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Application of polymerase chain reaction and restriction endonuclease analysis for the detection and differentiation of turkey-pox and fowl-pox virus infections

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ABSTRACT

Polymerase chain reaction (PCR) was standardized for the amplification of A-type inclusion body (ATI) gene using self-designed primers. Amplification was also achieved by using published primer for 39 K core protein gene of turkey-pox virus (TPV) and fowl-pox virus (FPV). Amplicons of 1.8 Kbp and 800 bp product were produced for ATI and 39 K gene, respectively, in both turkey-pox and fowl-pox virus infected samples. The applicability of the standardized PCR for the detection of FPV and TPV in a variety of experimental samples [infected chorioallantoic membrane (CAM), scabs, lymphocytes and chicken embryo fibroblast (CEF) cell cultures] indicated the usefulness of this novel technique for the diagnosis of pox virus infection at molecular level. Restriction endonuclease analysis (REA) revealed that the 800 bp amplicon of 39 K gene in both these viruses has no cleavage sites for *Bam* HI, *Hpa* II and *Eco* RI endonuclease enzymes. *Bam* HI enzyme cleaved FPV ATI gene amplicon at 2 sites giving 3 fragments of 1660, 100 and 40 bp size, but could not cleave TPV amplicon. *Hpa* II cleaved TPV ATI gene amplicon at 2 sites giving 3 fragments of 1400, 250 and 150 bp but did not cleave that of FPV. The enzyme *Eco* RI cleaved the ATI gene amplicon of both the viruses at 2 sites yielding 3 fragments of 1500, 200 and 100 bp in TPV, and 1110, 530 and 160 bp fragments in FPV. The RE analysis revealed the existence of genomic differences in ATI genes of turkey-pox virus and fowl-pox virus.

Key words: ATI gene, Fowl pox virus, PCR, REA, Turkey pox virus, 39 K gene

Avian pox causes severe economic losses in terms of poor growth, downgrading and increased cull rates due to meat condemnation in poultry (Wakenell 2001). Fowl-pox virus represents the type species of the genus *Avipoxvirus* (Tripathy and Reed 2003). The virus is currently considered as an emerging pathogen with world-wide distribution. Fowl pox and turkey pox, also known as contagious epithelioma or sorehead, are highly contagious diseases of chickens and turkeys and till now thought to be almost similar as these are serologically indistinguishable. Avian pox frequently occurs due to variant or more virulent strains as disease outbreaks occur in spite of proper vaccination (Singh *et al.* 2000, Dash *et al.* 2005). This may be either due to antigenic differences between avipoxviruses (Ghildhyal *et al.* 1989) or incomplete protection provided by prevailing vaccines (Garg *et al.* 1984) or the integration of reticuloendothelial virus in FPV genome resulting into increased virulence of the poxvirus (Fernandez 2003).

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The genome of avipoxvirus is about 300 Kbp in length bearing around 260 genes and being the largest animal virus has the potential to code for large number of proteins (Tripathy and Reed 2003). The genes located centrally are relatively conserved whereas the terminally located genes are variable (Binns *et al.* 1990). Most commonly studied genes of FPV are TK gene, 4b gene, F12L gene, CPD photolyase gene etc. Studies on the TPV are relatively limited (Dash *et al.* 2005). For the determination of unequivocal genomic variability between TPV and FPV by means of polymerase chain reaction – restriction endonuclease analysis (PCR-REA) and to gain a better understanding for detection and differentiation of the these 2 viral infections, 39K gene and FPV-190 gene/A-type inclusion body (ATI) genes were selected for the present study, which are responsible for potential immunogenicity and pathogenicity, respectively.

MATERIALS AND METHODS

Viruses

Turkey-pox virus (TPV) field isolate (511/AD/2001) and fowl-pox virus (FPV) field isolate (588/AD/2002) obtained from Virology Laboratory, Division of Avian Diseases, IVRI, in freeze dried chorioallantoic membrane (CAM)

homogenate form, were adapted on the CAM and chicken embryo fibroblast (CEF) cell cultures of susceptible chicken embryos up to 5 passages (Dash *et al.* 2004). The titres of FPV and TPV at fifth passage level were $10^{5.0}$ EID_{50}/ml and $10^{4.5}$ EID_{50}/ml, respectively, in embryonated chicken eggs and $10^{5.5}$ $TCID_{50}$/ml and $10^{4.25}$ $TCID_{50}$/ml, respectively, in CEF cell culture system.

