



Pathogenicity of *Avipoxviruses* in Chickens Isolated from Field Outbreaks Reported in Chhattisgarh

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Received: 18 June, 2018

Revised: 02 Oct., 2018

Accepted: 08 Oct., 2018

ABSTRACT

Virulence of field isolates of *Avipoxviruses* was assayed by pathogenicity test performed in 5 weeks old unvaccinated chickens. Viruses as dry scab were collected from naturally pox infected chickens, turkeys and pigeon and propagated in CAM of embryonated chickens upto various passages. In two separate trials 1 and 2, the chickens were infected with 5th and 20th passage CAM suspension, respectively by feather follicle method. All chicken groups in both trials (except control group) developed primary lesions as 'take' reaction from 48 to 72 hr PI and there after further progressive development of primary lesion did not differ among field isolates. In trial 1, secondary stage began with recovery from primary lesions at feather follicle, spread of infection to comb and wattles with development of secondary pox lesions and finally recovery from disease was observed after 15 days in FPV and TPV infected chickens, but not in PPV infected chickens. In trial 2, secondary pox lesions were not observed in any of the chickens, indicating that 20 passage virus induced 'take' at site without further spread of infection. All the recovered birds and controlled birds were challenged with the virulent FPV. The result has indicated 100% survival of birds except in control birds. Precipitating antibodies was confirmed in all birds group except control group using AGID and CIE test.

Keywords: Chicken, CAM, pathogenicity, viruses

Genus *Avipoxvirus* (APV) is a cluster of poxviruses infecting fowl, turkey, pigeon and many wild birds that may vary in their pathogenicity, host specificity and degree of cross-relationship. Fowlpox virus (FPV) represents the type species of genus APV subfamily *Chordopoxvirinae* of family *Poxviridae* (Gyuranecz *et al.*, 2013). Natural infection in susceptible birds occurs in cutaneous or diptheritic form or both (Fenner *et al.*, 1993). Host specificity is considered to be one of the important criteria for differentiation of APVs. At present, the exact number of existing APV species, strains and variants is unknown since very often the new isolates continued to be identified from number of avian species. Poxviruses are ubiquitous and it is debatable that how poxvirus infection has been transmitted and globally dispersed among wild and domestic birds. According to Gyuranecz *et al.* (2013), poxvirus infections have been found in 230 species of

wild and domestic birds worldwide, in both terrestrial and marine environment.

Fowlpox (FP) is of major importance and as the poultry population increased along with turkey and pigeons, other APV infections i.e. turkeypox (TP) and pigeonpox (PP) has also gained considerable economic importance. Losses due to major FP outbreaks are largely attributed to mortality; drop in egg production, meat condemnations and also to an unexpected vaccination failure, particularly in layers (Singh *et al.*, 2000). FPV is highly infectious for chickens and turkey rarely for pigeons and not all for ducks and canaries. The turkeypox virus (TPV) is virulent to ducks (Murphy *et al.*, 1999). Although it is assumed that though APVs are strongly species specific, FPV was also found associated with outbreaks in turkey (Hess *et al.*, 2011) which reveals the fact that FPV is emerging pathogen of turkey. TPV was considered more or less

similar to FPV but different from other APVs. It is still unclear that whether TPV possess definite biological differences with other APVs.

Mortality and morbidity related to PPV infection may be high in pigeons. There are relatively less reports of PPV in India. By and large the route of transmission, viral virulence and host susceptibility to the infecting APV strain might be responsible for the clinical sign of pox disease and also decide appearance of either cutaneous or diphtheritic form of avian pox. Macroscopic appearance of pocks on CAM by different APVs is one of the foremost criteria to describe proliferative nature of lesion as a result of consistency in viral growth after its adaptation to chicken embryos. To gather the information about local APV isolate is important step to formulate and execute an effective strategy for the preventive and control measures to be enforced against FP and TP to avoid economic loss to poultry production in the state. Keeping the above fact the present study was undertaken with the objective to evaluate pathogenicity of avipoxviruses in chickens.

