Detection of fowl poxvirus integrated with reticuloendotheliosis virus sequences from an outbreak in backyard chickens in India

Sanchay K. Biswas[1], Chandrakanta Jana[2], Karam Chand[1], Waseem Rehman[1] & Bimalendu Mondal[1]

Summary

Fowl poxvirus (FPV) infection was observed in unvaccinated backyard chickens. A total of 15 birds were affected in a flock of 37. Pock lesions were observed on the comb, eyelids, beak and wattles. The birds appeared sick with roughened feathers and stunted growth. No mortality was recorded. DNA was isolated from scabs and polymerase chain reaction (PCR) was performed to amplify the 4b core protein gene of FPV, the envelope (*env*) gene of reticuloendotheliosis virus (REV) and the region of FPV flanking REV 5′ long terminal repeat (LTR). Correct-size PCR products of 578 bp, 807 bp and 370 bp, respectively, were observed in agarose gel electrophoresis. Sequence analysis of these products suggests that the virus was an FPV with a genome containing an integrated near full-length REV provirus. Given the fact that REV has been associated with immunosuppression, its presence in the genome of FPV appears to play an important role in the pathogenesis of fowl pox and presumably prolongs persistence of FPV in bird populations. In the present case, fowl pox has been observed to have persisted for about three years in fowl that were reared in backyard systems in villages.

Identificazione del diftero-vaiolo aviario riassortito con sequenze del virus della reticoloendoteliosi in un focolaio in polli da cortile in India

Riassunto

È stata riscontrata un'infezione da virus del diftero-vaiolo aviario (FPV) in polli da cortile non vaccinati. Gli esemplari colpiti sono stati 15 su un gruppo di 37. Sono state osservate lesioni cutanee vescicolari su cresta, palpebre, becco e bargigli. Gli esemplari colpiti hanno presentato arruffamento del piumaggio e ritardo nella crescita. Non si sono registrati casi mortali. Il DNA è stato isolato dalle croste ed è stata effettuata la PCR (reazione a catena della polimerasi) per amplificare il gene 4b che codifica per la proteina del "core" del FPV, il gene env del virus della reticoloendoteliosi (REV) e la regione adiacente REV 5′LTR del FPV. L'elettroforesi su gel di agarosio ha evidenziato prodotti di PCR delle dimensioni previste

(1) Division of Virology, Indian Veterinary Research Institute, Mukteswar Campus, Dist. Nainital 263 138, Uttarakhand, India
bimalendu.m@email.com
(2) KVK Burdwan, Central Research Institute for Jute and Allied Fibers, Budbud, Dist. Burdwan 713403, West Bengal, India

Detection of fowl poxvirus integrated with reticuloendotheliosis virus sequences from an outbreak in backyard chickens in India

Sanchay K. Biswas, Chandrakanta Jana, Karam Chand, Waseem Rehman & Bimalendu Mondal

(rispettivamente 578 bp, 807 bp e 370 bp). L'analisi delle loro sequenze ha indicato che il virus è un FPV con un genoma contenente un provirus REV quasi completamente integrato. Poichè il REV è normalmente associato a immunosoppressione, la sua presenza nel genoma del FPV sembra giocare un ruolo importante nella patogenesi del diftero-vaiolo aviario e, presumibilmente, è in grado di prolungare la persistenza del FPV nelle popolazioni aviarie. Relativamente a questo aspetto, in polli allevati allo stato brado in villaggi, è stata osservata una persistenza del diftero-vaiolo aviario di circa 3 anni.

Parole chiave
Diftero-vaiolo aviario, India, Infezione ricorrente, PCR, Polli, Reazione a catena della polimerasi, REV, Vaiolo aviario, Virus, Virus della reticoendoteliosi.

