Integration of Reticuloendotheliosis Virus in Most of Tanzanian Fowl Pox Virus Isolates is not attributed to Imported Commercial Fowl Pox Vaccines

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Authors' contributions

This work was carried out in collaboration between all authors. Author SNM designed the study, did laboratory work, data editing, analysis of sequence data, literature search, and wrote the first draft of the manuscript. Author AM was involved in some laboratory work as well as in purchasing of consumables such as DNA extraction kits, primers, TBE buffer, nuclease-free water, loading dye and DNA molecular weight marker. Authors CJK and PNW read and corrected the entire manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aim: To investigate integration of reticuloendotheliosis virus (REV) in the Tanzanian fowlpox virus (FWPV) field isolates, and the imported commercial fowl pox vaccines currently used in the country.
Study Design: Experimental.
Place and Duration of Study: Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania; between June 2012 and October 2013.
Methodology: Fifty five samples of FWPV isolates from naturally infected chickens, and two isolates of FWPV from samples of the imported commercial fowl pox vaccines were analyzed for integration of REV envelope (*env*) gene and REV 5'long terminal repeat (5'LTR). The study involved polymerase chain reaction (PCR) amplification of FWPV P4b gene, REV *env* gene, and REV 5'LTR; agarose gel electrophoresis of PCR products,

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purification of PCR products, sequencing of the purified PCR products, and sequence analysis using standard procedures.

Results: Out of 55 analyzed field isolates 53 (96.36%) were found to have REV inserts. Most of them [38 (69.09%)] contained both REV *env* gene and REV 5'LTR inserts, 10 (18.18%) contained inserts of REV *env* gene only, and 5 (9.09%) contained inserts of REV 5'LTR only. Two isolates (3.64%) were found to be integrated with neither REV *env* gene nor REV 5'LTR. None of the screened FWPV isolates from the imported commercial vaccines was found to have REV inserts. Sequence analysis revealed that genomic fragments of REV integrated in the Tanzanian FWPV isolate were closely related (99–100% identity) to REV sequences integrated in FWPV isolates from other countries.

Conclusion: Currently there is a heterogeneous population of FWPV in Tanzania comprising of REV-integrated FWPV strains and REV-free FWPV strains. Since strain(s) of REV-integrated FWPV are more virulent than strain(s) of REV-free FWPV, further studies on the REV-integrated Tanzania FWPV isolates aiming at obtaining the appropriate isolate for development of autogenous fowl pox vaccine are highly recommended.

1. INTRODUCTION

Reticuloendotheliosis virus (REV) comprises a group of avian RNA viruses that belong to the genus *Gammaretrovirus*, sub-family *Orthoretrovirinae* and family *Retroviridae* [1]. Based on the replication ability in the host cells REV isolates are classified as either defective or non-defective. Defective REV refers to REV isolates which lack the ability to replicate in the cell cultures or naturally-infected host cells. Non-defective REV refers to those which can replicate in the cell cultures or naturally-infected host cells [2,3]. The group of defective REV comprises of strain T (REV-T) [4]; a laboratory strain of REV with deletions within the genome and possesses a viral oncogene, *v-rel*, inserted at some site within the genome [2,5]. This strain transforms haematopoietic cells and fibroblasts to tumourigenic cells in vitro [6]. REV-T causes acute reticulum cell neoplasia if experimentally infected to susceptible birds [2].

Strains that comprise the non-defective group of REV include REV strain A (REV-A), spleen necrosis virus (SNV), chick syncytial virus (CSV), and duck infectious anaemia virus (DIAV) [4]. Most field isolates of REV are non-defective [3] and have been reported to cause runting disease syndrome in chickens and ducks characterized by runting, bursal and thymic atrophy, enlarged peripheral nerves, abnormal feather development, proventriculitis, enteritis, anaemia, liver and spleen necrosis, cellular and humoral immunosuppression [2]. Apart from that non-defective REV cause chronic lymphoid neoplasms in chickens, ducks, geese, pheasants, quail, and turkeys [2].

Like other retroviruses non-defective REV have a simple genome that consists of four genes namely *gag*, which encodes for the synthesis of internal viral proteins which form the matrix, the capsid, and the nucleoprotein structure; *pol*, that encodes for an RNA-dependent DNA polymerase (also known as reverse transcriptase) and integrase enzymes; and *env*, which encodes for the viral envelope glycoproteins. In addition to these genes, a small gene known as *pro*, which encodes for viral protease is located within or adjacent to the *gag* gene

[2,7,8,9]. The structural genes (*env* and *gag*) are flanked by genomic sequences which regulate viral replication, which in the DNA provirus form the viral long terminal repeats (LTRs) that carry promoter and enhancer sequences [2].

