABU-P1 (Hungary)
CT-Par12 (JX434392)	Slaughterhouse sewage	Catalonia	VP1/VP2	98, ChPVABU-P1 (Hungary)
CT-Par14 (JX434393)	Slaughterhouse sewage	Catalonia	VP1/VP2	99, ChPV ABU-P1 (Hungary)
CT-Par15 (JX434394)	Chicken feces	Basque Country	VP1/VP2	99, ChPV ABU-P1 (Hungary)
CT-Par16 (JX434395)	Chicken feces	Basque Country	VP1/VP2	99, ChPV ABU-P1 (Hungary)
CT-Par17 (JX434396)	Chicken feces	Greece	VP1/VP2	99, ChPV ABU-P1 (Hungary)
CT-Par18 (JX434397)	Chicken feces	Greece	VP1/VP2	99, ChPV ABU-P1 (Hungary)
CT-Par19 (JX434398)	Chicken feces	Hungary	VP1/VP2	100, ChPV ABU-P1 (Hungary)

[a] NS, nonstructural protein; VP1, virion protein 1; VP2, virion protein 2.
[b] ChPVABU-P1, chicken parvovirus ABU-P1 (GenBank accession number GU214704.1); TuPV 260, turkey parvovirus 260 (GU214706.1); TuPV 1078, turkey parvovirus 1078 (GU214705.1).

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