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Comparative molecular studies on different isolates of IBR virus

Hassanein, S.A.*; Ibrahim, A.K.; Mervat, M.M.* and Nahed, K.A.***

Reference strains and field isolates of herpesviruses recovered from cattle, sheep and goat in Egypt were compared by virus neutralization, (VN), Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) analysis. Antigenic comparison using VN test and their effect on MDBK cell culture detected no difference, thus demonstrating the close antigenic relationship between the examined herpesviruses. Using the restriction endonucleases BamHI and EcoRI revealed no difference between the examined five viruses. But by the use of HindIII restriction endonuclease and RAPD, fingerprint patterns were capable of differentiating the analyzed five viruses by the presence or absence of amplification fragments and showed difference in fragments' sizes. On the basis of RFLP and RAPD comparisons, 3 main strains of bovine herpesvirus (BHV) were defined. These strains were (I) Abu-Hammad strain and Colorado strain, (II) Cattle isolate and Goat isolate and (III) sheep isolate. In conclusion, virus neutralization test could not differentiate bovine herpesvirus type 1 (BHV -1) from other strains of herepesviruses; on the contrary, RFLP and RAPD assays could be used to differentiate the virus. Thus, both techniques can be applied for the molecular epidemiology of BHV -1.

Key words: Herpesviruses, BHV -1, Restriction Fragment Length Polymorphism (RFLP), Restriction Endonuclease, Random amplified polymorphic DNA (RAP D) .

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is member of the family Herpesviridae, subfamily Alphaherpesvirinae **(Ashbaugh et al., 1997)**. Bovine herpesvirus 1 isolates were classified into

*ELISA Unit, Animal Health Research Institute, Dokki, Giza. , **Dep. Of Clinical Pathology, Fac. of Vet. Med., Cairo University

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subtypes 1 and 2, according to enzyme profiles **(Engels et al., 1986)**. BHV-1 is the causal agent of a variety of clinical syndromes in cattle, such as infectious bovine rhinotracheitis (IBR, BHV-1.1), a severe respiratory tract infection of great economic impact, as well as infectious pustular balanopostitis and infectious pustular vulvovaginitis (BHV-1.2). The virus was rarely associated with central nervous disorders **(Roels et al., 2000; and Abril et al., 2004)**. It is similar in structure to other herpesviruses, possesses a double stranded DNA genome about 100 X 10^6 in molecular weight **(Farley et al., 1980)**. Also like other herpesviruses, the virus can Restriction endonuclease analysis of herpes viral DNA has been used to demonstrate the existence of BHV-1 isolates that differ in their restriction fragment-migration patterns. These patterns are stable on viral passage in vitro, as well as through latency and reactivation **(Ludwig, 1983 and Thiry et al., 1984)**. Although BHV-1 isolates cannot be differentiated by biological or antigenic markers reliably, restriction endonuclease analysis can differentiate isolates into 2 major strain types **(Pauli et al., 1984 and Engels et al., 1981)**. become latent in animals probably in the trigeminal ganglion, and can be reactivated with ease **(Homan et al., 1980. and Misra et al., 1981)**.

BHV-1 is an economically important pathogen of cattle. It is associated with a wide variety of syndromes which, in addition to the more common infections of the upper respiratory and genital tracts **(Kendrick et al.,1958)**, also include encephalitis **(Barenfus et al., 1963)**, enteritis **(Gratzek et al., 1966 and Weelemass et al., 1974)**, abortion **(Chow et al., 1964)**, conjunctivitis **(Abinanti and Plummer, 1961)** and mastitis **(Greig and Bannister, 1965)**.

A correlation, has been detected between the clinical site of isolation and the restriction fragment pattern, the 2 major strain types have been described as IBR like and IPV-like **(Pauli et al., 1984)**. Although the epizootiologic importance of BHV-1 strains differences at the genomic level is still somewhat uncertain, restriction endonuclease analysis provides a reliable method of characterizing strains and isolates.

Random amplified polymorphic DNA (RAPD) typing, originally developed by **Welsh and**

McClelland in 1990. RAPD is a simple and easy method for detection of polymorphisms based on the amplification of random DNA segments with arbitrary short primers (usually 10 to 15 bp) to amplify nearly homologous sequences of the genomic DNA under low-stringency conditions. To date, RAPD has been used largely for population genetics, taxonomical and paternity studies **(Williams et al., 1990; Perez et al., 1998)**, and recently in ecotoxicology **(De Wolf et al., 2004)**. In microbiology, RAPD is used for phylogenetic analysis of bacteria and fungi **(Hahn et al., 2003 and Pinto et al., 2004)**, and this technique was first applied to virus studies in 2001, showing great accuracy for analysis of closely related orthopoxvirus isolates **(Stemmler et al., 2001)**, by **Comeau et al. (2004)** to access virus contaminants in environmental samples and most recently by **Afonso et al. (2007)** in Characterization of field bovine herpesvirus samples. It is, therefore, useful for determining whether two isolates of same species are epidemiologically related. So RAPD is probably the simplest DNA-based sub typing method to date if a temperature-cycling instrument is available, although the usefulness for epidemiological investigations remains to be determined, particularly with regard to reproducibility concerns.