MATERIALS AND METHODS

Viral isolates and virus isolation

Dry scab samples were collected from field outbreaks suspected for fowlpox, turkeypox and pigeonpox from chickens, turkey and pigeon respectively reported in some of the districts of Chhattisgarh. For the laboratory reference fowlpox virus isolates designated as FPV-1 (Raigarh), FPV-2 (Durg), FPV-3 (Antagarh), turkeypox virus as TPV-1 (Durg) and pigeonpox virus as PPV-1 (Durg) and maintained at Department of Veterinary Microbiology. Day old fertile chicken eggs (CE) of apparently healthy breeding hens tested negative for FP antibodies were obtained from the Government Poultry Farm, Durg (C.G). After 10 days incubation embryonated eggs were used for virus isolation. Ten per cent suspension of dry scabs in PBS (pH7.4) was made separately for each isolate. After three cycles of freezing and thawing suspensions were centrifuged for 15 min at 1500 rpm and supernatant was filtered in syringe filter of 0.45 μ m APD, 0.2 ml of filtrate was used for inoculating embryonated chicken eggs (ECE) by dropped chorioallantoic membrane (CAM) method as described by Cunningham (1966). The inoculated eggs were incubated at 37°C horizontally. After

5 days, live embryos were transferred to 4°C chamber for chilling at least for 5 hr. The CAM was detached from shell carefully then washed three times with cold PBS (pH, 7.4). Development of pock lesions on CAM was examined. Membranes not showing pock lesions in the first passage were given further passages till distinct pock lesions were obtained.

Lyophilized live FPV vaccine from Venkateshwara Hatcheries Private Limited, Pune was rehydrated and revived by three consecutive passages in ECE. The concentration of virus was 10^2 EID₅₀ per ml preserved in freeze dried powder. For the propagation of vaccine strain of FPV, each ECE received 0.2 ml (100 field doses/0.1ml of) virus suspension of vaccine dose by CAM route.

Titration of avipoxviruses (APVs)

Further, even after a minimum of five initial passages each of field strain of FPV, TPV and PPV was subjected to a definitive number serial passage (adjudged so upto 20) so as to ensure adaptation and consistent growth of the virus on the CAM in the form of typical pock lesions. Titration was performed with the respective CAMs suspensions of virus isolates (FPV-1, FPV-2, FPV-3, TPV-1 and PPV-1) following procedure described by Tripathy and Cunningham (1984). Egg infective dose 50 (EID₅₀ per ml) for 5th passage CAM and 20th passage CAM suspension was calculated as per method of Reed and Muench (1938).

Pathogenicity test in chickens

A total of 90 healthy unvaccinated chickens, one week of age were procured from the Government Poultry Farm, Durg and reared in separate pens/flocks in the experimental house of Veterinary Microbiology. The birds were provided with feed and other conditions as recommended by management requirements except vaccination and medication throughout the period of study. The capacity of chick embryo adapted APVs field isolates, propagated upto up 5th passage as well as 20th passage level, to produce disease with typical clinical signs have been studied so as to assess pathogenicity of the virus to chicken of five week of age. The pathogenicity test was carried out as per method summarized by El-Mahdy *et al.*, (2014) with some modification.

In trial 1, a minimum of 36 unvaccinated chickens were

selected and brought to the experimental house and the birds were kept for the purpose of acclimatization. The chicken were divided into six groups, the first group was consisted a minimum of six chickens that served as control. Each of remaining five groups contained a minimum of six birds and used for virus inoculation and subsequent pathogenicity test. On the basis of results of virus titration, the concentration of virus in the inoculum was evaluated and used accordingly. Table 1 summarize schedule of pathogenicity trial 1 in chickens by experimental infection with APVs propagated upto 5th passage.

Table 1: Schedule of pathogenicity Trial 1 in chicken by experimental infection with at CAM adapted APV at 5th passage

Group	Isolate of APV	No. of birds	Titre (EID ₅₀ / ml)	Dose (ml)	Route
Control	Uninfected CAM	6	—	0.2	Feather follicle
A-1	FPV-1	6	10 ^{4.24}	0.2	Feather follicle
B-1	FPV-2	6	10 ^{3.84}	0.2	Feather follicle
C-1	FPV-3	6	10 ^{4.37}	0.2	Feather follicle
D-1	TPV-1	6	10 ^{4.38}	0.2	Feather follicle
E-1	PPV-1	6	10 ^{4.37}	0.2	Feather follicle

The route of experimental infection for bird was by feather follicle method. Feather follicle of thigh region was plugged in opposite direction. Two hundred microlitre (0.2 ml) of virus suspension of 5th passage CAM was soaked in cotton then rubbed in plugged feather follicles gently on left leg. Control chickens received only 10 per cent suspension of uninfected CAM in PBS. Birds were kept in observation for minimum 20 days.

