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APPLICATION OF BIOTECHNOLOGY IN DIAGNOSTIC VETERINARY MEDICINE

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تطبيقات التكنولوجيا الحيوية في الطب البيطري التشخيصي

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الطرق التقليدية لعزل وتصنيف المسببات المرضية تمثل التشخيص الأمثل للعدوى الميكروبية النشطة. لكن هذه الطرق صعبه ومجهدة وتحتاج لوقت طويل. السيرولوجي مفيد للتعرف على العدوى السابقة في الدراسات الوبائية. تقنيات التشخيص جزيئى تشمل أستخدام الأجسام المناعية الأحادية في أختبار الأليزا التنافسي ومجسات الحامض النووي الثنائي وتكبير الحامض النووي المعروف بالتفاعل التبلمري المتسلسل، هذه التقنيات قد تحسن طرق التشخيص الحالية للتعرف على المجموعة والنوع السيرولوجي للمسبب المرضي الخاص. استخدام تقنيات البيولوجيا الجزئية يوفر الأساس لفهم أفضل لوبائية الأمراض التي لها أهمية بيطرية بالإضافة إلى أن تطبيق التكنولوجيا الحيوية في الطب البيطري التشخيصي سوف يحسن صحة القطعان والتحصين وبرامج الوقاية وسوف يسهل التعرف على المسببات المرضية النشطة أثناء حدوث وباء لمرض بين الحيوانات البرية والأليفة التي لها قابلية للإصابة.

SUMMARY

Conventional methods for isolation and identification of infectious agents represent the most accurate diagnosis for an active microbial infection. However, these methods are tedious laborious and time consuming. Serology is useful to identify a previous infection in epidemiological studies. Molecular diagnostic techniques including the use of monoclonal antibody (Mab) in competitive enzyme linked immunosorbent assay (cELISA), cDNA probes and nucleic acid amplification technology, commonly known as polymerase chain reaction (PCR) may improve the existing diagnostic techniques used for serogroup-specific detection and serotype-specific identification of specific infectious agent. The surge of new techniques molecular biology provides the basis for better understauding of the epidemiology of

diseases of veterinary importance. In addition, the application of biotechnology in diagnostic veterinary medicine will enhance herd health monitoring, vaccination and control programs, and will facilitate detection of an active microbial infections during an outbreak of the disease among susceptible wildlife and domestic livestock.

Key words: Biotecnology – ELISA – DNA probe – PCR.

INTRODUCTION

Epizootic hAemorrhagic disease (EHD) virus (EHDV), a member of the orbivirus genus; the family Reoviridae, is related to bluetongue virus (BTV) and palyam serogroup orbiviruses (Aradaib et al., 2000 and Fenner et al., 1974). The EHDV infects domestic and wild ruminants in many parts of the world including the Sudan (Hoff & Trainer, 1974; Fosgerg et al., 1977; Gibbs & Lawman, 1977; Metcalf et al., 1991; Work et al., 1992; Aradiab et al., 1997 and Aradiab et al. 2000). Ten serotypes of EHDV are recognized world-wide (Goman, 1992). EHDV serotype 4 and an untyped EHDV isolate designated (EHDV-318) are enzootic in the Sudan. Currently, little information is known about the epidemiology of these viruses. Sudanese zebu cattle are susceptible to EHDV infection but the disease is usually subclinical. Whereas the disease is fatal in North American White-tailed deer, no information is available about the disease in Sudanese deer populations (Hoff & Trainer, 1974; Nettles et al., 1991; Rabenau et al., 1993 and Shope et al., 1995). Several outbreaks of clinical Haemorrhagic disease were reported among deer population in the Sudan but the virus was not isolated Unfamilirity with the ecology, biology and molecular epidemiology of EHDV serogroup has led to major bariers in international trade of livestock and associated germplasm.

Biotechnology offers an excellent opportunity for understanding the molecular epidemiology of EHDV serogroup. The objective of the present investigation was to improve the detection of orbivirus infections, with special emphasis on EHDV, using a variety of molecular biological techniques.

Detection of Epizootic Haemorrhagic Disease Virus Infection:

I- Virologic techniques :

Virus Isolation and Identification:

Various labortatories have different methods for isolation of EHDV including intravenous (IV) inoculation of embryonated chicken

eggs (ECE) or isolation on cell lines. The IV technique is laborious, expensive, and time consuming and a final result required 2-4 weeks (Aradaib et al., 1994; Aradaib et al., 1994; Aradaib, 1995 and Aradaib et al., 1995).

