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Molecular Detection of Pox Virus in Pigeon

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SUMMARY

Pigeon pox virus causes a serious disease in pigeons and may threats the life of these birds. The pigeon pox virus is easily detected in clinical samples when the signs appeared but in that case the disease could affect the health of birds and may be complicated by secondary bacterial infections. So, the rapid and accurate detection of the virus is needed. In this study the application of molecular techniques based on nucleic acid was characterization done. Molecular characterization of pigeon pox virus was successful carried out by using PCR and real-time PCR techniques for the pox virus in samples from 8 clinical cases of suspected diseased pigeons from three provinces (Giza, Kafr El-Sheikh and Beni-Suef). DNA was extracted from skin lesions from each case, the primers and PCR conditions were used to amplify the FWPV P4b gene. Results of PCR amplification of pigeon poxvirus and agarose gel electrophoresis showed that there were five out of the eight suspected field samples were positive for the presence of pigeon pox virus with the expected correct size bands of 578 bp.

Real-time polymerase chain reactions (r-PCR) assay was also used for detection of the virus by using the same previously described primers with SYBR Green mix. Real time PCR dissociation curve of PCR products of the SYBR Green PCR assay indicated that the PCR products of melting temperature (Tm) at 75-77 °C were positive for 5 samples out of the 8 suspected cases of pigeon pox virus similar to the obtained results by conventional PCR.

In this study, the molecular methods used were able to detect virus in clinical

samples and showed the same sensitivity as virus isolation in case of obvious clinical signs but the molecular methods were more rapid and reliable when compared to conventional methods for virus isolation.

Key words: pigeon pox virus, conventional PCR, Real time SYBR Green PCR.

INTRODUCTION

Avian poxviruses belong to the genus Avipoxvirus of the Family Poxviridae within the subfamily Chordopoxvirinae (Thiel et al., 2005 and OIE, 2008). Pigeon poxvirus (PGPV) is antigenically related to fowl poxvirus (FPV), while the genomes of quail and canary poxvirus are different from FPV. Some pigeon poxviruses cause mild disease in chicken and turkeys. Pigeons experimentally susceptible to turkey, fowl and canary poxviruses (Tripathy and Reed, 1997).

There are at least 16 different species of avipoxviruses, pigeons being infected by their own poxvirus (Tripathy and Reed, 2003). Manuel et al., (2004) describes the first report of avian pox in white tailed laurel-pigeons in Canaries. Pox lesions involving feathered and unfeathered skin with characteristic intracytoplasmic inclusions were detected in the proliferative cells of all lesions and were phylogenitically related to wood pigeon poxvirus.

Protein and DNA trees show the avipoxviruses forming two major clades (A and B). The phylogenetic analysis of two PGPV, clade A and B can infect and cause disease in pigeons (Jarmin et al., 2006).

Morbidity may be as high as 90% but mortality is usually limited (Marlier and Vindevogel, 2006). Because of poxviruses stability outside the host, they can be transmitted from bird through contact with contaminated gloves, soil, perches or enclosures (Branson and Kip, 1995). In the naturally occurring disease, the incubation period is between 4 and 10 days. In the coetaneous form (dry pox), foci of epidermal hyperplasia are found on non-feathered areas of the body, usually on the head (eyelids, commeasure of the beak and caruncles), and may develop into dry scabs. In the mucosal or diphtheritic form (wet pox), diphtheritic membranes appear on the mucous membranes of the mouth, lung, pharynx, trachea and esophagus (Singh and Tripathy, 2003 and OIE, 2008). These lesions may develop into scabs that usually heal in 3–4 weeks (Chauhan et al., 1998 and Rupiper, 1998).

Diagnosis is generally made by the clinical presentation and the lesions; however, a differential diagnosis that should be considered for the dry form is bumble foot or staphylococcal infection (Schoemaker et al., 1998). Avian pox can be confirmed by histological identification of eosinophilic intracytoplasm inclusion bodies (Bollinger

bodies). Virus isolation can be attempted on the chorioallantoic membrane of embryonated chicken eggs or in duck embryo fibroblast cells (Ritchie, 1995).

Genomic DNA sequences of various sizes can be amplified by the polymerase chain reaction (PCR) using specific primers ((Huw Lee and Hwa Lee, 1997). Recently, a nested PCR was described for the detection of fowlpox. This technique is useful when there is only an extremely small amount of viral DNA in the sample (Fallavena et al., 2002).

The objective of this study is to apply simple, accurate and specific diagnostic molecular tests for avian poxvirus that could be adapted for use in the diagnosis of pigeon pox as rapid as PCR and real time PCR.

