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With 2 figures in the text

Detection of Reticuloendotheliosis Provirus in The Genome of Fowl Pox Virus Field Isolates in The Sudan Using PCR

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الملخص

أُجرى إختبار تفاعل البلمرة المتسلسل علي عينات من الحمض النووى المستخلص من دواجن مصابة بفيروسات جدري الدواجن والكناري والحمام. أستعمل نوعان من بادئات التفاعل هما: FPVORF 108 للكشف عن فيروسات الدواجن والكناري والحمام وبادى التفاعل REV LTR للكشف عن إدماج فيروس (REV) في عينات الحقل. وجد أن عينات الحمام والكناري موجبة باستخدام بادى التفاعل FPVORF 108 بينما عينات الحقل موجبة بإستخدام بادى التفاعل REV LTR و عينات الحمام والكناري سالبة. أظهرت هذه الدراسة أن إختبار تفاعل البلمرة المتسلسل يمكن إستخدامه لتشخيص الإصابة بجدري الدواجن وذلك لسهولة إجراء الإختبار وسرعته حيث يمكن إجراءه خلال يوم الي يومين و ذلك عند عدم ظهور علامات مميزة بالصوره الاكلنيكية و الصفة التشريحية و التشريح النسيجي. كما أن عزل الفيروس مُجهد ويستغرق زمن حيث يحتاج إلي 7-14 يوم. أظهرت هذه الدراسة والتي تمثل أول تقرير لظاهرة إدماج فيروس REV في جينوم جدري الدواجن في العينات الحقلية في السودان.

Summary

Polymerase chain reaction (PCR) was conducted on DNA samples from chicken infected with fowl pox, pigeon and canary pox viruses. Two set of primers were used, fowl pox major envelope antigen gene primer (FPVORF 108) for the detection of fowl, pigeon and canary pox viruses, while REV LTR primer was used for the detection of the REV provirus in local isolates of fowl, pigeon and canary pox virus. Pigeon and canary pox viruses isolates with FPV (ORF 108) gave positive results with a product size of 1,222 bp. Fowl pox field isolates with REV LTR primers, gave positive result with product size of 900 bp, while canary and pigeon pox virus were negative. This study shows that PCR is most suitable for the diagnosis of fowl pox infection for its simplicity and relative rapidity (1-2 days) when clinical signs, postmortem lesions and histopathological findings are not clear. In addition, virus isolation is laborious, time consuming and would take 7-14 days. To the best of our Knowledge, this is the first report of integration on RE provirus in fowl pox virus field isolates in the Sudan.

Introduction

Singh *et al.* (2003) have reported on the incidents of nine field strains of fowl pox virus (FPV) which were isolated from geographically different outbreaks of fowl pox during a 24 years period, in the United States. They were screened for the presence of Reticuloendotheliosis virus (REV) sequences in their genomes by PCR. Each isolate seemed to be heterogeneous in that it was either an almost entire provirus or only a 248–or 508–nucleotide fusion of portions of the integrated REV 5 and 3.

REV belongs to the family Retroviridae, genus *Gammaretrovirus* (Murphy *et al.,* 1999) and they are avian oncogenic retroviruses (Purchase *et al.,* 1973). Lymphoma is associated with integration of REV proviral genome adjacent to the c.myc gene (Fadly, 2000). Although losses in REV affected flocks can be significant due to tumor mortality and or immunosuppression, the principal economic concern of REV infection is contamination of poultry live virus vaccines or acting as barriers to export to certain countries (Fadly, 2004). The aim of this

study was the use of PCR for detection of REV in the fowl, pigeon and canary pox viruses field isolates.

Materials and Methods

Propagation of the viruses on the CAM of embryonated eggs

The propagation of the viruses was done on the chorioallantoic membrane (CAM) and performed as described by Tripathy and Cunningham (1984). Thirteen-day-old embryonated eggs were each inoculated onto the CAM with 0.2 ml of FPV. The eggs were incubated at 37˚C for five days before the development of the pock lesions was examined. The inoculation was repeated using canary and pigeon pox viruses separately.