Isolation of genomic viral DNA

The methods of Esposito *et al.* (1981) and Shinagawa *et al.* (1983) were used for the isolation of viral DNA from the FPV and TPV infected CEF cell monolayers, CAM and scabs, respectively. The DNA pellets were washed once with 70% ethanol and once with 100% ethanol. The pellets were dried completely, resuspended in 50 µl of TE buffer pH 8.0 (0.01M TRIS HCl, 0.001 M EDTA) and stored at –20°C. The peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation (Haddad and Mashly 1991), using histopaque (1.077 g/ml), from 3 ml of aseptically collected blood samples from chickens and turkeys, experimentally infected with FPV and TPV, respectively. Lymphocytes visualised in the form of a white band on the interface of pelleted RBCs and histopaque were collected carefully and washed thrice with phosphate buffer saline (PBS). A pellet of lymphocytes was achieved in the bottom of the centrifuge tube. This pellet was mixed in 1ml of PBS (pH 7.2), which was treated further as per Esposito *et al.* (1981) used earlier in DNA isolation from CAM of infected embryos. The quantity and purity of the DNA was estimated by the absorbance ratio at OD_{260}/OD_{280}. The intactness of the DNA was checked by electrophoresis on 1.5% agarose gel (Sambrook *et al.* 2001).

Polymerase chain reaction

DNA amplifications were performed in an automatic thermocycler in 25 µl reaction volume in 0.5 ml thin-walled polymerase chain reaction (PCR) tubes using primers for a part of ATI gene and 39K gene of FPV and TPV. The published primer sequence (Boulanger *et al.* 1998) of FPV against 39K core protein gene with some modifications was used in the present study. The primer against FPV-190 (ATI) gene was self-designed using computer software programme (Gene tool). The sequences of the primers for ATI gene : Forward – 5'-GCAAATCGATCTCTCACCAACA3', Reverse: 5'TGGGACAAATGTGGTATCGAA3'; and for 39K gene : Forward – 5'AGGAATAATAGCATCTCTGAG3', Reverse: 5'GCTGAGAACTTCCACAAAG3'. The amplification reactions were performed in volumes of 25 ml, each containing 10X *Taq* DNA polymerase assay buffer (2.5 µl), 10 mM dNTPs each per reaction (1µl), 5 units/µl *Taq* DNA polymerase (0.3 µl), 50 pmole of each of forward and reverse primer (1 µl each), nuclease free water (14.2 µl) and DNA template (5 µl).

For the amplification of ATI gene the reaction condition consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 2 min, followed by extension at 72°C for 1 min and a final extension at 72°C for 10 min. For 39K core protein gene, the amplification reaction condition consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 47°C for 1 min, followed by extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products generated were confirmed for their expected size in 1.5% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide in 0.5X Tris-acetate EDTA (TAE) buffer as per Sambrook *et al.* (2001) using horizontal gel electrophoresis apparatus and visualized/ photographed in gel documentation system. The standardized PCR was applied for the detection of FPV and TPV in a variety of experimental samples (infected CAM, scabs, lymphocytes and CEF cell cultures) to diagnose the pox-virus infection at molecular level.