MATERIAL AND METHODS

Field samples:

Pigeons with lesions resembling pox infections from 8 sporadic cases from three provinces (Giza, Kafer El-Sheikh and Beni-Suef). Each pigeon was examined closely for evidence of abnormalities, which primary consisted of proliferative lesions on the legs or face. If the lesions were in location and of a size that permitted sampling with minimal risk to the bird, a small portion was excised with a sterile scalpel, placed into an ependorf vial and frozen at -40°C for later analysis for lab diagnosis. Fresh dried lesions collected from live birds were prepared for PCR and real time PCR.

Avian embryo inoculation:

Virus isolation was done by the inoculation of suspected material into the chorio-allantoic membrane (CAM) of 11-13 day specific pathogen free (SPF) embryonated chicken eggs according OIE, (2008) with appropriate concentration of antibiotics. These are incubated at 37 °C for 5-7 days and then examined for focal white pock lesions or generalized thickening of CAMs.

Polymerase chain reaction (PCR) for diagnosis of pigeon pox virus:

Viral DNA were isolated from prepared skin lesions of infected pigeon by using QIAamp Viral RNA Mini Kit (Qiagen, Germany). The extracted viral DNA from each case were amplified using Thermo PCR master mix kit (Thermo, UK). Positive control was used from vaccine strain of Pigeon Poxvirus supplied from The Veterinary Serum & Vaccine Research Institute (VSVRI). Primers described previously ((Huw Lee and Hwa Lee, 1997) were used to amplify the FWPV P4b gene (fpv167). The sequences of the primers were as follows.

Table (1): PCR primers designed for diagnosis of pigeon pox virus.

Primer	Sequence (5'-3')	Gene	Location	Expected size of amplicons
M2925	CAGCAGGTGCTAAACAACAA	FWPV P4b gene (fpv167)	(*fpv*167 nt 459–478)	578 bp
M2926	CGGTAGCTTAACGCCGAATA		1016–1035	

The amplification was conducted using a PCR thermacycler (ABI-2720) by using thermal profile of initial denaturation at 94 °C for 5 m, 35 cycles of 94°C/1 m, 56°C/1 m and 72°C/1 m then followed by final extension at 72°C/10m. The PCR products were processed for electrophoresis gel with wide range GelPilot DNA ladder (Cat No.239125, Qiagen, Germany).

Real-time PCR:

Real –time PCR was done by using the same previously described primers with SYBR Green mix (Cat # K0229, Fermentas) on real-time PCR machine (Stratagen Mx3005P). Dissociation curve was conducted on PCR product after the thermal profile of SYBR Green PCR assay on the Stratagen software as follows: initial denaturation at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds, at 55°C for 1 minute and at 72°C for 1 minute then followed by the dissociation curve cycle of 95°C for 1 minute, at 55°C for 30 seconds and 95°C for 30 seconds where the measurement of SYBR green emission was done. The PCR product of melting temperature (Tm) at 75-77°C considered positive for pigeon pox virus.

RESULTS

Among affected birds, some had lesions involving eyelid, mouth and some of them had lesions on the legs or feet. Most of the affected birds had one or more lesions on their feet and may coalescent lesions.

Results of Avian embryo inoculation:

Focal white pock lesions or generalized thickening of CAMs had developed after the inoculation of embryonated chicken eggs in 5 out of the 8 suspected cases.

Results of PCR:

Amplification of pigeon poxvirus and agarose gel electrophoresis showed that there were five out of the eight suspected field samples were positive for the presence of pigeon pox virus where the primers amplified a product of 578 bp (photo 1).

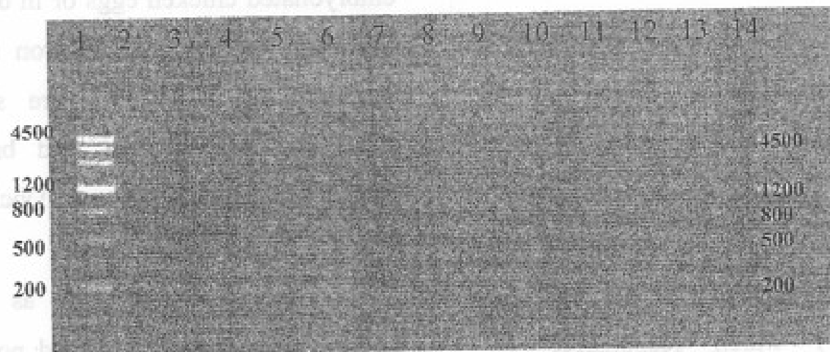

Photo (1): Results of PCR amplification of pigeon poxvirus and agarose gel electrophoresis; lanes 1 and 14 are the DNA ladder, lanes 2-11 represent field samples where the positive result indicated at 578 bp, lanes (4, 8, 9, 10 and 11). Lane 12 is the positive control and lane 13 is the negative control.