Table 2: Schedule of pathogenicity trial 2 in chicken by experimental infection with at CAM adapted APV at 20th passage

Group	Isolate of APV	No. of birds	Titre (EID ₅₀ / ml)	Dose (ml)	Route
Control	Uninfected CAM	6	—	0.2	Feather follicle
A-2	FPV-1	6	10 ^{6.14}	0.2	Feather follicle
B-2	FPV-2	6	10 ^{5.37}	0.2	Feather follicle
C-2	FPV-3	6	10 ^{6.36}	0.2	Feather follicle
D-2	TPV-1	6	10 ^{6.28}	0.2	Feather follicle
E-2	PPV-1	6	10 ^{6.40}	0.2	Feather follicle

The similar approach was also employed for another separate pathogenicity trial 2 in chicken by experimental infection with APVs at 20th passage level. The number of birds, route of administration, dose and schedule was mentioned in Table 2. Except control birds other group was inoculated with 0.2 ml suspension of 20th passage CAM infected with each field isolate.

In both trials, the birds were examined at regular intervals for primary lesions as well as spread of infection in the form of secondary lesions.

Challenge with FPV

A challenge test was conducted on birds recovered in Trial 2 only. Briefly, after 30 days birds was challenged with 5th passage CAM virus infected with field isolates of FPV-1 in the respective groups. Five hundred microlitre virus suspension was injected by subcutaneous route on the neck at multiple points of the birds (back of neck). Control birds received 10 per cent suspension of uninfected CAM in PBS only. Birds were kept under observation for three weeks. Occurrence of disease and/or severity of clinical signs of FPV were observed.

Collection of serum from experimentally infected birds

In both trial 1 and 2 serum was obtained from blood collected by jugular puncture from experimental birds before virus exposure and 15 days after exposure to the virus. Serum was clarified if required, by centrifugation at 1500 rpm for 15 min and stored at -20°C till tested. The sera were subjected to detection of specific antibody against APV.

Preparation of hyperimmune serum in rabbits

Hyperimmune serum against FPV vaccine was raised. Each isolate was inoculated into two rabbits. In a primary dose, 1 ml of 10 per cent suspension of FPV vaccine infected CAM (known antigen) emulsified with equal volume of Freund's Complete Adjuvant (FCA) was given sub-cutaneously. On day 14 and 28 booster dose with Freund's Incomplete Adjuvant (FIA) was given. A week after the last vaccination test serum was obtained from each rabbits.

Agar gel immuno diffusion test

To detect APVs specific antibody AGID test was performed according to OIE protocol (OIE, 2008). In gel diffusion medium 30µl known antigen (in central well) allowed to react with 30µl test serum (in peripheral wells) along with positive control (30µl hyperimmune serum) and negative control (serum from control bird) at 37°C for 24 hr.

Counter immune-electrophoresis

The test was performed as described by OIE (OIE, 2008). The medium was composed of 1g agarose dissolved in 75 mM tris buffer and 25 distilled water. Using tris buffer (0.2M, pH, 7.4) the slide was incubated in the buffer tank at a constant current (10 mA). Reference antigen (vaccine strain) was (30 µl) filled towards cathode and the test serum (30 µl) towards anode. Positive control (hyperimmune serum) and negative control (serum from control bird) was also maintained. After one hr of incubation the slide was examined for the presence of precipitation lines.

RESULTS AND DISCUSSION

Avian poxviruses (APVs) infection occurs in all birds species and outbreaks are reported consistently not only in poultry (chicken, turkey and pigeon) but also in other birds including wild birds (Godoy *et al.*, 2013). In at least 278 of approximately 9800 bird species under 23 orders, APV infections have been reported and it is believed that all avian species are susceptible (Kane *et al.*, 2012). The literature revealed that almost every year one APV infection in new bird species is added to the long list of APV infection.