Restriction endonuclease analysis

The PCR products, generated from ATI gene and 39K gene of FPV and TPV, were purified using PCR purification kit. Purified amplicons were subjected to restriction endonuclease enzyme digestion using enzymes, viz. *Bam* HI, *Hpa* II and *Eco* RI as per the manufacturer's protocol employing specific assay conditions and the buffers supplied. For digestion, 0.5 to 1 µg of purified PCR product checked on the gel, was taken in a 10 µl reaction mix in a 0.5 ml eppendorf tube, containing 1.0 µl (10U/µl) of restriction enzyme and 1.0 µl of 10X compatible RE buffer, the final volume being made up with nuclease free water. The reaction was performed at 37°C overnight in digital water-bath, and stopped either by adding loading dye or stored at –20°C until it was subjected to agarose gel electrophoresis. Digested products along with 100 bp DNA ladder were electrophoresed in 2% (w/v) agarose gel in 0.5X TBE buffer at 80V for 3 h using horizontal submarine electrophoresis apparatus. The gels were examined on UV transilluminator and photographed by gel documentation system. The size of various fragments of digested viral DNA were determined using Fortran Software and expressed in base pairs (bp).

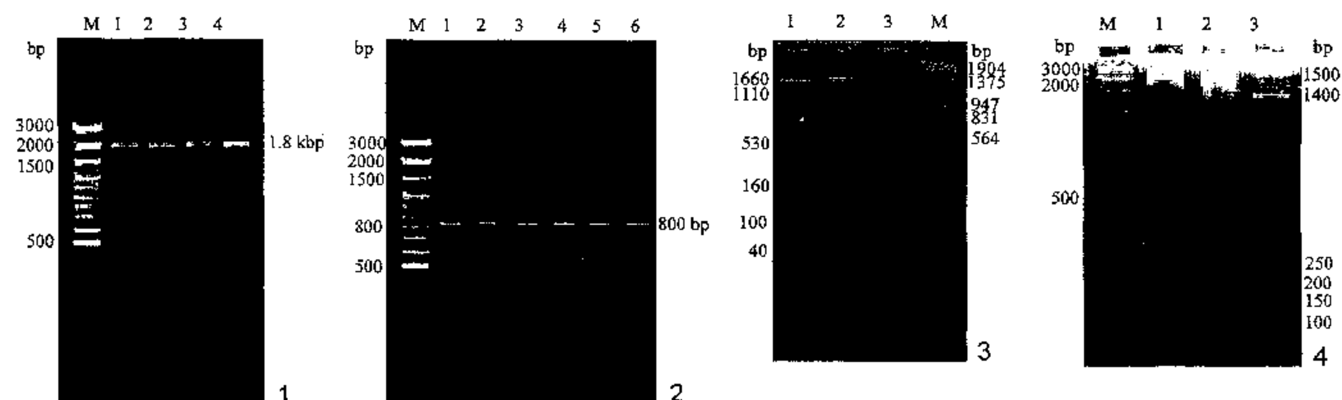
RESULTS AND DISCUSSION

Avian pox is a contagious and slow spreading viral disease, generally observed in chickens, but other avian species, viz. turkey, quail, pigeons etc. are also found to be susceptible to Avi-poxviruses (APVs, Tripathy and Reed 2003). The rapid detection with high specificity and differentiation of APVs infection is required for effective management of the economically important disease. Serological and histopathological methods for diagnosing the pox diseases are generally time consuming, labor intensive and less sensitive due to extensive cross reactions observed between the members of *Avi-poxvirus* genus. Also, these methods are

not very reliable for differentiation of different APV strains. Thus the conventional detection methods are perceived to be having limitations and moreover require *in-vivo* or *in-vitro* assay systems. To circumvent the difficulties in detection, epidemiological investigations, molecular characterization and differentiation of poxviruses, the present study was envisaged for detection and differentiation of fowl-poxvirus (FPV) and turkey-poxvirus (TPV), the members of *Avi-poxvirus* genus, based on polymerase chain reaction – restriction endonuclease analysis (PCR-REA). The viral genomic DNA from FPV and/or TPV infected chorioallantoic membrane (CAM), scabs/skin, lymphocytes and chicken embryo fibroblast (CEF) cell cultures were isolated. The ratio of $OD_{260/280}$ for isolated DNAs was between 1.7 and 1.8, which indicated the purity of the DNA samples. The total amount of viral DNA extracted from each sample as estimated from A_{260} nm value was between 30.5 µg and 45.2 µg per ml. On agarose gel electrophoresis the isolated DNAs showed a single band indicating their purity and intactness. The ATI gene and 39K core protein gene of FPV and TPV isolates were PCR amplified using specific sets of self-designed primers. The thermocycling conditions utilized in the PCR assay were found to be optimum and yielded virus specific amplicons. The size of the amplicon generated against ATI gene and 39K core protein gene was found to be 1.8 Kbp (Fig. 1) and 800 bp (Fig. 2), respectively, for both of the viruses. All the amplicons obtained from different samples (infected CAM, scabs, lymphocytes and CEF) were of almost the same intensity and size.