Introduction

Fowl pox, a common viral disease of chickens and turkeys, is responsible for substantial economic losses to poultry farming. It has a worldwide distribution and is caused by fowl poxvirus (FPV), the prototypical member of the genus *Avipoxvirus*, family *Poxviridae* (15). FPV produces lesions on the skin (cutaneous form) and/or in the mouth, pharynx, larynx, oesophagus and trachea (diphtheric form) of affected birds. Both forms of the disease can occur in a single bird. The prognosis of the diphtheric form of the disease is poor due to lesions that often cause death by asphyxiation (20). In areas where fowl pox is endemic, birds are vaccinated with live virus vaccines of FPV. Despite regular vaccination, the disease tends to persist for long periods, especially in multiple-age group flocks. In recent years, the disease has re-emerged as a new threat to poultry production and a number of outbreaks have been reported in vaccinated flocks (5, 24). The diphtheric form of the disease has been diagnosed in previously vaccinated flocks causing high mortality (10). In this regard, the pathogenicity of vaccine strains is an important concern and studies revealed that most of the FPV field strains show higher pathogenicity than vaccine viruses for susceptible chickens (17, 21, 22, 23).

Characterisation of these field strains revealed that a near intact reticuloendotheliosis provirus is integrated in their genomes (6, 8, 18, 19). Presumably the expression of one or more of the acquired retroviral genes could alter the biological properties of the host and result in the emergence of a modified virus against which current vaccines would not afford adequate protection. As reticulo-endotheliosis virus (REV) has been shown to cause immunosuppression, lymphomas and runting-stunting syndrome (27), its presence in the genome of FPV appears to play an important role in the pathogenesis of fowl pox, presumably prolonging the persistence of FPV in bird populations.

Fowl pox is endemic in India and it is one of the more important poultry diseases that cause considerable economic losses to the poultry industries and to the poorer farmers who rear poultry in backyard management systems. Many field veterinarians and poultry owners in India have noticed persistent FPV infection in unvaccinated flocks and in spite of vaccination, fowl pox outbreaks have been observed in vaccinated flocks (3, 7, 9, 13, 16). This paper describes a recurring FPV infection in backyard poultry and the integration of REV provirus in the genome of the FPV strain as revealed by PCR and sequencing.

Materials and methods

Fowl pox outbreak

The outbreak of fowl pox described here occurred in mid-May 2009 in the Rhode Island Red (RIR) breed of poultry (at 8 weeks of age) reared in backyard management conditions in a village (Galsi Block-I, Burdwan District, West Bengal). Fifteen birds were affected in the flock of 37. Pock lesions were observed on the comb, eyelids, beak (Fig. 1, inset) and wattles. The birds appeared sick with roughened feathers and stunted growth (Fig. 1). There was no mortality but one affected bird was euthanised and examined for pock lesions in the internal organs. Pinhead-size raised lesions were observed on the mucosal surface of the pharynx. There was no lesion in the lungs or in other organs. Earlier, in December 2006 fowl

pox outbreaks were recorded in the same and nearby villages in non-descript poultry reared in backyard management conditions. Subsequently, in the winter of 2007 and 2008, outbreaks were also reported in adult non-descript and RIR birds. In March 2009, non-descript adult and grower birds were affected and the infection probably spread from this flock to RIR birds where sampling was performed. These birds were not vaccinated against fowl pox. Pock lesions from the beaks and pharynx were collected for laboratory investigations.

Figure 1
Pock lesions on the beak of a roughened feathered chick

DNA isolation from scab and infected tissues

The tissues (scab and pharyngeal lesions) were triturated and homogenised to make a 10% suspension in phosphate buffered saline (PBS). DNA was extracted according to standard procedures and recovered by ethanol precipitation. Briefly, 0.5 ml of tissue homogenate was treated with sodium dodecyl sulfate (SDS) (1% final concentration) at 37°C for 30 min and then digested with protinase K (1 mg/ml final concentration) in a buffer (0.01 M Tris-HCl, 0.1 M NaCl, 0.001 M ethylenediaminetetraacetic acid [EDTA], pH7.6) at 56°C for 2 h. DNA was extracted using equal volumes of the phenol/chloroform mixture and precipitated by 2.5 volume of ethanol. After washing and drying, DNA was dissolved in 20 μl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Polymerase chain reaction amplification of fowl poxvirus gene, reticuloendotheliosis virus gene and integration site