DNA isolation

Extraction of viral DNA from the lesions on the comb of infected chickens and from pocks on virus infected CAMs, was made according to Kim *et al.* (2003) using phenol-chloroform extraction method with some modifications. Two ml of cell lysis solution (10 mM Tris-HCl ph 7.4, 100 mM NaCl, 10mM EDTA, and 0.5% SDS), and 2% B mercaptoethanol was added to 200-500 µg of infected CAMs or scabs and homogenized in mortar and pestle. Then 15 µl of proteinase K solution (10mg/ml) was added and the mixture was vortexed for few seconds, incubated at 55 °C for 4 hrs or at 37 °C for overnight. Ten µl of Rnase was added and the tube was inverted 25 times, and then incubated at 37 °C for 15 mins. DNA was extracted from the digest in the presence of equal volume of phenol-chloroform-isoamylalcohol (50:2:48). The extraction lasted for 30 second with mixing by inverting Eppendorf tube followed by centrifugation at 10.000 rpm for l min. Three M sodium acetate was added in a volume equivalent to 1:10 of aqueous phase and equal volume of isopropanol alcohol was added, mixed and stored at -20 °C overnight. The DNA was pelleted by centrifugation at 13000 rpm for 45 min at 4 °C. Then the DNA pellet was washed with 300 µl of 70% ethanol with centrifugation at 13000 rpm for 15 min at 4 °C. The tube was then allowed to dry for 10-15 min. Fifty µl of DNA hydration solution was added to the DNA which was dissolved by incubating at 65 °C for 1 hr before it was stored at 4 °C until used.

PCR amplification

The primers used in this study were specific for the major envelope antigen gene of FPV (FPV ORF 108) (Calvert *et al.,* 1993; Afonso *et al.,* 2000). They were FPV-For primer: 5'-CATACATTACTCTT AATTCGTTTC-3 Rev primer: 5'-TTGTA ACTGTCTATTAGTGCC-3'. These primers are expected to give 1222bp fragment. In addition, specific primers to detect the envelope and 5'LTR sequences of REV (Kim *et al.,* 2003) were used. The primers are expected to give 900bp in amplifications; they were REV-For primer: 5'-GAAGCA GACAATAGGATGG-3' and Rev REV primer: 5'-CCTCGAGGTCAAATGAT GACCT AGG-3'.

PCR of fowl, pigeon and canary pox viruses

PCR was performed in a final volume of 50 µl including 3 µl of template DNA, 2 µl Taq DNA polymerase (Invitrogen), 2 µl FPV 108 forward and reverse primers each and REV specific primer, 5 µl of NH_4So_4, 1 µl dNTP, 3 µl $MgCl_2$, 15 µl of 5m Mg_2So_4 and then 18 µl of distilled water were added to complete the volume to 50 µl. After the initial denaturation for 30s at 94 ^{0}C the DNA was amplified for 35 cycles of 30s of denaturation at 94 ^{0}C, 30s of annealing at 56 ^{0}C and 9 min of extension at 68 ^{0}C, with a final extension step of 10 min at 68 ^{0}C. As positive control, 1 µl of the original REV genome was also included. Double distilled water is used as negative control. The amplicons were analyzed by gel electrophoresis using 1 % agarose gel.

Results

PCR with primer pair FPV ORF (108)

The three DNAs samples under test gave positive result for the major envelope gene (FPV ORF 108). Strong bands were detected in the ethidium bromide stained-gel that corresponds exactly to the expected DNA band size of the DNA positive control (1222bp) (Fig. 1).

PCR with primer pair REV-F/REV-R

The DNA of fowl pox isolate under test gave positive results for the REV env gene and the bands corresponded exactly to the expected DNA band size of the REV genome positive control (900bp) (Fig. 2). No PCR products were obtained from the amplification of pigeon and canary pox DNAs using REV/F and R primers.