Fowlpox virus (FWPV) is a DNA virus that belongs to the *Poxviridae* family, *Chordopoxvirinae* subfamily and genus *Avipoxvirus* [10]. FWPV and other avipoxviruses (APVs) cause pox in birds [11]. Unlike REV, FWPV have a large genome that consists of 260 genes [12].

Previous studies [9,13,14,15,16,17,18,19,20] demonstrated the integration of various genomic fragments of REV in the genomes of field strains of FWPV isolated from chickens in various countries outside the African continent. Furthermore, Singh et al. [18] demonstrated that integration of genomic fragments of REV into the FWPV genome leads to increased virulence of the later (REV-integrated FWPV). Apart from that Singh et al. [18] demonstrated that in a country where fowl pox is endemic with a heterogeneous population of FWPV consisting of REV-integrated FWPV strains and REV-free FWPV strains, appropriate autogenous fowl pox vaccine can be developed from an appropriate REV-integrated FWPV isolate after attenuating it by removing the REV provirus provided that the removal of the REV provirus from the FWPV genome does not alter immunogenicity and antigenicity of the REV-less FWPV. According to Hertig et al. [13] infection of susceptible chickens with REV-integrated FWPV may lead to dissemination of REV if a full length or near-full length REV provirus is integrated into the FWPV genome.

In the recent years incidences and prevalence of fowl pox in chickens have increased in Tanzania, characterized with high mortalities of chicks and growers [21]. Increased virulence of field strains of FWPV currently prevalent in Tanzania due to integration of genomic fragments of REV in the FWPV genomes is one of the probable attributing factors implicated to be behind the increased incidences and prevalence of fowl pox in the country.

REV is known to be a potential contaminant of fowl pox vaccine [22]. In 1966 to 1999 cases of a neoplastic disease were encountered in turkeys in Israel after vaccinating the birds against fowl pox using REV-contaminated fowl pox vaccine [14]. In the United States and Australia outbreaks of REV occurred after vaccination of chickens using REV-contaminated FWPV vaccine [8]. Currently imported commercial fowl pox vaccines are used in Tanzania for control of fowl pox in chickens and turkeys. Although to date no cases of runting disease syndrome or chronic lymphoid neoplasms have been reported in domestic and/or wild birds in Tanzania, examination of the vaccines to determine their REV contamination status is important in order to make the Tanzanians (poultry keepers in particular) be certain with the safety of the vaccines. The objective of this study was to investigate integration of genomic fragments of REV in the Tanzanian FWPV field isolates, and the imported commercial fowl pox vaccines currently used in the country.

2. MATERIALS AND METHODS

2.1 Study Location

DNA samples analyzed in this study were extracted from samples of chorioallantoic membrane (CAM) containing virus cultures after inoculation of 10 day-old embryonated chicken eggs with inoculums prepared from samples of cutaneous nodular lesions collected from chickens suspected to have fowl pox in Eastern Tanzania (Dar es Salaam and

Morogoro regions), Central Tanzania (Dodoma region), Western Tanzania (Kigoma and Tabora regions), North-western Tanzania (Mwanza region), Northern Tanzania (Mara region) and Southern Tanzania (Mbeya and Iringa regions). Laboratory work was carried out at the Faculty of Veterinary Medicine (FVM), Sokoine University of Agriculture (SUA), Morogoro, Tanzania. The country is located in Eastern Africa between latitudes 1º-12º South and longitudes 29º-41º East [23,24].

2.2 DNA Samples

Fifty seven samples of genomic DNA which were confirmed to contain FWPV-specific DNA in the previous work by Masola et al. [25] were used in this study. Fifty five samples were extracted from samples of CAM containing virus cultures after inoculation of 10 day-old embryonated chicken eggs with inoculums prepared from samples of cutaneous nodular lesions collected from chickens suspected to have fowl pox. The other two samples were extracted from CAM containing virus cultures after inoculation of 10 day-old embryonated chicken eggs with inoculums prepared from two samples of imported commercial fowl pox vaccines (each sample from a different batch).

2.3 Amplification of FWPV P4b Gene, REV *env* Gene and REV 5'LTR

Three polymerase chain reaction (PCR) assays were conducted in Takara Thermal Cycler (Takara Bio Inc., Japan) using three sets of primers (Table 1). A PCR assay amplifying a P4b gene (578bp) of FWPV was carried out using the first set of primers, P1 and P2. The second assay was conducted to amplify an 807bp region of REV provirus *env* gene using primers P3 and P4. The third set of primers, P5 and P6, was used in the third assay for amplification of REV 5'LTR (370bp), an REV integration site in the FWPV genome.