PCR, an *in-vitro* technique for amplifying a specific DNA fragment, is an exquisitely sensitive and rapid molecular diagnostic technique, being used widely for the diagnosis of a variety of avian pathogens (Cavanagh 2001, Kataria *et al.* 2005). In the present study, by using the primers for ATI and 39K gene, amplicons of 1.8 Kbp and 800 bp were generated, respectively, providing the confirmatory presence of the FPV and TPV infections in the infected tissues, culture fluids and blood samples. Corresponding differences in the size of the amplicon were observed by the amplification of 39K gene from genomes of fowl-poxvirus (FPV) and pigeon poxvirus (PPV) strains due to the difference in the size of the central part of the protein (Singh *et al.* 2000). Based on it, primers for 39K gene and ATI gene from genomes of FPV and TPV were taken for the study so as to detect variabilities, if any, between these 2 species-specific viruses. PCR exploits species-specific differences in A-type inclusion (ATI) body gene located in highly conserved region of FPV and TPV genome with the possibilities of sequence alterations in certain positions of hypervariable region of ATI gene (Tripathy and Reed 2003, Gubser *et al.* 2004). Some sequence alterations in certain positions of hypervariable region of ATI gene have been reported in camel-pox virus (Meyer *et al.* 1994). The applicability of the standardized PCR for the detection of FPV and TPV in a variety of experimental samples indicated the usefulness of this novel technique for the diagnosis of poxvirus infection at molecular level.

Further, for molecular characterization of the FPV and TPV, the PCR amplified products of ATI gene (1.8 Kbp) and 39K gene (800 bp) of both the viruses were subjected to restriction endonuclease analysis (REA) using restriction enzymes, viz. *Bam* HI, *Eco* RI and *Hpa* II. RE digestion of the ATI gene amplicon of both the viruses, with all the 3 enzymes revealed different restriction patterns, while 39K gene could not be digested. Digestion of FPV ATI gene amplicon with *Bam* HI enzyme revealed 3 fragments of 1660, 100 and 40 bp, indicating the presence of 2 cutting sites for this enzyme but the TPV ATI gene amplicon remained undigested. *Eco* RI enzyme digestion of ATI gene of both FPV and TPV indicated 2 sites for this enzyme in both the viruses but the three fragments obtained were of different molecular weights viz. 1110, 530 and 160 bp; and 1500, 200 and 100 bp, respectively. Amplicon digestion with *Hpa* II



Figs 1–4. **1.** PCR amplification of ATI gene. Lanes indicate: M-100bp DNA ladder; 1-TPV (CAM); 2-FPV (CAM); 3-TPV (Scab) and 4-TPV (CEF); **2.** PCR amplification of 39 K core protein gene. Lane M: 100 bp DNA ladder; Lane1-6: 1.TPV (CAM); 2.FPV (CAM); 3.TPV (scab); 4. Lymphocytes from fowl-poxvirus infected bird; 5. Lymphocytes from turkey-poxvirus infected bird; 6. Turkey-poxvirus (CEF). **3.** RE analysis of ATI gene of fowl-poxvirus. Lane M: Marker, DNA double digested with Hind III and *EcoRI*; Lane 1: *Hpa II*; Lane 2: *Bam HI*; Lane 3: *Eco RI*. **4.** RE analysis of ATI gene of turkey-pox virus. Lane M: 100 bp DNA ladder plus; Lane 1: *Bam HI*; Lane 2: *Eco RI*; Lane 3: *Hpa II*.

enzyme yielded 3 fragments of 1400, 250 and 150 bp in TPV ATI gene, while in FPV it remain undigested due to lack of the available restriction site for the enzyme to act. The RE analysis of the 39K gene amplicon in both FPV and TPV showed no cleavage sites for all the 3 enzymes. Thus the cleavage sites for these enzymes were found restricted to ATI gene sequences. Results of RE analysis are depicted in Figs 3 and 4.