FPV-specific PCR was performed in accordance with the method described by Lee and Lee (11). The primers were designed by these workers based on the 4b gene sequence of an FPV strain reported by Binns *et al.* (1). The sequences of the primer set were as follows:

- P1, 5′-CAG CAG GTG CTA AA CAA CAA-3′ (nucleotide 459-478 of 4b gene)
- P2, 5′-CGG TAG CTT AAA CGC CGA ATA-3′ (nucleotide 1016-1035).

The size of the amplified DNA fragment using these two primers was expected to be 578 bp in length. PCR was performed in a total volume of 50 μl, containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.08% NP40, 1.5 mM MgCl$_2$, 200 μM each dNTP, 5 μl DNA, 0.2 μM primer and 1.5 units of *Taq* DNA polymerase (Fermentas, Vilnius). Amplification was performed in a PCR thermal cycler under the following conditions for 30 cycles: initial denaturing at 94°C for 5 min, subsequent denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. The final extension was conducted at 72°C for 15 min to ease cloning in the TA cloning vector. A volume of 5 μl of the PCR products was separated on 1.2% agarose gel electrophoresis.

A PCR assay amplifying an 807 bp region of REV envelope (*env*) gene was employed (2) using primers P3 (5′-TGA CCA GGC GGG CAA AAC C-3′) and P4 (5′-CGA AAG GGA GGC TAA GAC T-3′) designed on the REV-A *env* gene sequence spanning the region 5 999-6 806 bp (26). Another PCR for detection of the REV integration site in FPV was also performed. The primers, P5 (5′-ACC TAT GCC TCT TAT TCC AC-3′) and P6 (5′-CTG ATG CTT GCC TTC AAC-3′), were designed by Wang *et al.* (25) on the basis of nucleotide sequence of FPV (Australian vaccine strain) DNA region, flanking the 5′REV long terminal repeat (LTR) (8). The predicted size of the product was 370 bp. The annealing

Detection of fowl poxvirus integrated with reticuloendotheliosis virus sequences from an outbreak in backyard chickens in India

Sanchay K. Biswas, Chandrakanta Jana, Karam Chand, Waseem Rehman & Bimalendu Mondal

temperature for both the PCR reactions was 52°C.

Cloning of polymerase chain reaction products, sequencing and analysis

The expected size products generated from three PCR assays were gel-purified and cloned into pGEM®-T Easy Vector (Promega, Madison, Wisconsin) in accordance with the manufacturer's instructions. The vector was grown in *Escherichia coli* DH5α and the presence of the insert was confirmed by *Eco* RI digestion of recombinant plasmids. Nucleotide sequencing of three positive clones, generated from three different PCR reactions, was performed in both orientations by an automated sequencer (Applied Biosystems 3130 Genetic Analyzer, Foster, City, California) using the Big dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc,) in accordance with the instructions of the manufacturer. The sequence homology searches were conducted using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST). The percentage of sequence identity was calculated with the BLAST two sequences program (National Center for Biotechnology Information: NCBI) that produces alignment of two given sequences.

Results

To confirm the presence of FPV in scab materials and pharyngeal lesions, an FPV-specific PCR was performed. This generated expected size (578 bp) amplification (Fig. 2: lanes 2 and 3). Nucleotide sequences of these PCR products was found to be cognate (99% nucleotide identity) with the 4b core protein gene of several isolates viz. FPV Munich isolate HP438 (GenBank Accession No. AJ581527), FPV strain HP444 (M25781), FPV USA Animal and Plant Health Inspection Service (APHIS) (AF198100).