The components of each amplification reaction were as described previously by Masola et al. [25]. The cycling parameters described earlier by Masola et al. [25] were used in the first PCR assay. For the second assay the cycling parameters included initial denaturation step at 94ºC for 1 minute, for 35 cycles; followed by heat denaturation at 94ºC for 1 minute, primer annealing at 57ºC for 2 minutes, DNA extension at 72ºC for 1 minute. The final extension cycle was performed at 72ºC for 6 minutes. The cycling parameters for the third assay were similar to those of the second assay except that the annealing temperature was 52ºC instead of 57ºC.

2.4 Agarose Gel Electrophoresis

Samples of PCR products were run in a 1.5% agarose gel as described previously by Masola et al. [25].

2.5 Purification of PCR Products, Sequencing of Purified PCR Products and Sequence Analysis

Two samples of PCR products, one with fragment size of 370bp and the other with fragment size of 807bp, were selected. The selected samples were purified followed by sequencing and assembling of sequences (forward sequence and reverse sequence) of each sample to get a consensus sequence using procedures described by Masola et al. [25]. Thereafter each sequence was analyzed by blasting it in the GenBank using the BLAST algorithm [20,28].

Table 1. Primers used in this study

Primer specific for	Primer sequence (5'→3')	Expected fragment size (bp)	Reference
APV P4b gene	Forward (P1): CAGCAGGTGCTAAACAACAA Reverse (P2): CGGTAGCTTAACGCCGAATA	578	[10,11,19,20,25, 26,27,28,29,30]
REV *env* gene	Forward (P3): TGACCAGGCGGGCAAAACC Reverse (P4): CGAAAGGGAGGCTAAGACT	807	[16,20]
REV 5'LTR	Forward (P5): ACCTATGCCTCTTATTCCAC Reverse (P6): CTGATGCTTGCCTTCAAC	370	[20]

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Amplification of FWPV P4b gene, REV *env* gene and REV 5'LTR

Selected samples of genomic DNA known to contain FWPV-specific DNA were subjected to three conventional PCRs in order to amplify FWPV P4b gene, REV *env* gene and REV 5'LTR; followed by agarose gel electrophoresis. Integration of REV *env* gene and REV 5'LTR in the FWPV genome of the analyzed field isolates was indicated by migration of amplicons to approximately 807bp and 370bp, respectively (Fig. 1). Out of 55 field FWPV isolates screened for REV *env* gene and REV 5'LTR; 2 (3.64%) were found to be integrated with neither REV env gene nor REV 5'LTR, 10 (18.18%) were found to be integrated with REV *env* gene only, 5 (9.09%) were found to be integrated with REV 5'LTR only, and 38 (69.09%) were found to be integrated with both REV *env* gene and REV 5'LTR (Table 2). FWPV isolates from the two samples of fowl pox vaccine currently used in Tanzania were found to contain no inserts of REV env gene and/or REV 5'LTR.

3.1.2 Sequence analysis

In order to analyze the obtained sequences the later were blasted in the GenBank. Blast results indicated that the sequence of a PCR product with fragment size 807 bp showed 95 – 100% identity to sequences of several REV *env* gene obtained in the GenBank (accession numbers GU012641, GU012645, GU012646, GU012642, GU012639, GU012638, GU222416, KC884563, KC884554, GQ415646, GQ415647, GQ415645, GQ415644, GQ415643, GQ375848, FJ439120, FJ439119, AF246698, EU246946, DQ925492, KF305089, KC884562, GU222420, M22223, AY842951, DQ237903 and X01455); and 100% identity to sequence of *env* gene of REV provirus integrated in a FWPV isolate (GenBank accession number AF246698).
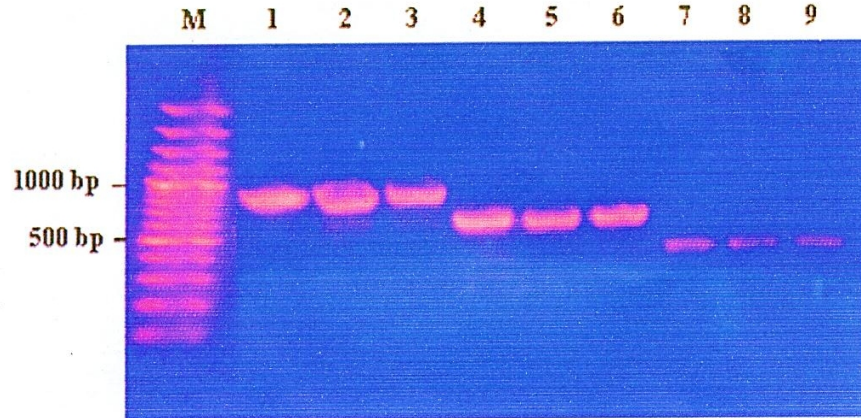
Fig. 1. Agarose gel electrophoresis of FWPV-specific and REV-specific PCRs showing migration of amplicons of REV provirus *env* gene (807 bp) (lanes 1, 2, and 3), FWPV P4b gene (578bp) (lanes 4, 5, and 6) and REV provirus 5'LTR (370bp) (lanes 7, 8 and 9). Lane M is a DNA ladder with 100-bp increments

