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Evidence for Virus Population Heterogeneity in the Sheep and Goat Pox Vaccine Strain 0240

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Abstract: Sheep and goat pox vaccine strain 0240 was characterized. We designed a pair of primers flanking 3 ORFs including thymidine kinase (tk) gene of Kenya sheep-1. Polymerase chain reaction (PCR) was performed and PCR products of approximately 908 base pair (bp) in size were obtained for all cloned plaques and the vaccine strain. Lamb testicle (LT) DNA (negative control) as expected did not produce any PCR products. PCR products were digested with Ssp1 and Dra1 restriction enzymes and analyzed using agarose gel and polyacrylamide gel electrophoresis (PAGE), respectively. Generation of the expected banding pattern following digestion confirmed virus identity. The analysis revealed the presence of restriction fragment length polymorphisms (RFLPs) between some plaques at temperatures of 33°C and 37°C and the vaccine strain. An *in vitro* recombination experiment was performed for the 0240 vaccine strain, which revealed its viral sequence heterogeneity. The pattern of heat inactivation indicated the presence of at least two virus populations differing in heat sensitivity. In conclusion, these experiments indicated the presence of two different virus populations.

Key words: Virus, heterogeneity, pox, vaccine, strain 0240

Introduction

Capripoxviruses are the most important pox viruses of animals, causing generalized pox diseases of sheep, goats and cattle. The diseases are characterized by pyrexia, generalized skin and internal pox lesions and adenopathy (Kitching and Taylor, 1985a and Carn, 1993). The diseases are restricted to Asia and Africa and caused major economic losses, associated with trade restrictions following disease epizootics. In the Sudan, sheep and goat poxes are assuming importance associated with animals exported to the Gulf countries. The virus persists in the scabs, developed from the papules following recovery for over 3 months (Kitching and Taylor, 1985a).

The sheep and goat pox vaccine strain 0240 was introduced into the Sudan in the late 1980s. This vaccine is used to control sheep and goat pox and lumpy skin diseases in the country. Originally the 0240 strain was passaged twice on lamb testis cell monolayer (LT) and when inoculated intradermally into susceptible British sheep and goats, caused only a local lesion without development of pyrexia (cited by Kitching *et al.*,1986). When passaged three more times on LT cell monolayer this strain produced a milder local reaction and was shown to protect sheep and goats against challenge with virulent capripoxvirus isolates (Kitching *et al.*, 1986) and developed as a vaccine against sheep and goat pox.

The present investigation was designed to provide evidence for virus populations in the 0240 vaccine strain which might explain its rapid development after only 5 passages .

Materials and Methods

Virus and Cells: Virus seed of Kenya sheep and goat pox vaccine strain 0240 was obtained from the vaccine seed bank, at the viral vaccines production unit of the Central Veterinary Research Laboratories (C V R L), Khartoum, Sudan.

Lamb Testis Cells (LT): The cells were obtained from healthy lambs as described by Freshney (1987) and used for virus propagation. Total DNA was extracted from noninfected LT cells and used as a negative control during PCR reactions.

Isolation of Virus Clones: The plaque assay was used to obtain pure virus clones as described by Cooper (1961) for cultures grown both at 33°C and 37°C.

Virus Purification: Virus purification was carried out as described by Talavera and Rodriguez (1991a)..

Heat Treatment of Plaques: Heat treatment of the purified plaques was performed as described by El-Awar and El-Zein (1986).

Virus Titrations: Ten fold serial dilutions were carried out for each virus preparation and titrated in LT cells in microtiter plates (Kitching and Carn, 1996). The virus preparations were then heated in a water bath at 56°C for 3min or the multiple of 3, as time interval, immersed in ice-water and microtitrations were carried out at each time interval. Virus titer in each sample was calculated using Karber method (Villegas and Purchase, 1980).

DNA Extraction from Virus Preparations: The procedure cited by Talavera and Rodriguez (1991a) was adopted.

Thymidine Kinase Gene Nucleotide Sequence: The nucleotide sequence of Kenya sheep -1 (KS-1) poxvirus, thymidine kinase (tk) gene was obtained from European molecular biology laboratory (EMBL) GenBank under the accession number D00423. The amplicon is composed of 3 open reading frames (Gershon and Black, 1989).

Primers Design: A pair of primers was manually designed, at the Department of Molecular Biology, Institute of Endemic Diseases, flanking the tk gene from 14 – 922bp and then it was provided by Sigma –Genosys ltd,USA. The sequences of the primers were as follows:
Positive sense: 5 GTGGGTTACCTAATACTA 3 (14- 31 bp).
Negative sense:5? TGAAACGTGCTATCTAGT3 (904- 922 bp).
The primers were used at a concentration of 15 (picomole) pmole. The designed size of the PCR product is 908bp.