A 807 bp PCR product was generated from the scab and pharyngeal lesions (Fig. 2) and sequence analysis of this product showed 93.9% sequence similarities to *env* gene of REV A (GenBank Accession No. X01455). The PCR for the region of FPV flanking REV 5′LTR

resulted in the detection of amplified products of expected size (Fig. 2). Analysis of the sequence of 370 bp PCR products from each tissue sample revealed a fragment of REV 5′LTR (183 bp) at the 3′ end (Fig. 3), which showed 96%-98% sequence homology to that of several REV isolates (GenBank Accession Nos GQ870289, AF198100, AY255633). Upon alignment with the REV 5′LTR of near full-length provirus present in the FPV S (Australian standard vaccine strain) (8), two nucleotide mismatches and three deletions were observed in the present virus (designated FPV I).
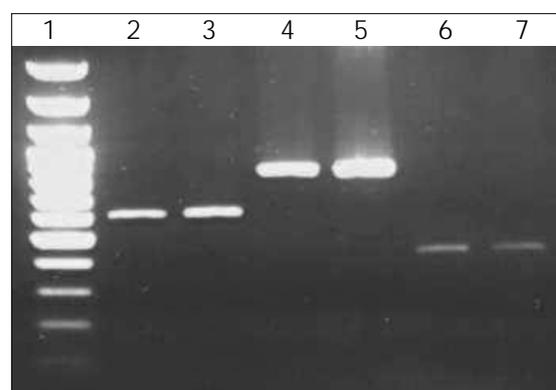
Figure 2
Polymerase chain reaction amplification of fowl poxvirus (FPV) 4b gene, reticuloendotheliosis virus (REV) *env* gene and FPV-REV junction site
FPV 4b gene, REV *env* gene and FPV-REV 5′ long terminal repeat (LTR) were amplified from DNA extracted from scab material which yielded 576 bp (lane 2), 807 bp (lane 4) and 370 bp (lane 6) products, respectively
Similar amplifications were observed (lanes 3, 5 and 7, respectively) with the DNA isolated from pharyngeal lesion
Lane 1: 100 bp DNA molecular weight marker SM#0321 Fermentas

Discussion

This study reports on a recurring FPV infection in backyard poultry farms in villages of the West Bengal area of India. The infection was recurring in birds of various age groups, particularly during the winter months, and for several years. The location of infection was limited to a radius of 15-20 km. The severity of the disease was negligible but the growth rate of chicks and other productive performances were seriously affected (poor nutrition,

```
                                              ← FPV │ U3 →
FPV S 5'LTR  aacqgaacaqtaacaatataaaaaqTGTGGGAGGGAGCTCCGGGGGGAAT  50
FPV I 5'LTR  tacqgaacaqtaacaatataaaaaq.....-....................  49

FPV S 5'LTR  AGCGCTGGCTCGCTAACTGCCATATTAGCTTCTGTAATCATGCTTGCTTG  100
FPV I 5'LTR  ...........................T......................  99

FPV S 5'LTR  CCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCATTTCTCGGAA  150
FPV I 5'LTR  ..................................................  149

FPV S 5'LTR  TCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAG  200
FPV I 5'LTR  ..........--......................................  197

FPV S 5'LTR  GCAAGCATCAG                                         211
FPV I 5'LTR  .........A                                         208
```

Conserved nucleotides are indicated by dots
Deletions are indicated by a dash
Mismatched nucleotides are presented in bold
Flanking FPV sequences are shown in lower case
The unique sequences (U3) of the REV long terminal repeat (LTR) are indicated

Figure 3
Alignment of reticuloendotheliosis virus (REV) long terminal repeat and flanking fowl poxvirus (FPV)
sequences present in FPV strains
The REV 5′ LTR present in FPV S (Australian vaccine strain) (8) was aligned with the REV 5′ LTR sequence of the near
full-length provirus present in the genome of FPV I (Indian strain)

parasitic load, immunosuppressive factors etc.).