The sequence of a PCR product with fragment size 370 bp showed 88 – 99% identity to sequences of several REV LTR obtained in the GenBank (accession numbers KF305089, GQ415646, JQ804915, FJ439120, FJ439119, GQ375848, FJ496333, DQ387450, JX912710, M22224, M22223, S70398, GQ870289, GQ870290, AY842951, KC018475, KF709431 and DQ003591) (Fig. 2); and 99 – 100% identity to sequences of LTR of REV provirus integrated in several FWPV isolates from other countries (GenBank accession numbers AY255633, AY255632, AF246698, AJ581527, AF198100, AF006065, AF006064 and HQ111429) (Fig. 3). After analysis sequences of *env* gene and 5'LTR of REV provirus integrated in a Tanzanian FWPV isolate were deposited in the GenBank data base under accession numbers KF225480 and KF268024, respectively.

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Tzfwpv  186  TGT-GGAGGGAGCTCCGGGGGGAATAGCGCTGGCTCGCTAACTGCCATATTAGCTTCTGT  244
             ||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
HA1101  3    TGTGGGAGGGAGCTCCGGGGGGAATAGCGCTGGCTCGCTAACTGCCATATTAGCTTCTGT  62


Tzfwpv  245  AATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCATTTCT  304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HA1101  63   AATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCATTTCT  122


Tzfwpv  305  CGGAATCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAGGCAAG  364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HA1101  123  CGGAATCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAGGCAAG  182


Tzfwpv  365  CATCAG  370
             ||||||
HA1101  183  CATCAG  188
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Fig. 2. Alignment of the sequence of LTR of REV provirus integrated in the genome of a Tanzanian FWPV isolate (designated as Tzfwpv) with sequence of LTR of REV "strain HA1101" obtained in the GenBank (accession number KF305089) (designated as HA1101) showing 99% identity

Table 2. REV integration in the Tanzanian FWPV isolates in various geographical locations and regions

Geographical location	Region	Number of FWPV isolates screened for REV *env* gene and REV 5'LTR	Number of FWPV isolates integrated with[a]				Total percentage of FWPV isolates integrated with REV
			Neither REV *env* gene nor REV 5'LTR	REV *env* gene only	REV 5'LTR only	Both REV *env* gene and REV 5'LTR	
Eastern Tanzania	Dar es Salaam	7	0 (0.00)	2 (28.57)	1 (14.29)	4 (57.14)	100
Eastern Tanzania	Morogoro	4	0 (0.00)	0 (0.00)	0 (0.00)	4 (100.00)	100
Central Tanzania	Dodoma	10	0 (0.00)	2 (20.00)	0 (0.00)	8 (80.00)	100
Western Tanzania	Kigoma	3	0 (0.00)	0 (0.00)	1 (33.33)	2 (66.67)	100
Western Tanzania	Tabora	2	0 (0.00)	2 (100.00)	0 (0.00)	0 (0.00)	100
North-western Tanzania	Mwanza	8	0 (0.00)	0 (0.00)	2 (25.00)	6 (75.00)	100
Northern Tanzania	Mara	4	0 (0.00)	0 (0.00)	0 (0.00)	4 (100.00)	100
SouthernTanzania	Mbeya	14	1 (7.14)	2 (14.29)	1 (7.14)	10 (71.43)	92.86
Souther Tanzania	Iringa	3	1 (33.33)	2 (66.67)	0 (0.00)	0 (0.00)	66.67
Total		**55**	**2 (3.64)**	**10 (18.18)**	**5 (9.09)**	**38 (69.09)**	**96.36**

[a]*Numbers in brackets are the percentage of FWPV isolates integrated or not integrated with a particular genomic fragment(s) of REV*

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Tz fwpv          1    ACCTATGCCTCTTATTCCACTATCGAAGTACGATTTTACATTTACTGATTTGAGTGTTAT    60
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich] 221553 ACCTATGCCTCTTATTCCACTATCGAAGTACGATTTTACATTTACTGATTTGAGTGTTAT    221612