The purposes of the study reported here were to examine, by VN, RFLP and RAPD IBR virus isolates (one from cattle, sheep, and goat with naturally occurring herpes virus infection, reference Colorado strain and vaccinal strain Abu-Hammad) and to investigate what similarities might exist among these viruses and trying to define precisely the biotype to which each strain belongs. Of particular interest was the comparison of isolates from sheep and goat and those of cattle isolates, may be important in the epidemiology of BHV-1 infection. Using the vaccinal strain restriction enzyme patterns and RAPD pattern for comparison, we have presented data concerning the usefulness of REA and RAPD as a diagnostic technique in differentiating vaccine-induced IBR from IBR associated with naturally occurring isolates of IBR virus.

MATERIAL AND METHODS

Viruses: IBR isolates, originally obtained from cattle, sheep and

goat (kindly supplied from Viral Strains Bank and ELISA Research Unit, Animal Health Research Institute (AHRI)) with a reference IBR Colorado strain (kindly supplied from Virology department faculty of Vet. Med. Cairo Univ.) and IBR vaccinal Abu-Hammad strain (kindly supplied from Serum and Vaccine Research Institute).

Cell culture methods: The MDBK cells were grown in tissue culture flasks with Eagle's minimum essential medium containing 60 ug of penicillin/ml, 140 ug of streptomycin sulfate/ml, and supplemented with 10% heat-inactivated fetal serum (FCS) for cell growth or 2% horse serum for cell culture maintenance and viral inoculation. Cell cultures were incubated at 37 ^{0}C in a humidified 5% Co_2 incubator.

Virus neutralization: All viruses were compared by VN tests, using 10-fold serial dilutions of virus and constant (1:20) anti-BHV-1 serum (kindly supplied from Viral Strains Bank and ELISA Research Unit, Animal Health Research Institute). Tests were performed in 96 well Microtiter plates seeded with MDBK cells, using standard techniques (Cottral, 1978).

DNA extraction: Each virus was grown in MDBK cells. The infection was allowed to proceed until the cultures exhibited 80% of cytopathic effects. Then the cells freeze and thawed three times then centrifuged at low speed (3000 rpm) for 20 minutes and the supernatant was collected and the cell debris was discarded. For the purpose of RFLP and RAPD analysis, viral DNA was extracted from this supernatant, using Qiagen DNA Extraction Kit , Diagen incorporation, Germany

Random Fragment Length Polymorph RFLP: DNAs were enzymatically cleaved either with Hind III, Bam HI and EcoRI. Takara Bio Inc. (Shiga, Japan) where the reaction mix prepared by adding 10 u of Enzyme /reaction(2ul), 2.5 ul of 5 x reaction buffer and 5 u DNA and completed to 25 ul by Deionized water. The total digested DNA materials were then analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide.

Random amplified polymorphic DNA: RAPD was done using two primers (Primer1: TGTCTACAC-GTT and Primer2: GACCACACT-ACC) in the same reaction. The following low stringency cycling profile was used: amplification involved initial denaturation of DNA template (20 ng per 25 µl

reaction mix) at 94°C for 3 min, followed by 33 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C in thermocycler "Tertsik" (Russia). Amplification was finished with incubation at 72°C for 7 min. DNA fragments were separated on a 2 % agarose gel at 7,5 V/cm for 2,5 hours.

Results analysis: A binary data matrix was constructed for each restriction enzymes and RAPD profile based on the presence (1) and absence (0) of the fragment. UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5 was used to analyze results of each restriction enzymes and RAPD.

RESULTS

Results of virus neutralization: Results of virus neutralization revealed no difference in the effect of constant anti-BHV-1 serum on the decreasing infectivity titer of the five IBR viruses examined.

Results of RFLP analysis: The RFLP pattern obtained after digestion of the five IBR viruses using BamHI and EcoRI endonuclease showing no difference between the viruses except some minor difference in some bands size. The RFLP pattern obtained after digestion of the five IBR viruses using Hind IIII endonuclease shown in Fig (1) revealed that it can cleaved the examined five IBR viruses DNA into 5-6 fragments that molecular weight sizes are shown in table (1). The Hind III pattern could be differentiated into three main categories: pattern (A) that contains Abu-Hammad and Colorado strain, pattern (B) that contains cattle and goat isolates and pattern (C) that contains sheep isolate. So the examined five IBR viruses could be divided into three main strains I, II, and III.