PCR Conditions: Denaturation was carried out at 94°C for 30 sec, followed by annealing at 50°C for 2 min then extension was performed at 72°C for 1 min. Final extension was done for one cycle at 72°C for 10 min.
The thermal profiles were performed in an MJ Research PTC-225 Thermocycler for 35 cycles. Products of the PCR were detected in 1.5% agarose gel (Sambrook *et al.*, 1989b).

Generation of Restriction Fragment Length Polymorphism (RFLP)
Determination of Restriction Sites for Endonucleases: The Internet Program (Web Cutter 2) was utilized to determine restriction sites on tk gene of K-1 sheep poxvirus. Ssp1 and Dra1 were chosen for this purpose, because the sheep pox genome is A + T rich and the recognition sequence of both enzymes is composed of A + T bases (aatatt, tttaaa respectively) i.e. they are frequent cutters.Digestion of the PCR fragments were performed according to manufacturers instructions (Promega , Madison- USA). The digestion products were analysed using 2% agarose and 12% polyacrylamide gel electrophoresis (Sambrook *et al.*,1989c).

Results

Plaque Assay: A total of 45 plaques were analysed including 20 plaques from 33°C and 25 plaques from 37°C.

Heat Treatment Profiles
Titration Results: Results of the microtitrations assays are shown in Tables 1 and 2. Plaques raised at 37°C produced a smooth decline of titre similar to some plaques raised at 33°C e.g. 2, 3, 4 and 5. Some plaques raised at 33°C produced an abrupt decline of titre and dropped to zero e.g. at 6min for plaque 11 and at 9min for 7. None of the plaques produced a biphasic decline of titre, which indicated their purity and ruled out the effect of cells debris and virus aggregates.These results indicated the presence of at least two virus populations differing in heat sensitivity.

PCR Reactions: PCR products having a size of approximately 908 bp were obtained for each plaque and for the vaccine strain. Total genomic DNA extracted from LT cells failed to produce the specific 908bp PCR product.

Table 1: Titres of plaques isolated from the 0240 sheep pox vaccine strain after heat treatment at 56oC taking 3 min as time interval

	Titre(Log_{10} $TCID_{50}$/ml)				
Plaque No.	0min	3min.	6min	9min	12min
44(37°C)	5.2	5.0	5.0	4.6	4.6
28	4.8	4.6	4.6	4.8	4.4
62	5.2	5.0	4.8	4.6	4.4
11(33°C)	5.6	3.0	0.0	0.0	0.0
2	4.0	3.8	3.4	3.4	3.2
7	3.6	3.4	3.6	0.0	0.0

Table 2: Titres of plaques isolated from 0240 sheep pox vaccine strain after heat treatment at 56°C, taking the multiple of 3min as interval

Plaque No.	Titre (Log_{10} $TCID_{50}$/ml)		
	0min	6min	15min
A(37°C)	6.7	5.3	4.9
3(33°c)	5.5	5.9	3.9
11	5.9	0.0	0.0
4	6.7	6.1	4.3
10	4.9	4.1	0.0
9	5.9	4.1	0.0
5	6.9	6.3	4.5

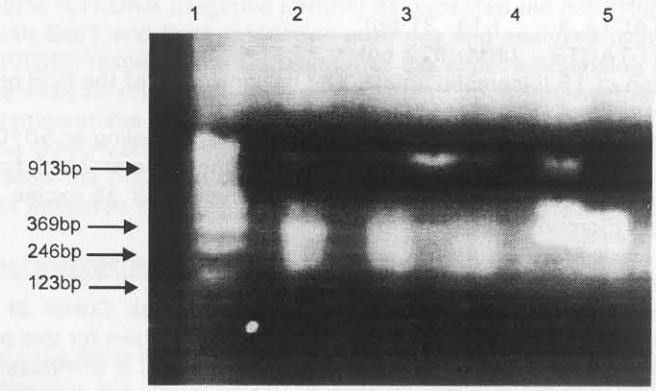

Fig. 1: PCR products of some plaques of sheep and goat pox vaccine strain 0240 digested with Ssp1 restriction enzyme and analyzed with 2% Agarose gel. M, Molecular weight marker 123bp ladder. (1) Sheep and goat pox vaccine strain0240 (a). (2) P37, 10. (3) P37, 12. (4)Sheep and goat pox vaccine strain 0240 (b). (5) P37, 6.

