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REGULAR ARTICLES

Existence of variant strains *Fowlpox virus* integrated with *Reticuloendotheliosis virus* in its genome in field isolates in Tanzania

Alexanda Mzula · Selemani N. Masola · Christopher J. Kasanga · Philemon N. Wambura

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Abstract *Fowlpox virus* (FPV) is one example of poultry viruses which undergoes recombination with *Reticuloendotheliosis virus* (REV). Trepidation had been raised, and it was well established on augmented pathogenicity of the FPV upon integration of the full intact REV. In this study, we therefore intended at assessing the integration of REV into FPV genome of the field isolates obtained in samples collected from different regions of Tanzania. DNA extraction of 85 samples (scabs) was performed, and FPV-specific PCR was done by the amplification of the highly conserved P4b gene. Evaluation of FPV–REV recombination was done to FPV-specific PCR positively identified samples by amplifying the *env* gene and REV long terminal repeats (5′ LTR). A 578-bp PCR product was amplified from 43 samples. We are reporting for the first time in Tanzania the existence of variant stains of FPV integrated with REV in its genome as 65 % of FPV identified isolates were having full intact REV integration, 21 % had partial FPV–REV*env* gene integration and 5 % had partial 5′ LTR integration. Despite of the fact that FPV–REV integrated stains prevailed, FPV–REV-free isolates (9 %) also existed. In view of the fact that full intact REV integration is connected with increased pathogenicity of FPV, its existence in the FPV genome of most field isolates could have played a role in increased endemic, sporadic and recurring outbreaks in selected areas in Tanzania.

Keywords *Fowlpox virus* · *Reticuloendotheliosis virus* · PCR

A. Mzula (✉) · C. J. Kasanga · P. N. Wambura
Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture, P.O. Box 3019, Morogoro, Tanzania
e-mail: amzula@yahoo.com

S. N. Masola
Mpwapwa Veterinary Investigation Centre, P.O. Box 202, Dodoma, Tanzania

Introduction

Fowlpox (FP) is the common poultry disease caused by the double-stranded DNA *Fowlpox virus* (FPV) of the genus *Avipoxvirus*. For a very long intermission of time till now, FP has no treatment rather than vaccination with live vaccines (Awad 1998) which serves as a control of the disease in the poultry industry to minimize economic losses that were to encountered (Diallo et al. 1998). However, in current years, this disease re-emerged as a new menace to poultry production in other countries such as Australia and USA (Silva et al. 2009; Arathy et al. 2010). The incidence is much higher in tropical and subtropical countries (Beytut and Haligur 2007) including Tanzania. Many sporadic, endemic cases and outbreaks of FP were occurring in unvaccinated flocks of free range kept chickens in Tanzania recent years. Ten thousand one hundred one chickens were clinically seen to be affected with Fowlpox, and 637 chickens died of the disease between November 2011 and February 2012 in Mbeya, Tabora, Iringa, Kigoma and Dodoma region. This study collected specimens in outbreak, sporadic and endemic areas of the country (Table 1) to investigate on the cases as most of the affected chickens were free range growers of 1 to 2 weeks. In other countries such as Australia and USA, it appeared that the current vaccines were unable to provide efficient protection against the disease. The outbreaks were due to the emergence of antigenically ‘variant strains’ of FPV which contain a provirus in their genomes, an intact *Reticuloendotheliosis virus* (REV) (Kim and Tripathy 2001: Singh et al. 2000; Wang et al. 2006; Davidson et al. 2008). REV has the ability to integrate partially or entirely in large DNA viruses. Although the mechanisms for acquiring such host genes and evolutionary alterations in the genome of pox viruses are not well known, analysis of the REV integration site in wild-type field FPV isolates and in FPV vaccine strains revealed that all insertion events occurred in a hot spot of the FPV genome, located between the FPV open reading frame (ORF) 201 and

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Table 1 Outbreak of FP with their respective regions in Tanzania

Date	Region	Flock size	Age group of birds	No. of birds with signs of FP	Chicken dead
Jan 2012	Mbeya	60	Adults	15	7
Dec 2011	Dodoma	406	Growers and adults	380	320
Dec 2011	Dodoma	41	Growers	20	10
Feb 2012	Morogoro	20	Adults	10	0
	Total	527		425	337