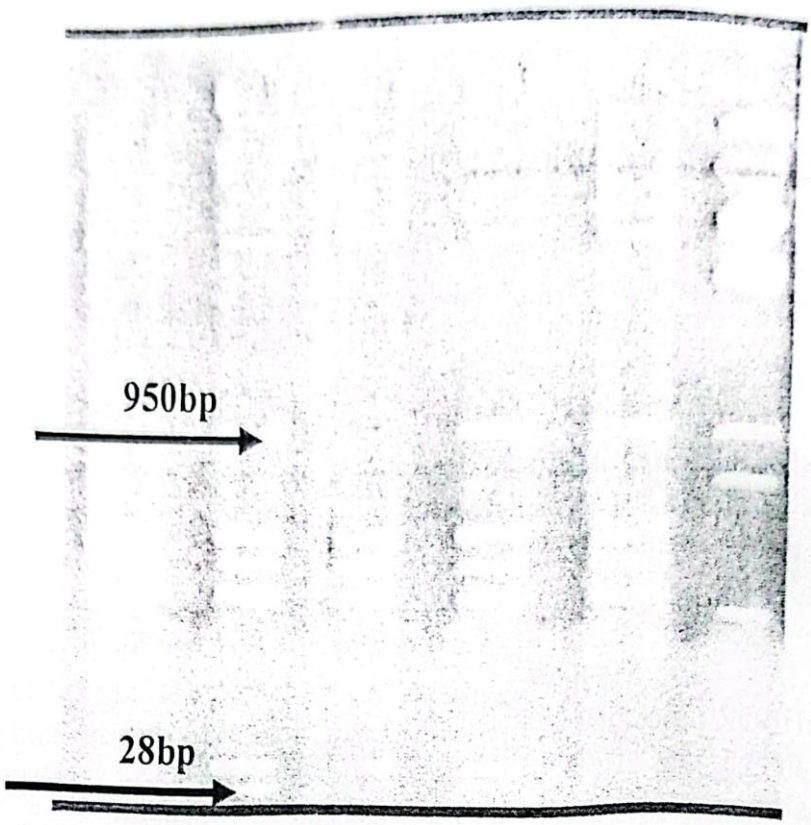

Fig. (1): Electerophoretic pattern of Hind III restriction enzyme, (Abu-Hammad lane 1, Colorado lane 2, sheep isolate lane 3, cattle isolate lane 4, goat isolate lane 5 and The *Hind* III-λ phage lane 6 as a marker.

Table (1): The molecular weight sizes of the examined viruses by Hind III enzyme

Lanes / Bands	Lane 1 (mol.w.) Abu-Hammad	Lane 2 (mol.w.) Colorado strain	Lane 3 (mol.w.) sheep isolate	Lane 4 (mol.w.) Cattle isolate	Lane 5 (mol.w.) Goat isolate	Lane 6 (mol.w.) Marker
1	5672	5672	5672	5741	5536	23130
2	2290	2285	1213	2300	2312	9416
3	1195	1204	960	1221	1204	6557
4	950	943	-------	929	957	4361
5	687	718	715	687	677	2322
6	42	41	40	41	40	2027
7						564
8	28	27	-------	-------	--------	125

Results of RAPD: RAPD fingerprint pattern Fig (2) was capable of differentiating the analyzed five viruses by the presence or absence of amplification fragments and showed difference in fragments size. The amplified RAPD profile using two primers in a same reaction shows conserved amplified fragments between the five viruses. According to fig (2), RAPD fingerprint pattern clearly showed that this method is able to comprise the examined five viruses. On the basis of RAPD assay followed by phylogenetic analysis using UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5 Fig (3), differentiated the examined five IBR viruses into two groups: group I (Abu-Hammad, Colorado, cattle and goat viruses) and group II (sheep isolates). The group I was branched into two subgroups: subgroup I.1 contains Abu-Hammad and Colorado strains together and subgroup I.2 contains cattle and goat viruses together.