The importance of ATI gene has been explored by many workers for diagnosis and molecular characterisation of cowpox, vaccinia, variola, camel-pox and mouse-pox viruses by Restriction endonuclear mapping, sequencing and dot blot hybridisation (Funahashi *et al.* 1988, Meyer *et al.* 1994). α-type inclusion (ATI) bodies are found in cells infected by poxviruses like cowpox, ectromelia, racoonpox, canarypox and fowlpox. This gene encodes antigenically similar proteins with apparent Mw of 94 Kd in vaccinia, 155 Kd in racoonpox, 92 Kd in monkeypox, 130 Kd in ecteromelia and 160 Kd in fowl poxvirus, which are characteristic of the virus type (Kitamato *et al.* 1986, Patel *et al.* 1986). By now no reports are available regarding the PCR-REA of ATI gene amplicon in turkey-poxvirus and fowl-poxviruses. Therefore, in the present study the A-type inclusion (ATI) body gene and 39K immunodominant gene, responsible for the pathogenicity and immunogenicity, respectively were selected for carrying out PCR-RE analysis of Indian FPV and TPV isolates.

In the present study 39K gene of TPV and FPV was amplified using slightly modified primers from the published sequences, generating a 800 bp size amplicon, which revealed no cleavage sites for *Bam* HI, *Eco* RI, and *Hpa* II endonuclease enzymes. However, Boulanger *et al.* (1998) amplified 39K gene of FPV using the original set of primers which yielded fragments of 1404 bp which got cleaved by *Bam* HI and *Hind* III enzymes. Corresponding differences in the size of the amplicons observed in the intact 39 Kd protein gene when amplified by PCR from genomes of FPV and PPV strains, which may be due to difference in the size of the protein expressed by this gene. The molecular mass of this protein varies in different strains of fowl-pox viruses from 36 to 42Kda (Boulanger *et al.* 1998). It may be suggested that the site for *Bam* HI endonuclease enzyme may be present in the 39K gene away from 800 bp, that may fall between 800 and 1404 bases. It can also be noticed in the present study that during the course of modification during designing of the primers, 11 bases in the flanking region at 5' end from both forward as well as reverse primer were deleted from the published primers for its use in amplification of 39K gene, which might have resulted in the generation of amplicon of small size with lack of cleavage site for *Bam* HI enzyme.

Several workers have reported the combination of PCR and RE to be a rapid, sensitive, specific and effective system for routine identification and differentiation of closely related genomes (Cavanagh 2001, Luschow *et al.* 2004). The well-established PCR technology, which can be used to detect very low copy numbers of viral nucleic acids and having several other unique advantages of detection of subclinical infection, quick diagnosis etc, combined with fast and routinely accomplished technique like RE analysis of amplified products is a sensitive tool for characterization and strain differentiation. Diagnosis and differentiation of poxvirus infections was studied by utilising PCR-REA (Ghildyal *et al.* 1989, Mockett *et al.* 1992, Lee and Lee 1997, Tripathy and Reed 1998, Tripathy 2000, Hosmani *et al.* 2004). Genomes of fowl-pox virus and quail-pox virus were compared by REA using *Bam* H1, *Eco* R1 and *Hind* III endonucleases revealing genomic difference between the 2 viruses (Ghildyal *et al.* 1989). Mockett *et al.* (1992) identified the restriction sites for *Bam* H1, *Pvu* II, *Pst* I and *Nco* I enzymes in the FPV, FP9 strain using REA method. Investigation for detection and differentiation of nine APVs were carried out by the use of PCR-REA for the region within FPV 4b core protein gene (Luschow *et al.* 2004), where *Mse* I and *Eco* RV enzyme were found to be useful in differentiating *Avi-poxvirus* species. Boulanger *et al.* (1998) and Singh *et al.* (2000) amplified the 39K gene from purified FPV genome of Munich origin and thus emphasized the diagnostic role of 39K gene in PCR and further by using REA method for differentiation of APVs. These research findings of recent past suggest that REA is a sensitive and useful technique for identification and classification of poxvirus isolates by comparing their genomes. In the same fashion, the REA of TPV and FPV carried out in the present study using three enzymes, viz. *Bam* HI, *Eco* RI and *Hpa* II to digest the PCR amplicons of ATI and 39K gene revealed the presence of sequence variations in ATI gene. Further sequence analysis of ATI gene of FPV and TPV will confirm the genetic changes at nucleotide level. Utility of these three enzymes can be explored for differentiation of avian-pox viruses.