The immunosuppressive effects of REV is well documented and a major mode of transmission of this virus is through FPV as integration of REV provirus in the FPV genome is widely acknowledged and recorded as having occurred prior to 1949 (10). Almost all field isolates were found to contain near intact REV provirus in the genome of FPV. In contrast, in most of the commercial vaccines, integration is limited to the presence of incomplete LTRs (8, 14). In the present field strain of FPV, the FPV-5′REV LTR sequence and REV *env* sequence were detected, which suggests the integration of a near full-length REV provirus in the FPV genome. It is likely that the integrated near intact REV provirus can give rise to infectious REV which, in turn, can cause immunosuppression and/or runting-stunting syndrome. In the present outbreak, feathering defects (Fig. 1), which is one of the clinical signs of reticuloendotheliosis (4) was observed in chickens; however, there was no clinical immunosuppression, probably, due to the older age (8 weeks or more) of the chickens. It is to be noted that the age of infection is important as birds that are infected transovarially or in their first week of life are immunosuppressed, while those that are infected at three weeks of age or older have little evidence of clinical immunosuppression (12).

In conclusion, an FPV infection was reported to have persisted in unvaccinated backyard chickens for about three years. PCR on scab samples, together with results from sequencing revealed that the genome of the FPV field strain contained a near intact integrated REV provirus. Further work is being conducted to isolate the FPV in cell culture and to characterise the integrated REV provirus. It is also felt that these backyard poultry should be vaccinated with an attenuated FPV strain that is free of integrated REV.

Detection of fowl poxvirus integrated with reticuloendotheliosis virus sequences
from an outbreak in backyard chickens in India

Sanchay K. Biswas, Chandrakanta Jana, Karam Chand,
Waseem Rehman & Bimalendu Mondal

Acknowledgments

References

1. Binns M.M., Boursnell M.E.G., Tomley F.M. & Campbell J. 1989. Analysis of the fowlpoxvirus gene encoding the 4b core polypeptide and demonstration that it possesses efficient promoter sequences. *Virology*, **170**, 288-291.

2. Davidson I. & Malkinson M. 1996. A non-radioactive method for identifying enzyme-amplified products of the reticuloendotheliosis proviral env and LTR genes using psoralen-biotin labelled probes. *J Virol Methods*, **59**, 113-119.

3. Dhanesar N.S., Sharma S.N. & Malik B.S. 1980. Fowlpox outbreaks in vaccinated flocks and isolation of virus strains. *Ind Vet Med J*, **4**, 144-148.

4. Diallo I.S., MacKenzie M.A., Spradbrow P.B. & Robinson W.F. 1998. Field isolates of fowlpox virus contaminated with reticuloendotheliosis virus. *Avian Pathol*, **27**, 60-66 (www.informaworld. com/smpp/content~db=all~content=a784671941~frm=abslink accessed on 25 March 2011).

5. Fatunmbi O.O. & Reed M.W. 1998. Vaccination against fowl pox may not result in protection. *World Poultry*, **14**, 67.

6. Garcia M., Narang N., Reed W.M. & Fadly A.M. 2003. Molecular characterization of reticulo-endotheliosis virus insertions in the genome of field and vaccine strains of fowl poxvirus. *Avian Dis*, **47**, 343-354.

7. Garg S.K., Joshi H.C. & Shukla H.C. 1984. Vaccination failure in fowlpox. *Ind J Vet Med*, **4**, 60-61.

8. Hertig C., Coupar B.E.H., Gould A.R. & Boyle D.B. 1997. Field and vaccine strains of fowl pox virus carry integrated sequences from the avian retrovirus, reticuloendotheliosis virus. *Virology*, **235**, 367-376.

9. Joshi R.K., Shakya S. & Gupta N. 1995. Isolation of fowl pox virus from fowl pox vaccinated layer flock. *Ind J Comp Microbiol Immunol Infect Dis*, **16**, 139-140.

10. Kim T.-J. & Tripathy D.N. 2001. Reticuloendotheliosis virus integration in the fowl poxvirus genome: not a recent event. *Avian Dis*, **45**, 663-669.

11. Lee L.H. & Lee K.H. 1997. Application of polymerase chain reaction for the diagnosis of fowl poxvirus infection. *J Virol Methods*, **63**, 113-119.