FPV ORF 203 (Davidson et al. 2008). Fowlpox field isolates normally contain complete REV provirus by having fragments of 3′ and 5′ REV long terminal repeats (LTR) in addition of other REV fragments such as REV *env* gene (Davidson et al. 2008). Incorporation of REV in FPV genome causes various syndromes in multiple avian species, including runting, an acute non-neoplastic syndrome leading to high transience, severe immune suppression and T and/or B cell lymphomas (Cheng et al. 2011; Koo et al. 2013). REV infection causes theatrical economic losses from a runting syndrome or chronic neoplasia with mortality (Fadly et al. 2008; Cheng et al. 2011). Momentous losses can occur when REV-contaminated vaccines are administered to very young chickens. The body weights of infected chickens may be 20–50 % lower than uninfected controls by 3–5 weeks after infection (Motha et al. 1984). Furthermore, the integration of REV in the FPV genome contributes to the enhanced pathogenicity of FP in infected chickens (Singh et al. 2000). There is also a crisis of weight gain reduction which has also been reported in infected ducks (Purchase et al. 1973). It has also been noted that poor growth and anomalous feather development in chickens are the physical characteristics which suggest infection of FPV having intact REV in its genome as observed by Biswas et al. (2011). The condition is termed as 'Nakanuke' where barbs of the primary and secondary wing feathers are abnormally stuck to the rachises which look like a stick-like appearance. No any study that has been conducted in Tanzania addresses the status of REV integration in FPV field isolates. Therefore, following outbreaks and sporadic cases of FP in the country, this study aimed at assessing the integration of REV into FPV genome of the field isolates obtained from the collected samples from endemic and sporadic outbreaks in Tanzania.

Materials and methods

Sample collection

A total of 85 fresh suspected FPV chicken scabs from sporadic outbreaks and endemic cases from Mbeya, Iringa, Dar es Salaam, Morogoro, Dodoma, Kigoma and Tabora regions were collected. These samples were directly collected from village poultry farmers and transported to Virology Laboratory at the Faculty of Veterinary Medicine at Sokoine University of Agriculture (SUA) where laboratory analysis was performed.

DNA extraction

DNA was extracted from 25 mg of the skin lesions of all the samples using Sepa Gene Extraction kit (according to manufacturer's instruction). Briefly, tissues were chopped and then ground and placed in 1.5 ml clean sterile Eppendorf tubes and dissolved by sterile phosphate-buffered saline (PBS) and then centrifuged at low speed of 3,000 rpm for 10 min at 4 °C. The supernatant was transferred to new Eppendorf tubes followed by centrifugation at 13,000 rpm for 30 min at 4 °C to recover the pellet. Solution I was added to pellets and re-suspended by pipetting. The suspensions were then incubated at room temperature for 10 min before the addition of solutions II, III and IV. All tubes were vortexed and centrifuged at 12,000 for 15 min at 4 °C, and supernatants were transferred to new tubes before addition of solution V. Isopropanol was added to precipitate the DNA followed by incubation at −30 °C for 1 h. Pellets were collected following centrifugation of incubated tubes at 13,000 for 30 min at 4 °C. Seventy percent ethanol was used to wash the DNA pellet and then air dried by blotting on a clean bench and stored in TE at −30 °C until when used.

PCR amplification for P4b gene core protein

Gradient PCR reactions for FPV identification were done in TAKARA Thermal cycler Dice by amplifying the specific Avipox core protein gene (P4b gene) using specific primers P15′-CAGCAGGTGCTAAACAACAA-3′ and P2 5′-CGGT AGCTTAACGCCGAATA-3′. PCR consisted of 25 μl PCR buffer containing 1.5 mM of MgCl2, 6 pmol of each primer, 200 μM of each dNTP and 1.25 U Taq polymerase as described by Long and Kuo (1997). Amplification was done according to Manarolla et al. (2010) but with modification where initial denaturation was performed at 94 °C for 2 min; 40 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 minute and final extension at 72 °C for 2 min.