REA coupled with PCR detection has provided useful information in determining the differences between vaccine and field strains of FPV (Singh *et al.* 2000, Tadese *et al.* 2003). Similarly, the serologically indistinguishable viruses, viz. FPV and TPV needs to be elaborated at molecular level as FPV vaccine is not providing proper protection against TPV infection in turkeys (Dash *et al.* 2005), which indicated that there might be species-specific genetic differences between these two pox viruses. Accurate and timely diagnosis of turkey pox and fowl pox by PCR-RE is of paramount importance for their prevention and control. Rapid diagnosis with these special methods aids in surveillance to limit the spread of disease and to identify the carriers/pathogens.

In conclusion, the ATI gene and 39K gene of APV strains (FPV and TPV) of Indian origin were amplified by PCR and the amplicons were further subjected to PCR-RE analysis to differentiate the strains at genomic level. The RE analysis of the amplicon generated by ATI gene amplification of FPV and TPV proved that genomic differences exist in this region. The

results indicated that the technique of PCR in combination with restriction endonuclease (RE) analysis can be helpful for genetic detection, differentiation and characterization of APVs. Its utility as a sensitive and rapid diagnostic test for easy differentiation of APVs and as a reliable molecular epidemiological tool needs to be explored further.

RE mapping of the PCR product generated by ATI gene amplification of FPV and TPV was helpful in identifying the differences existing in their genomes by employing *Bam* HI, *Eco* RI and *Hpa* II restriction enzymes.

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REFERENCES

Binns M, Mason C and Boursnell M. 1990. A 39000 molecular weight immuno-dominant protein of fowl-pox virus containing multiple copies of a 12 amino-acid repeat sequences. *Journal of Virology* **71**: 2883–88.

Boulanger D, Green P, Smith T, Czerny C and Michael S A. 1998. The 131 amino acid repeat region of the essential 39 kd core protein of fowl-pox virus FP9, equivalent to vaccinia virus A4L protein, is non-essential and highly immunogenic. *Journal of Virology* **72**: 170–79.

Cavanagh D. 2001. Innovation and discovery: the application of nucleic acid-based technology to avian virus detection and characterization. *Avian Pathology* **30**: 581–98.

Dash, B B, Kataria J M, Dhama K and Mazoomdar S. 2004. Isolation and identification of turkey-pox virus. *Proceedings XII Conference of Indian Poultry Science Association* (IPSA), Palampur (Himachal Pradesh).

Dash, B B, Mahendran M, Kataria J M, Dhama K and Praveen B N. 2005. *Avian Pox-An Overview*. Paper presented in National Symposium of Deptt. of Animal Husbandry & Dairying, Govt. of India at IVRI, Izatnagar (Uttar Pradesh).

Esposito J, Condit R and Obijeski J. 1981. The preparation of Orthopoxvirus DNA. *Journal of Virological Methods* **2**: 175–79.

Fernandez A. 2003. Emergence and reemergence of poultry diseases. *Revista Cubana De Ciencia Avicola* **27**: 95–101.

Funahashi S, Sato T and Shida H. 1988. Cloning and characterisation of the gene encoding the major protein of the A-Type Inclusion body of cow-pox virus. *Journal of General Virology* **69**: 35–47.

Garg S K, Joshi H C and Shukla S K. 1984. Vaccination failure in fowl pox. *Indian Journal Veterinary Medicine* **4**: 60–61.