In present study, APV field isolates of namely fowlpox virus (FPV), turkeypox virus (TPV) and pigeonpox virus (PPV) were obtained from AP suspected field outbreaks, respectively, in flocks of chickens, turkeys and pigeon that occurred in different district of Chhattisgarh state which indicated that all three APVs studied (FPV, TPV and PPV) were prevalent in Chhattisgarh state. The occurrence of FPV was more as compared to TP followed by PP.

Virus isolation in chicken embryos

In the present study five sample i.e. FPV-1, FPV-2, FPV-3, TPV-1 and PPV-1 were isolated in CAM of 10 days

CE by dropped CAM method. The study showed more marked generalized thickening of membrane at initial passage and white opaque raised area of necrosis called 'pock' noticed onto virus infected CAM by subsequent passages as the level of passage increased. Focal as well as diffuse pocks were noticed in CAM at different passage levels. Pock morphology is one of the foremost criteria for characterization of viral growth of different APVs on CAM. The virus could be easily isolated from dry scabs obtained from affected flocks by chicken embryos inoculation (Promukund *et al.*, 2003; Balachandran *et al.*, 2012; Roy *et al.*, 2013; El-Mahdy *et al.*, 2014 and Kabir *et al.*, 2015). Some reports by Diallo *et al.* (1998) and Das *et al.* (2007) reported failure of APV to grow on CAM in initial passages and recommended more serial blind passages and/or choice of other host system for isolation of APV

Titration of virus in chicken embryos

In the present study EID_{50} of virus isolates was calculated in chicken embryos at 5th passage and 20th passages level. At 5th passage level the FPV-2 has lowest titre of $10^{3.84}$ and for other four field isolates i.e. FPV-1, FPV-3, TPV-1 and PPV-1 the titre was $10^{4.24}$, $10^{4.37}$, $10^{4.38}$ and $10^{4.37}$ respectively. The result at 20th passage level have indicated that except for FPV-2 ($10^{5.37}/ml$) the EID_{50} for other four isolates was more or less similar i.e. $10^{6.14}$, $10^{6.36}$, $10^{6.28}$ and $10^{6.4}$ for FPV-1, FPV-3, TPV-1 and PPV-1, respectively. Earlier workers have also conducted similar studies on titration of APVs and reported different concentration of virus with respect to passage and strain of APVs. Odoya *et al.* (2006) found virus titre $10^{5.6} EID_{50}$ per 0.1ml at 20th passage CAM suspension for field isolate. Various APV strain viz FPV, TPV, QPV and PPV were found to have titre of $10^{6.4} EID_{50}/ml$, $10^{5.0} EID_{50}/ml$, $10^{4.5} EID_{50}/ml$ and $10^{5.5} EID_{50}/ml$, respectively at 5th passage level in CAM (Yadav *et al.*, 2007). Shil *et al.* (2007) computed $10^6 EID_{50}/0.1 ml$ virus titre for field isolates of fowl pox virus at 5th passages.

In the above discussion it can be concluded that development of pock lesions and concentration of virus is unpredictable throughout adaptation and consistent propagation of virus in initial passage in CEE. The difference in pock morphology and EID_{50} depends on concentration and virulence of field strain which may carry

inherent difference in degree of pathogenicity to variety of host system. Hence, to ensure maximum attenuation of field strain it is required to undertake adequate passages that render field strain of APV less pathogenic but highly immunogenic. Nevertheless, maximum degree of consistency in pock morphology on CAM coupled with increase in concentration of mature virion particle with respect to serial passage should be ensured before predicting degree of attenuation of any field strain of APV.

Pathogenicity test and host specificity in chicken

To determine the virulence of fields isolates pathogenicity test was performed in 5 weeks old unvaccinated chickens. Two separate trials were conducted. In Trial 1 and Trial 2, the chicken were experimentally infected with each of field strains of FPV-1, FPV-2, FPV-3, TPV-1 and PPV-1 propagated upto 5th passage and 20th passage level, respectively. It was observed that all chicken groups except control group developed ‘takes’ as early as 48 hr but not later than 72 hr PI. Progressive developmental stage of primary lesion (Table 3) at the inoculated site was almost similar for each field isolate. Recovery of primary lesions at feather follicles was observed from 10 day onward.