Detection of REV integration by PCR

All genomic DNA samples positive to P4b gene PCR were subjected to a second-stage PCR for detection of REV integration. A PCR assay amplifying an 807-bp region of REV provirus *env* gene (Wang et al. 2006) was used. The primers were as follows: forward (P3) 5′-TGACCAGGCGGGCAAA ACC-3′; reverse (P4) 5′-CGAAAGGGAGGCTAAGACT-3′. A PCR for detection of the REV integration site in FPV

(Biswas et al. 2011) was also performed. Primers corresponding to the FPV DNA regions flanking the REV integration site were designed to be P5 5′-ACCTATGCCTCTTATTCCAC-3′ and P6 5′-CTGATGCTTGCCTTCAAC-3′. The amplification reactions consisted of the following components in a total volume of 50 μl: 1× reaction buffer; 1.5 mM MgCl2; 10 mM each of dATP, dCTP, dGTP and dTTP; 0.5 mM of each primer; 1.25 U of Taq DNA polymerase and 3 μl of DNA. The PCR reactions were conducted in a TAKARA Thermocycler. The PCR cycling parameters included a denaturation step at 94 °C for 2 min, followed by 35 cycles followed by 94 °C for 1 min, 57 °C for 2 min and 72 °C for 1 min. A final extension cycle was performed at 72 °C for 6 min. The annealing temperature for the second set of primer, P5 and P6, was 52 °C.

Agarose gel electrophoresis

Electrophoresis was performed using specified volume of DNA diluted in bromophenol blue loading dye (1 U of 10× loading dye to 9 U of DNA). A pre-stained 1 kilobase (kb) molecular weight marker (100 bp) was always run in conjunction with other DNA amplicons. All DNA amplicons were run in 2 % agarose gel stained with 3 μl of ethidium bromide at 100 V for 45 min in 1× Tris–borate–EDTA (TBE) buffer. Gels were visualized under UV light using UVi tec transilluminator.

Pathogenicity study

Preparation of the inoculum

Virus isolation was done according to Manarolla et al. (2010). Briefly, cutaneous lesions of samples proved to be FPV–REV free by PCR were chopped and ground with sterile sand using a mortar and pestle and suspended in a PBS. Then 1 ml of 50 IU/ml penicillin and 50 μg/ml streptomycin was added to each tube to prevent bacterial contamination. Approximately 0.1 ml of the supernatant from each sample was separately inoculated into 10-day-old specific pathogen free (SPF) developing chicken embryo eggs (obtained from a SPF farm in Morogoro, Tanzania) through chorioallantoic membrane (CAM). The same procedures were adopted for FPV–REV integrated isolates. Thereafter incubation, allantoic fluid was harvested and clarified by centrifugation and used to inoculate experimental chickens.

Source of experimental chickens and management

A total of 105 6-day-old chicks which were used in the study were purchased from a poultry farm in Morogoro. These chickens were screened for FP and reticuloendotheliosis antibodies and were found to be seronegative. The chicks were randomly assigned to three groups of 35 chicks each and kept in an animal house near to our laboratory. They were fed standard growers mash and given water ad libitum.

Experimental design

The chickens in group 1 were inoculated with allantoic fluid having FPV–REV-free isolate at a single dose of 10^6 EID_{50}/0.1 ml. The chickens in group 2 were inoculated with allantoic fluid containing FPV–REV integrated isolate at the same dose as above while the control group was given normal saline. Chickens were observed for physical characteristics such as weight loss/weight gain, poor growth and anomalous feather development as well as pathological changes for 4 weeks post-inoculation.

Sequencing and comparison of *env* gene and 5′ LTR products

PCR products for *env* gene and 5′ LTR of selected FPV–REV isolates were sequenced by ABI 3500 Genetic analyser (Applied Biosystems, Inqaba Biotec Ltd, South Africa) in agreement with the manufacturer's protocol. The sequence comparison was performed by BLASTING the sequences in www.ncbi.nlm.nih.gov/BLAST, and alignment of the *env* gene (KF225480) and LTR (KF268024) sequences from this study and GU012641.1; AY255633.1 from the gene bank was conducted using Clustal W in MEGA 5 software.