Tz fwpv         61    CAAATCAGAGAATGTTAAAACGGTACTCTCTAAAGTTCATACGAGTATGAAATCGTACTA   120
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich] 221613 CAAATCAGAGAATGTTAAAACGGTACTCTCTAAAGTTCATACGAGTATGAAATCGTACTA   221672


Tz fwpv         121   CAACAATGATACGTCTCTTCCTGTCGCCGTTAAGGTGATTTACGGAACAGTAACAATATA   180
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich] 221673 CAACAATGATACGTCTCTTCCTGTCGCCGTTAAGGTGATTTACGGAACAGTAACAATATA   221732


Tz fwpv         181   AAAAGTGTGGAGGGAGCTCCGGGGGGAATAGCGCTGGCTCGCTAACTGCCATATTAGCTT   240
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich] 221733 AAAAGTGTGGAGGGAGCTCCGGGGGGAATAGCGCTGGCTCGCTAACTGCCATATTAGCTT   221792


Tz fwpv         241   CTGTAATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCAT   300
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich] 221793 CTGTAATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTTCGCTGATATCAT   221852


Tz fwpv         301   TTCTCGGAATCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAGG   360
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HP-438[Munich] 221853 TTCTCGGAATCGGCATCAAGAGCAGGCTCATAAACCATAAAAGGAAATGTTTGTTGAAGG   221912


Tz fwpv         361   CAAGCATCAG   370
                      ||||||||||
HP-438[Munich] 221913 CAAGCATCAG   221922
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Fig. 3. Alignment of the sequence of LTR of REV provirus integrated in the genome of a Tanzanian FWPV isolate (designated as Tz fwpv) with sequence of LTR of REV provirus integrated in a FWPV "isolate HP-438[Munich]" (GenBank accession number AJ581527) (designated as HP-438[Munich]) showing 100% identity.

3.2 Discussion

In the present study the Tanzanian FWPV isolates were analyzed between open reading frame (ORF) 201 and ORF 203 for REV integration [9,17]. The analysis revealed integration of various genomic fragments of REV in the genome of most of the FWPV isolates. This implies that currently in Tanzania there is a heterogeneous population of FWPV circulating in the field due to integration of various genomic fragments of REV in their genome. This has led to emergence of variant strains of FWPV currently prevalent in the country, which according to Singh et al. [18] are more virulent than REV-free FWPV strain(s).

Prior to this study integration of genomic fragments of REV in the genome of field isolates of FWPV had been reported in Australia [13,14], Croatia [19], India [20], Israel [9] and the United States [15,16,17,18,31]. The high proportion of near-full length REV integration in the genome of field isolates of FWPV observed in the present study (Table 2) is in agreement with a previous report by Biswas et al. [20].

Fowl pox is mainly controlled by vaccination of susceptible chickens or turkeys using fowl pox vaccines of FWPV- or pigeonpox virus (PGPV)-origin [32]. The findings of this study are a step toward development of autogenous fowl pox vaccine for control of fowl pox in Tanzania and other countries with populations of FWPV which are genetically and antigenically similar to FWPV strains currently prevalent in Tanzania.

Lack of genomic fragments of REV in the FWPV isolates from samples of the imported commercial fowl pox vaccines currently used in Tanzania, and demonstration of the presence of REV *env* gene in most (90.57%) of REV-integrated Tanzanian FWPV isolates indicated that the vaccines are not a source of the variant strains of FWPV revealed in this study. The genetic diversity of the Tanzanian FWPV revealed in the present study could be a result of recombination between field strains of FWPV and field strains of REV [33].

4. CONCLUSION

Based on the findings of this study the following conclusions are drawn:

i)   Currently there is a heterogeneous population of FWPV in Tanzania comprising of REV-integrated FWPV strains and REV-free FWPV strains.
ii) The imported commercial fowl pox vaccine currently used in Tanzania is not contaminated with REV.
iii) The increased incidences and prevalence of fowl pox currently experienced in Tanzania, characterized with high mortalities of chicks and growers, is attributed to increased virulence of variant FWPV strains due to integration of genomic fragments of REV in their genome.

Further studies on REV-integrated Tanzanian FWPV isolates, aiming at obtaining the appropriate strain (isolate) for development of appropriate autogenous fowl pox vaccine for control of fowl pox in Tanzania, and other countries with populations of FWPV which are genetically and antigenically similar to FWPV currently prevalent in Tanzania are highly recommended. This recommendation is based on the fact that strains of REV-integrated FWPV are more virulent than strains of REV-free FWPV [18].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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