Table 3: Progressive development of primary lesion in chickens after exposure of virus

Sl. No.	Times after post inoculation	Changes on inoculated sites (take reaction)
1	24 hr	Site became hyperemic
2	2-3 day	Small papules formation
3	3-5 days	Pustules formation occurred
4	6-10 days	Pustules ruptured, yellowish colour fluid oozes, scar formation started.
5	10 days onward	Recovery of lesion and refeathering

Spread of infection and development of secondary pock lesions in the form of small nodules on comb, wattle, eye and beak was observed after 15 days and onwards. Secondary pox was observed in all those groups which received 5th passage CAM except in chicken inoculated with PPV-1. All chickens were recovered within four weeks after inoculation. Similar finding was also reported earlier by several workers (Tamador *et al.*, 2001; Promkuntod *et al.*, 2003; Odoya *et al.*, 2006; Shil *et al.*, 2007; Siddique *et al.*, 2011; Zhao *et al.*, 2014; El-Mahdy *et al.*, 2014; Kabir *et al.*, 2015; Hassanin *et al.*, 2015). These workers have reported ‘take’ reaction in primary inoculation site in 3-10 days of post infection indicated virus multiplication on those areas.

Our findings showed that FPV field isolates (FPV-1, FPV-2, FPV-3) and TPV field isolate (TPV-1) were pathogenic to chickens indicated that these may be of similar host origin or show cross reactivity among these isolates. Our finding supports those reported earlier by several workers (Promkuntod *et al.*, 2003; Siddique *et al.*, 2011; Kabir *et al.*, 2015; Hassanin *et al.*, 2015) who have concluded that FPV was not strict host specific and it can infect other host species. Odoya and coworker (2006) reported that poxvirus isolated from turkey poult could reproduce disease in chickens.

Pigeon pox field isolate was not pathogenic for chickens. This might be due to relatively higher degree of host specificity to infective dose of PPV. Similar finding was also reported by other worker (Siddique *et al.*, 2011 and Kabir *et al.*, 2015). However, Khodir and Mikhail (2006) reported that FPV is not extremely host specific and PPV also produces pox lesion in chicken and quail. However, our findings on PPV indicate that PPV used in the study was highly host specific. Cross pathogenicity trials in variety of birds using PPV challenge will be needed to draw some more conclusions.

In trial 2, all chicken groups received 20th passage CAM suspension. There was ‘mild take’ in all birds but the spread of infection and subsequent secondary lesions did not appear in any of the groups. Thus, loss in virulence, if any as a result of serial passage of field isolates onto CAM upto 20th passage was assessed by challenge of these birds with virulent 5th passage virus FPV at day 30 post inoculation. The chicken did not develop disease and found refractory to challenge of 5th passage FPV. Our study is supported by finding of Odoya *et al.* (2006) who observed that field isolates of TPV propagated in CAM upto 20th passage protect strongly both chicken and turkeys from pox infection, resistance of turkey poults to challenge after vaccination with attenuated TPV confirmed the immunogenicity of turkey pox antigen in turkeys.

Antibodies status of experimental chickens

In the present study, antibody status in experimental birds

were assayed by AGID and CIE. Homology (precipitin) was observed between known antigen and test serum along with hyperimmune serum indicated presence of APVs specific antigen in all group birds except control one. Similar work was also done by other workers (Al-Falluji *et al.*, 1979 and Attar *et al.*, 2007) who have confirmed poxvirus infection in peacocks by observing precipitin between infected CAM and antiserum against FP and PP vaccine strain. Adebajo *et al.* (2012) surveyed current antibody status to FPV in unvaccinated free range chickens using AGID. Tamador and coworker (2001) concluded that CIE is more sensitive than AGID because it detected low levels of antibodies to FP vaccine even 7 weeks of post vaccination.

CONCLUSION

Pathogenicity trials in chicken experimentally infected by all field strains of APVs propagated at low passage level (five passages) and high passage level (20th passages) revealed differences in host specificity among APVs. While FPV and TPV did not differ in host specificity, the PPV found to be host specific to pigeons. Some degree of attenuation due to serial passage was also recorded. Loss of virulence of field isolates was observed on higher passage but immunogenicity of the viruses remained.

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