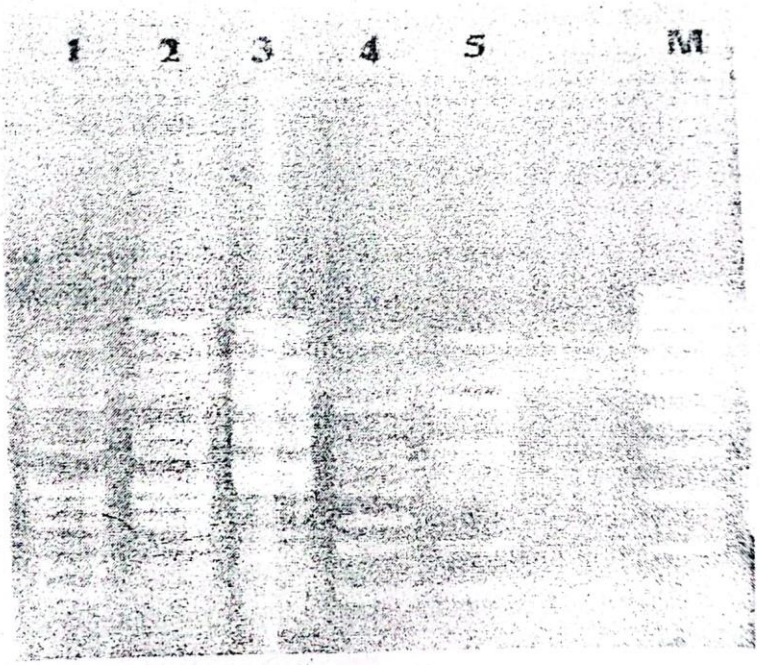

Fig. (2): Electerophoretic pattern of RAPD analysis (Abu-Hammad lane 1, Colorado lane 2, sheep isolate lane 3, cattle isolate lane 4, goat isolate lane 5 and The *Hind* III-λ phage lane 6 as a marker.

On the basis of Hind III RFLP profile and RAPD assay profile, the examined five viruses can be differentiated into 3 main strains I, II, and III strain I and II could be further subdivided into two sub-strains depending on the minor difference in the size of the fragments Table (2).

Table (2) proposed strains designation for examined IBR viruses:

Proposed IBR virus strain designation	Hind III pattern	RAPD assay	Virus name
I.1	A	Group I.1a	Abu-Hammad strain
I.2	A	Group I.1b	Colorado strain
II.1	B	Group I.2a	Cattle isolate
II.2	B	Group I.2b	Goat isolate
III	C	Group II	Sheep isolate

DISCUSSION

BHV-1 has often been isolated from respiratory manifestation of cattle and buffaloes in Egypt but it also isolated from respiratory manifestation of sheep and goat (Ahmed, 2002). In search for a better understanding of Bovine Herpes Viruses epidemiology in Egypt, cattle, sheep and goat local isolates were characterized and compared with standard references Abu-Hammad strain and Colorado strain by antigenic virus neutralization test and molecular RFLP and RAPD assay of the viral DNA in this study.

Comparison of the examined five viruses on the basis of antigenic properties using virus neutralization test and their effect on MDBK cell culture detected no difference and this demonstrate the close antigenic relation ship between the examind viruses. This was expected, because BHV-1 strains are closely related and therefore can not differentiated reliably by antigenic and biological markers (Gillespie et al., 1959; House, 1972; Steck et al., 1975; Engels et al., 1986 and Kennedy et al., 1986).

Using of restriction endonuclease BamHI and EcoRI revealed no difference between the examined five viruses. But using of HindIII restriction endonuclease revealed difference between the viruses and this agree with results of Engels et al., 1986; Kennedy et al., 1986; Christensen et al., 1996 and D'Arce et al., 2002.

HindIII restriction endonuclease yield DNA patterns that were divided the examined viruses into three patterns: pattern (A) that contains Abu-Hammad and Colorado strain, pattern (B) that contains cattle and goat isolates and pattern (C) that contains sheep isolates. On the basis of these patterns the examined five BHV-1 viruses could be divided into three main strains I, II, and III strain I and II could be further subdivided into two sub-strains depending on the minor difference the size of the fragments.

RAPD fingerprint pattern was capable of differentiating the analyzed five viruses by the presence or absence of amplification fragments and showed difference in fragments size (Afonso et al., 2007). The amplified RAPD profile using two primers in a same reaction shows conserved amplified fragments between the five viruses. RAPD fingerprint pattern clearly showed that this methods is able to comprise the examined five viruses. On the basis of RAPD assay followed by phylogenetic analysis using UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5, the examined five IBR viruses can be into two groups: group I (Abu-Hammad, Colorado, cattle and goat viruses) and group II (sheep isolates). The group I was branched into two subgroups: subgroup I.1 contains Abu-Hammad and Colorado strains together and subgroup I.2 contains cattle and goat viruses together.

On the basis of Hind III RFLP profile and RAPD assay profile, the examined five viruses can be differentiated into 3 main strains I, II, and III strain I and II could be further subdivided into two sub-strains depending on the minor difference in the size of the fragments.

In conclusion: virus neutralization test can not differentiate BHV-1 but RFLP and RAPD assays can use to differentiate the virus. The two techniques (RFLP and RAPD) can use in epidemiological study of BHV-1.

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