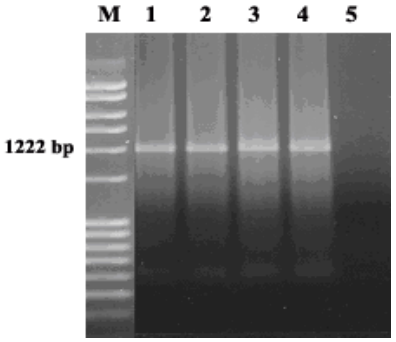

Fig. 1: Visualization of PCR product of fowl, pigeon and canary pox viruses using major envelope gene showing the target 1222 bp.

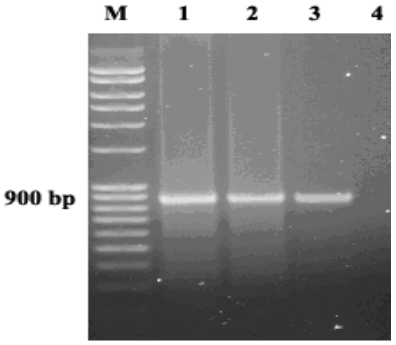

Fig. 2: Ethidium bromide stained-gel of PCR products of DNAs of fowl pox (11), pigeon (2) and canary (13) pox viruses using *env* gene showing the target 900 bp.

Discussion

Recently, molecular biological methods such as polymerase chain reaction (PCR) have proved to be useful in the detection of genomes of several avian viruses (Cavanagh, 2001). PCR is considered to be one of the most sensitive techniques for the routine diagnosis. PCR with primers that are designed for a well conserved region within FPV (ORF 108), the major envelop antigen gene of fowl pox virus DNA was reported (Calvert *et al.,* 1993; Afonso *et al.*, 2000; Kim *et al.*, 2003). In the present investigation, we used the primers set (FPV ORF 108) which amplifies the gene in the DNAs of fowl, pigeon and canary pox viruses and DNA positive control and gives a PCR product size of 1222bp. The successful amplification of this gene, obtained from pigeon and canary pox DNAs, points to the fact that the major envelop antigen gene (FPV ORF 108) is a gene common to the three viruses. This finding is promising and may prepare the ground for future production of a recombinant vaccine that has the major envelop antigen gene to cover the three avipox viruses. The increased prevalence of FPV isolates with genomic integrated REV sequences has raised the interest in adoption of current REV detection method to screen stock cultures of FPV for the presence of infectious provirus. In the present study, we were able to detect the env gene of the Reticuloendotheliosis virus in the genome of the field FPV isolates. The primers set used in this study (Kim *et al.,* 2003) specifically amplifies Reticuloendotheliosis virus provirus env gene. It has been shown that REV is immune-suppressive for the lymphoid organ (Kimura *et al.,* 1976) and the susceptibility of vaccinated chickens to FPV-REV could be explained by the lack of adequate immunity due to immunosuppression by FPV-REV (Coupar *et al.,* 1990). No amplifications with REV primer were obtained from the genomes of pigeon and canary pox viruses. This result is in agreement with Moore *et al.* (2000) who reported that no REV sequences have been found in the DNAs of either canary, quail, or a field isolate

of pigeon pox viruses. The presence of REV LTR sequences within the FPV strains is not a random event, but an indication of a functional or an evolutionary relationship between the viruses. Specific-pathogen-free and congenic chicken lines have been used to detect infectious REV in the FPV vaccine stocks (Fadly and Witter, 1998). However, these studies are not easy to reproduce because REV titres cannot be standardized. Therefore, at this point, PCR is the preferred tool to screen FPV samples for REV provirus. To the best of our Knowledge, this is the first study in the Sudan to report the phenomenon of integration of REV provirus in the genome of FPV field isolate.

It may be concluded that use of PCR for laboratory diagnosis of fowl pox in the Sudan should be implemented.

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