Ghildyal N, Schnitzlein W M and Tripathy D N. 1989. Genetic and Antigenic differences between Fowl-pox virus and Quail-pox viruses. *Archives of Virology* **186**: 85–92.

Gubser C, Hue S, Kellam P and Smith G L. 2004. Poxvirus genomes: a phylogenetic analysis. *Journal of General Virology* **85**: 105–17.

Haddad E E and Mashly M M. 1991. Chicken growth hormone, triodo-thyronine and thyrotropin releasing hormone modulation of the levels of chicken natural cell-mediated cytotoxicity *Developmental Comparative Immunology* **15** : 65–71.

Hosmani M, Mondal B, Tembhurne P A B, Santanu K, Singh R and Rasool T J. 2004. Differentiation of sheep-pox and goat-pox viruses by sequence analysis and PCR-RFLP of p32 gene. *Virus Genes* **29**: 73–80.

Kataria J M, Madan C M, Dey S, Dash B B and Dhama K. 2005. Diagnosis and immunoprophylaxis of economically important poultry diseases: A Review. *Indian Journal of Animal Sciences* **75**: 555–67.

Kitamato N, Tanimoto S, Hiroi K, Miyamoto H, Wakamiya N, Ueda S and Kato S. 1986. Cross reactivity among cow-pox, ecteromelia and vaccinia viruses with monoclonal antibodies recognising distinct antigenic determinants in A-type inclusion bodies. *Archives of Virology* **91**: 357–66.

Lee L H and Lee K H. 1997. Application of polymerase chain reaction for the diagnosis of fowl-poxvirus infection. *Journal of Virological Methods* **63**: 113–19.

Luschow D, Hoffmann T and Hafez H M. 2004. Differentiation of avian-poxvirus strains on the basis of nucleotide sequences of 4b gene fragment. *Avian Diseases* **48**: 453–62.

Meyer H, Pfeffer M and Rziha H J. 1994. Sequence alterations within and downstream of A-Type inclusion protein genes allow differentiation of orthopox virus species by PCR. *Journal of General Virology* **75**: 1975–81.

Mockett B, Binns M, Boursnell M and Skinner M. 1992. Comparison of the locations of homologous fowl pox and vaccinia virus genes reveal major genome organisation. *Journal of General Virology* **73**: 2661–68.

Patel D D, Pickup D J and Jokli K W K. 1986. Isolation of Cowpox virus A-type inclusions and characterisation of their major protein component. *Virology* **149**: 174–89.

Sambrook J, Fristch, E F and Miniatus T C. 2001. *Molecular Cloning-A Laboratory Manual*. 3rd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Shinagawa M, Matsuda A, Ishiyama T, Goto H and Sato G. 1983. A rapid and simple method for preparation of Adenovirus DNA from infected cells. *Microbiology and Immunology* **27**: 817–22.

Singh P, Kim T J and Tripathy D N. 2000. Re-emerging fowl pox: evaluation of isolates from vaccinated flocks. *Avian Pathology* **29**: 449–55.

Tadese T, Potter E A and Reed W M. 2003. Development of a mixed antigen agar gel enzyme assay (AGEA) for the detection of antibodies to pox virus in chicken and turkey sera. *Journal of Veterinary Medical Science* **65**: 255–58.

Tripathy D N. 2000. Molecular techniques for the diagnosis of fowl pox. *137th AVMA Convention Notes*, pp. 655–56.

Tripathy D N and Reed W M. 1998. Pox. A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th edn, pp. 137–43. (Eds) Swayne D E, Glisson J R, Jackwood M W, Pearson J E and Reed W M. American Assosciation of Avian Pathologists, Kennett Square, PA.

Tripathy D N and Reed W M. 2003. Pox. *Diseases of Poultry*. 11th edn. (Eds) Saif Y M, Barnes H J, Glisson, J R, Fadly AM, McDougald L R and Swayne D E. pp. 253–69. Iowa State University Press, Ames, IA, USA.

Wakenell S P. 2001. Avian poxvirus. 'Zootechnica'. *World Poultry Journal*. pp. 48–52.