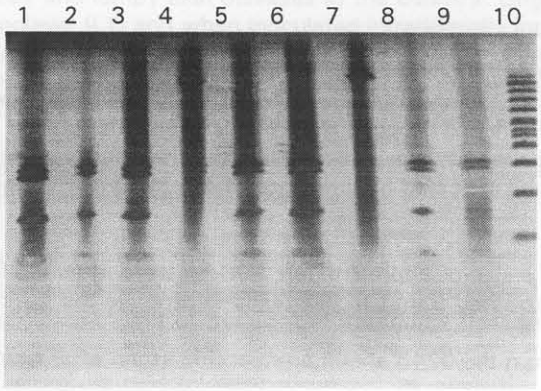

Fig. 2: PCR products of some plaques of sheep and goat pox vaccine strain 0240 digested with Dra1 and analyzed with 12% polyacrylamide gel. This figure shows the differences in bands size between p37/3 and others shown in the plate. (1) P37, 8. (2) P37, 3. (.3) P37, 16. (4) P37, 30 uncut. (5) P33,7.(6) P37,25.(7) P37,34 uncut. (8) P37,1.(9)Vaccine strain.(10) Molecular weight marker 100bp ladder

Restriction Fragment Length Polymorphism (RFLP): The RFLPs generated with Ssp1 and resolved in agarose showed differences in band size between the sheep and goat pox vaccine strain 0240 samples (a, b) and one plaque (p37/6) obtained at 37°C., as shown in (Fig.1), where the band above 300bp marker of the vaccine has greater molecular weight than that of P37/6 and vaccine strain (b).However, the vaccine strain sample (a) is similar to p37/10 and p37/12 PCR product.

RFLPs generated by Dra1 were resolved in 12% polyacrylamide gel electrophoresis. Fig. 2 reflected differences in bands size between (P37, 3) the vaccine strain and other plaques obtained at 33°C and 37°C. All bands of plaque 37/3 appeared to be shifted up.
These fragment patterns indicated the presence of at least two different virus populations.

Discussion

The plaque assay is a convenient method for cloning the virus, as well as for isolating virus variants that are the product of spontaneous mutation or due to artificial manipulation of the genome (Talavera and Rodriguez, 1991 and McKeating, 1991). In the present study plaques were developed at 33°C and 37°C and only well isolated plaques were picked and propagated on LT cells.
The purity of the plaques was clearly indicated by the fact that no biphasic decline in the virus titers was observed when these plaques were heat treated at 56°C for various time intervals. Heat sensitivity treatments also indicated the presence of at least 2 different virus populations in the vaccine strain. This is in conformity with the results of El Awar and El Zein (1986).
It is known that in pox viruses virus variants in which the tk gene has been inactivated can successfully infect normal cells (Talavera and Rodriguez, 1991 and Carstens, 1999). This justifies selection of tk gene as an amplicon. Primers were designed flanking the tk gene together with 2 ORFs of Kenya (KS-1) sheep pox virus and were used for PCR amplification (Gershon and Black, 1989). In the present study digestion of tk PCR products of plaques with Ssp1 and Dra1 revealed differences in resultant bands sizes. The difference in band size may be due to presence of different virus populations arising from spontaneous mutations (e.g. due to errors by the replicating polymerase or as a result of incorporation of the tautomeric forms of the bases. This indicates that such differences are most probably due to differences in nucleotide sequences among plaques due to spontaneous mutations. Most viral nucleic acid polymerases lack proofreading capability. These different populations were stable as PCR produced the same pattern
Some plaques grown at 33°C were similar in restriction fragment length polymorphism (RFLP) to some plaques raised at 37°C , because the parental vaccine virus can grow at both temperatures, while others were different from their sister plaques grown at both temperatures. Wild type animal viruses can generally multiply over a temperature range of 20°C to 39.5°C.Temperature sensitive mutants code for some viral protein that cannot assume or maintain structural conformation.
The most significant problem with amplification of heterogeneous viral sequences by PCR is artifactual recombination. This occurs during the later cycles, where there is insufficient DNA polymerase to complete the synthesis of all primed DNA strands in the allotted 10 min-elongation step (Simmonds and Chan, 1993). To verify this was not the case, in our study *in vitro* recombination experiments were carried out for the sheep and goat pox vaccine strain 0240, using different concentrations of Taq polymerase .PCR products were digested with Dra1 enzyme and analysed by polyacrylamide gel electrophoresis RFLPs were different (data not shown). Hence the generation of different profiles is most probably a consequence of sequence heterogeneity of the 0240 vaccine strain, which also indicates the presence of different virus populations.
The outcome of PCR analysis (RFLPs) agreed with that of the heat treatment experiments, in that some plaques obtained at 33°C are similar to others obtained at 37°C (smooth decline of titre) while other plaques raised up at 33°C retained distinct identities (abrupt decline of titre).
Wild- type virus (wt) is passaged in culture or eggs until a mutant arises , usually after high passage level, unlike 0240 vaccine strain which became attenuated after a low passage level. The presence of different populations of virus may explain the rapidity with which the original virus was attenuated, which made the 0240 strain a vaccine.
The sheep and goat pox vaccine strain 0240 is composed of at least two virus populations having different heat sensitivity. It would be interesting to investigate the pathogenecity of these virus populations and to detect whether a pathogenic wt virus is still present.
The use of temperature stable plaques for the production of a thermos- table vaccine may also worth further investigation.

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