12. Li J., Calnek B.W., Schat K.A. & Graham D.L. 1983. Pathogenesis of reticuloendotheliosis virus infection in ducks. *Avian Dis*, **27**, 1090-1105.

13. Mishra S.S. & Mallik B.B. 1996. Comparative immunological and genomic characterization of fowlpox virus isolates. *Ind J Exp Biol*, **34**, 11-17.

14. Moore K.M., Davis J.R., Sato T. & Yasuda A. 2000. Reticuloendotheliosis virus (REV) long terminal repeats incorporated in the genomes of commercial fowl poxvirus vaccines and pigeon poxviruses without indication of the presence of infectious REV. *Avian Dis*, **44**, 827-841.

15. Moss B. 1996. *Poxviridae*: the viruses and their replication. *In* Field's virology (B.N. Field, D.M. Knipe, P.M. Howley, R.M Chanock, J.L. Melnick, T.P Monath, B. Roizman & S.E. Straus, eds.). Lippincott-Raven, Philadelphia, Pennsylvania, 2637-2671.

16. Sharma S.N., Grewal G.S., & Baxi K.K. 1981. Respiratory form of fowlpox among vaccinated flocks. *J Research*, Punjab Agricultural University, Ludhiana, **18**, 53-54.

17. Singh P., Kim T.-J. & Tripathy D.N. 2000. Re-emerging fowlpox: evaluation of isolates from vaccinated flocks. *Avian Pathol*, **29**, 449-455.

18. Singh P., Schnitzlein W.M. & Tripathy D.N. 2003. Reticuloendotheliosis virus sequences present in the genomes of field strains of fowlpox virus display variability. *J Virol*, **77**, 5855-5862.

19. Tadese T. & Reed W.M. 2003. Detection of specific reticuloendotheliosis virus sequences and protein from REV-integrated fowlpox virus strains. *J Virol Methods*, **110**, 99-104.

20. Tripathy D.N. 1991. Pox. *In* Diseases of poultry, 9th Ed (B.W. Calnek, H.J. Barnes, C.W Beard, W.M. Reid & H.W. Yoder Jr, eds). Iowa State University Press, Ames, Iowa, 583-596.

Sanchay K. Biswas, Chandrakanta Jana, Karam Chand,
Waseem Rehman & Bimalendu Mondal

Detection of fowl poxvirus integrated with reticuloendotheliosis virus sequences
from an outbreak in backyard chickens in India

21. Tripathy D.N., Hanson L.E. & Killinger A.H. 1974. Atypical fowlpox in a farm in Illinois. *Avian Dis*, **18**, 84-90.

22. Tripathy D.N. Sells D.M. & Hanson L.E. 1975. Natural pox and herpes as a dual viral infection in chickens. *Avian Dis*, **19**, 75-81.

23. Tripathy D.N. & Hanson L.E. 1978. Pathogenesis of fowl pox in laying hens. *Avian Dis*, **22**, 259-265.

24. Tripathy D.N., Singh P. & Schnitzlein W.M. 1998. Fowl pox: a re-emerging disease in chickens. *In* Proc. 49th North Central Avian Disease Conference and Symposium on enteric and emerging diseases, 27-29 September, Indianapolis, Indiana. Purdue University, Purdue, 36-37.

25. Wang J., Meers J., Spradbrow P.B. & Robinson W.F. 2006. Evaluation of immune effects of fowl pox vaccine strains and field isolates. *Vet Microbiol*, **116**, 106-119.

26. Wilhelmsen K.C., Eggleton K. & Temin H.M. 1984. Nucleic acid sequences of the oncogene v-rel in reticuloendotheliosis virus strain T and its cellular homolog, the proto-oncogene c-rel. *J Virol*, **52**, 172-182.

27. Witter R.L. 1997. Reticuloendotheliosis. *In* Diseases of poultry, 10th Ed. (B.W. Calnek, H.J. Barnes, C.W. Beard, R.L. McDougald & Y.M. Saif, eds). Iowa State University Press, Ames, Iowa, 467-484.