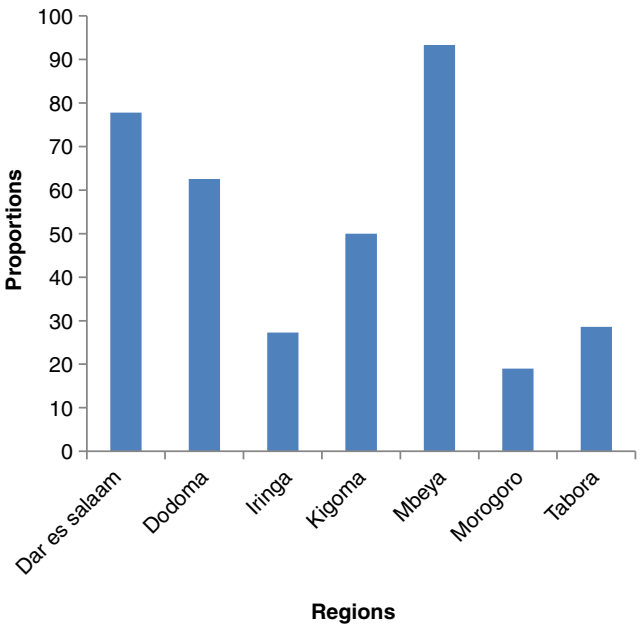

Fig. 1 Proportions (%) of FP isolates positively detected by PCR in selected regions in Tanzania

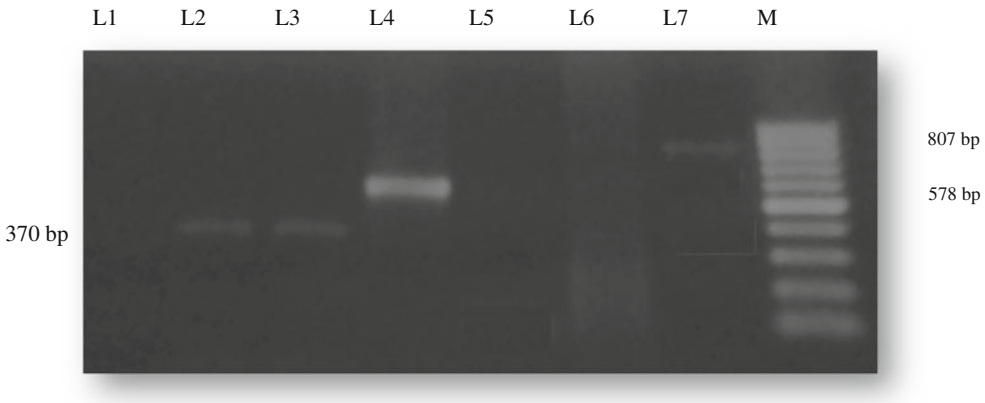

Fig. 2 PCR products of FPV–REV junction site terminal repeat (LTR), FPV P4b gene and REV *env* gene which yielded 370 bp (*lanes 2–3*), 578 bp (*lane 4*) and 807 bp (*lane 7*) products, respectively. *Lane 1* is negative control, *lanes 5* and *6* are a REV-free sample for LTR and REV*env* gene and *lane M* is a DNA molecular size marker (100 bp)

Results

Specific FPV and REV integration PCR results

A total of 43 samples (50.6 %) out of 85 samples analysed were confirmed to be FPV by amplification of a 578-bp DNA fragment of the gene. The 43 positive samples came from different regions of different geographical zones of the country (Fig. 1). Of the 43 field isolate DNA samples positive to FPV-specific PCR, 39 were observed to contain REV provirus. The 807- and 370-bp PCR products were generated from the FPV-positive DNA samples (Fig. 2). Twenty-seven field isolates had full-length REV in their genomes by having both regions amplified (*env* gene and REV 5′ LTR fragment). The full intact REV integrated FPV has proven to exist in almost all studied regions of Tanzania. In this study, two regions, namely Iringa and Tabora, had no full intact REV integration. Also 12 isolates had partial integration (either of the two regions got amplified). Only four REV-free isolates existed. The PCR results for the *env* gene region and that franking REV 5′ LTR are shown in Table 2.

Analysis of *env* gene and 5′ LTR sequences

The LTR and *env* gene from the isolates showed sequence homologue of 99–100 % with several REV sequences from the GenBank. No any nucleotide variation was observed in the near full-length provirus from the present study (KF268024 and KF225480, sequences not shown) and 5′ LTR (AY255633.1) and *env* gene (GU012641.1) from the GenBank.

Pathogenicity of FPV–REV integrated isolates

In examination of the pathogenicity of the experimental groups, poor and anomalous feather development characterised by very soft joined feathers around the head, neck and wings was observed in chickens in group 2 contrary to group 1 and control group (Fig. 3). The growth rate indexes showed retarded growth in chickens inoculated with FPV–REV integrated allantoic fluid (chickens in group 2) (Table 3). The pathological examination of the immune organs—spleen, thymus, bursa and liver—revealed severe atrophy in chickens in group 2 beside of the common pathological changes observed in chickens in group 1 (Table 4).