Real time PCR dissociation curve of PCR products of the qualitative SYBR Green PCR assay indicated that the PCR products of melting temperature (Tm) at 75-77 C were positive for 5 samples of out of the 8 suspected cases of pigeon pox virus.

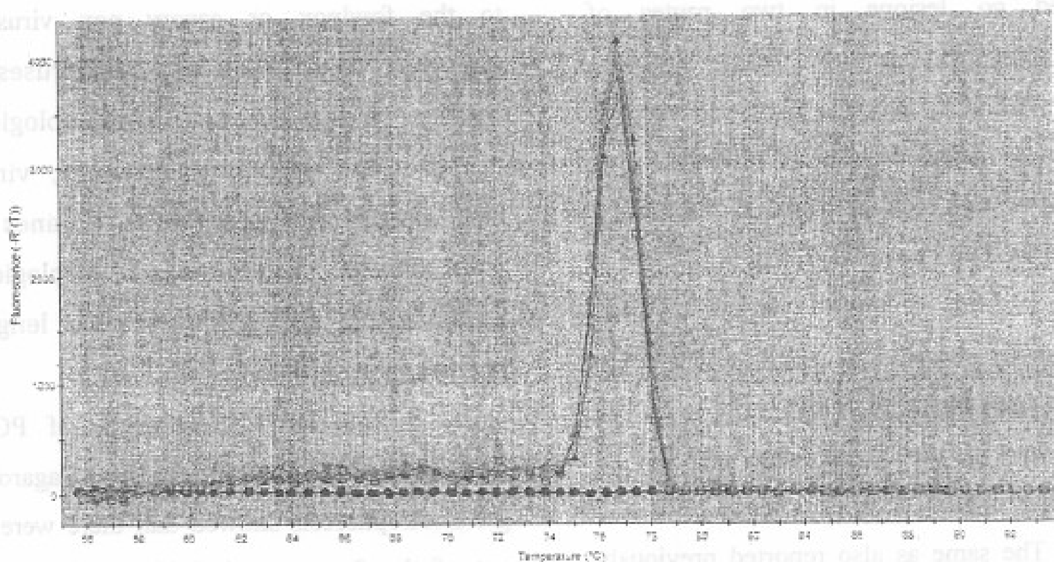

Photo (2): Real time PCR dissociation curve of PCR products of the qualitative SYBR Green PCR assay. The PCR products of melting temperature (Tm) at 75-77°C considered positive for 5 samples, positive control of pigeon pox virus and negative amplification for 3 samples and negative control.

DISCUSSION

Fowl pox is unique throughout the world, but because its host-range is limited to birds it is not considered to be a public health hazard. (Marlier and Vindevogel, 2006). It is a serious disease of poultry caused by Fowl poxvirus (FPV) which responsible for considerable economic loss in poultry industry in many countries (Soad et al., 2005). Serology has revealed cross- reactivity among several of the viral species, this disease affects more than 232 species of 23 orders of birds (Murphy et al., 1995) including fowl, turkey, pigeon, canary and quail (Bolte et al., 1999). Experimental infection of avian pox in pigeon showed no lesions in two routes of inoculation (Al-Attar et al., 2007).

Pox virus- induced disease is common in unvaccinated flocks of canaries and pheasants, and infections occur sporadically in pigeons (Saif et al. 2003).

In this study, the virus was successfully detected in 5 cases where the clinical sings appeared, white pocks or diffuse thickening of the CAM observed in the inoculated eggs by these samples.

The same as also reported previously by Tripathy and Reed (2003). Virus propagation on CAMs of chicken embryo is one of the conventional methods of confirming poxvirus (Van Riper et al., 2002 and OIE, 2008). Virus isolation can be attempted on the chorioallantoic membrane of embryonated chicken eggs or in duck embryo fibroblast cells. Under electron microscopic examination, the virions are seen to be enveloped, pleomorphic, and brick-shaped, with a diameter of 200 nm (Docherty et al., 1991).

Serologic tests such as AGID are helpful for thoroughly studied poxviruses of domestic species, but the test suffers from limitations. Because of cross reaction, one can not differentiate different species or strains of poxvirus (Tadese and Reed, 2003).

Smits et al., (2005) and Thiel et al., (2005) describe a simple polymerase chain reaction–based method for identification and discrimination of avipoxvirus strains similar to the fowlpox or canary pox viruses. Conventional diagnosis of avipoxviruses is usually carried out by histopathological examination, electron microscopy, virus isolation on chorioallantoic membranes of embryonated chicken eggs, serological methods and restriction-fragment length polymorphism (RFLP).

In this study, the results of PCR amplification of pigeon poxvirus and agarose gel electrophoresis showed that there were 5 out of the 8 suspected field samples were positive for the presence of the virus where the positive result indicated at 578 bp (photo 1).

The detection methods by using virus isolation are being supplemented by the use of PCR using primers for a 578 bp product of

fpv167 gene, that encodes P4b (Huw Lee and Hwa Lee, 1997). P4b gene (fpv167) locus PCR analysis of the highly conserved P4b gene was initially used as a diagnostic marker for FWPV infections, generating a 578 bp product (Tadese and Reed, 2003).

These results were confirmed with Real time PCR dissociation curve of PCR products of the qualitative SYBR Green PCR assay and the results indicated that the PCR products of melting temperature (Tm) at 75-77 °C were positive for the 5 samples of out of the 8 suspected cases of pigeon pox virus (photo 2). Results of PCR and Real time SYBR Green PCR showed the same results when a trail of virus isolation by inoculated embryonated chicken eggs was carried out. There were 3 cases did not induce pock lesion in eggs and also not detected by either PCR or Real-time PCR in the clinical samples. This is may be due to inappropriate sampling or very low virus present in clinical samples under the detection limit of PCR assays.

Because the genomes of fowl, pigeon and junco poxviruses are similar, DNA probes designed for use in chickens might also work in pigeons and juncos (Schnitzlein et al., 1988). PCR and real time PCR are confirmed for the presence of pigeon pox virus (Lüschow et al., 2004).

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In conclusion, we described in this study the application of PCR based method (conventional PCR and real-time PCR) for identification of pigeon pox virus in clinical samples.

The molecular methods were more rapid, accurate and reliable when compared to conventional methods for virus isolation.

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الكشف الجزيئى لفيروس الجدرى فى الحمام

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يعتبر فيروس جدرى الحمام من مسببات الأمراض الخطيرة لطائر الحمام وقد تهدد حياة الطائر.
من الممكن الكشف بسهولة عن فيروس جدرى الحمام فى العينات المصابة اكلينيكيا عن طريق الأعراض الظاهرة التى تؤثر سلباً علي صحة الطائر والتي قد يتبعها اصابات بكتيرية ثانوية, لذلك نحتاج للكشف السريع والدقيق لهذا الفيروس.

فى هذا البحث تم تطبيق التجارب الجزيئية التي تعتمد على وجود الأحماض النووية عن طريق اجراء اختبار تفاعل البلمرة المتسلسل وكذلك تفاعل البلمرة المتسلسل فى الوقت الحقيقى لعدد ثمانية عينات لحمام مصاب اكلينيكيا من ثلاث محافظات مصرية هى الجيزة وكفر الشيخ وبنى سويف.

تم استخراج الحامض النووي (د ن أ) من الإصابات الجلدية لكل حالة باستخدام البادىء لتكاثر جين FWPVP4b وكانت نتائج هذا التفاعل فى جل الأجاروز المصبوغ بالإيثيديم بروميد ايجابية لعدد خمسة من ثمانية اصابات لوجود فيروس جدرى الحمام عند 578 وحدة مزدوجة بالمقارنة بضوابط ايجابية وسلبية.

كما تم الكشف عن طريق تفاعل البلمرة المتسلسل فى الوقت الحقيقى باستخدام نفس البادىء وصبغة سيبر الخضراء وكان منحنى التفاعل للصبغة عند درجة حرارة الذوبان 75-77° م ايجابيا لعدد خمسة من أصل ثمانية اصابات لحالات مشابهة كما فى نتيجة تفاعل البلمرة المتسلسل العادى.

وخلصت الدراسة إلى نجاح طرق الكشف الجزيئى لفيروس الجدرى فى الحمام عن طريق تفاعل البلمرة المتسلسل وكذلك تفاعل البلمرة المتسلسل فى الوقت الحقيقى بنفس كفاءة العزل الفيروسي على أجنة بيض و لكنها تميزت بدقتها و حساسيتها للكشف المحدد للفيروس و كذلك سرعتها في التشخيص.