Discussion

In the poultry industry, control measures against FPV are achieved primarily by vaccination with live FPV or antigenically similar *Pigeonpox virus* strains produced in CEF cells or SPF chicken embryonated eggs. In the past two decades, numerous

Table 2 REV integration pattern in FPV isolates from selected regions of Tanzania

Regions	Dodoma	Morogoro	Iringa	Mbeya	Dares salaam	Tabora	Kigoma	Total
Amplified products								
env gene only	2	–	2	2	1	2	–	9
5′ LTR only	–	–	–	1	–	–	1	2
env gene and 5′ LTR	8	4	–	10	4	–	2	28
REV free	–	–	1	1	2	–	–	4

Fig. 3 Roughed feathered chickens infected with FPV–REV isolate (see *arrows*)

outbreaks have been reported in vaccinated flocks, suggesting that vaccines used against the disease were not effective (Fatunmbi and Reed 1996; Tripathy et al. 1998; Singh et al. 2000). In the USA, a commercial FPV vaccine was shown to be contaminated with REV and caused lymphoma among broiler chickens and hence immunosuppression (Weli and Tryland 2011). This study reports an occurrence and existence of field FPV isolates having REV provirus integration for the first time in Tanzania. As it had been described by Kim and Tripathy (2001) and Biswas et al. (2011), this study has also shown the same scenario that REV has been integrated into the DNA of field FPV isolates, and the size of the integrated fragments was 370 bp for LTRs and 807 bp for *env* gene.

As it has been shown by other workers, majority of FPV field isolates in this study contained near full intact REV integration in its genome. Based on experiment done in this study on pathogenicity, it can be ascertained that the contribution of high morbidity and mortality of chickens especially growers in Tanzania is a result of infection of FPV isolates containing intact REV genome. This is in agreement with what Singh et al. (2000) reported that the integration of full intact REV in the FPV genome contributes to the enhanced pathogenicity FP in infected chickens. Wei et al. (2012) also reported deaths of embryos bred by broiler breeders after 19 days of hatching due to REV infection that was congenitally transmitted from broiler breeders that were vaccinated with contaminated vaccine. The high proportion of full intact REV integration in FPV field isolates was also reported by Biswas et al. (2011). It is likely that the integrated near intact REV provirus can give rise to infectious REV which in turn may have resulted to immunosuppression and hence increased pathogenicity. REV genomic insertion is important, as these REV insertions may change the biological properties of the recipient virus and infectious clones of REV packaged in other viruses may provide a novel mechanism for transmission of REV (Mays et al. 2010).

Despite the fact that field strain of *Gallid herpesvirus* 2 (GHV 2) causing Marek's disease is reported to have novel REV LTR insertion (Niewiadomska and Gifford 2013), this study reports existence of field isolates of FPV having partial REV integration by having only 5′ LTR fragment amplified, lacking the *env* gene fragment. This countermands the description given by Davidson et al. (2008) that a FPV virus isolated in 1956 is the only field isolate FPV known for having 5′ LTR remnant only. We also identified isolates with partial integration having only additional proviral genome (*env* gene fragment).

Table 3 Mean body weight of chickens involved in pathogenicity study within the period of study

No. of days	Experimental groups		
	G1	G2	G3
5	42.3	41.9	42.2
7	50.01	50.1	51.6
14	105.76	61.4	125.5
28	197	157	286

G1 chickens inoculated with FPV isolate, *G2* chicken inoculated with FPV–REV integrated isolate, *G3* chickens saved as control croup inoculated with normal saline

Table 4 Mean weight of the immune organs in grams 28 days post-inoculation

Chicken groups	Spleen	Thymus	Bursa	Liver
Group 1 (FPV only)	1.16	2.01	0.8	2.1
Group 2 (FPV–REV integrated isolate)	0.7	0.8	0.45	1.76
Group 3 (control group, normal saline)	2.6	2.3	1.03	2.45

Conclusion

This study confirmed existence of variants of FPV integrated with REV in its genome. The increased pathogenicity due to full intact REV integration in FPV genome is well recognized. The re-emerged outbreaks and increasing endemic cases of FP disease in Tanzania may be associated with increased pathogenicity of FPV due to REV integration in its genome.

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Conflict of interest The authors declare that there is no conflict of interest.

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