The serotype-specific identification of the virus is usually based on serum neutralization test using known reference antiserum (Pearson et al., 1994). Plaque inhibition test could also be used instead as described by Stott et al. (1978).

II- Serological Techniques:

Serology does not necessarily identify an active infection. However, it is useful to determine past infection in a seroepidemiological survey (Aradaib & Osbum, 1995 and Aradaib et al., 2000). Several serodiagnostic techniques have been validated for the diagnosis of orbiviruses. The main bulk of the work, so far, has been on the Bluetongue, a closely related orbivirus. The serological techniques applied for diagnosis of EHDV include agar gel immunodiffusion (AGID); the complement fixation test (CFT); and the indirect labeled antibody technique such as the indirect immunofluorescence antibody technique (IFAT); the indirect enzyme linked immunosorbent assay (ELISA); the competitive ELISA (cELISA) using monoclonal antibody (Mab).

III- Molecular Diagnostic Techniques:

For the last 10 years, the major thrust of our research was directed towards the improvement of the existing techniques used for diagnosis of EHDV infection. The development of a rapid sensitive specific and inexpensive method for diagnosis of the disease would greatly facilitate clinical disease investigations, epidemiological investigation, and treatment of the infected animals, and would enhance vaccination and control programs (Aradaib et al., 1994).

1- Subunit core and outer coat proteins:

Recent techniques in cellular immunology have made possible the development of monoclonal antibody (Mab) directed against specific-EHDV epitope. We were able to use this Mab in competitive ELLSA (cELLSA) for detection and specific identification of EHDV-1 and EHDV-2 (Aradaib et al., 1995).

Immunoblotting Technique (Western blotting):

Details of the technique were described elsewhere by Aradaib and Osbum (1995). This technique has been recently introduced for detection of viral protein 2, the viral structural protein of the outer coat (VP2), of Bluetongue virus recombinant vaccine expressed in

baculovirus or vaccinia virus against specific IgG antibodies. The viral proteins are run in polyacrylamide gel and transferred to nylon (Zeta) membranes. The blotted proteins are then incubated with antisera at room temperature and then with labeled antibody at room temperature and finally a substrate is added for visualization of the antigen-antibody reaction at the expected molecular weight of the protein using molecular weight marker. This western blot is highly sensitive and specific. The technique is useful to study the immune response of animals to Orbivirus vaccines as well as correlation between antibody production and protection (Roy et al., 1990).

Serological tecnniques mentioned previously, despite their advantage in detecting infected individuals, are complicated by cross reactions between EHDV serogroups and the other members of the Orbivirus genus. Although the use of monoclonal antibodies (MAb) in competitive ELISA (cELISA) technique has improved sensitivity and specificity at the serotypes level (Aradaib et al., 1994 and Nettles et al., 1991), this technique is applicable only to blood samples (serum) and requires at lest 14 days post infection for the production of anti-EHDV antibodies by the susceptible host (Aradaib et al., 1994).

2- Polyacrylamide Gel Electrophoresis (PAGE):

The polyacrylamide gel electrophoresis (PACE) technique and the staining of the gels with silver nitrate was performed as described previously (Hammami and Osbum, 1992). The nucleic acid analysis of EHDV-318 by PAGE showed 10 distinct dsRNA genome segments, which represent the characteristic pattern of Orbiviruses (Mohammed & Taylor, 1987; Mecham & Dean, 1988; Nel & Huismans, 1990 and Nel et al., 1996). Tentative diagnosis of an EHDV infection could be made using PAGE technique. However, different serotypes of EHDV serogroup may have the same electropherotype and different orbiviruses may have indistinguishable elecropherotypes (Aradaib et al., 2000). Thus, electrophoretic pattern themselves have a definitive diagnostic value for the detection of EHDV infection or even of the serotype of the particular EHDV. In a previous study, the genomes of EHDV-318 (Africa), EHDV-1 and EHDV-2 (north America) serotypes migrated to the same length despite the different electropherotypic patterns of their dsRNA. This finding suggests that it would be interesting to compare the lengths of EHDV electropherotypes from North America or Africa with their Asian and Australian counterparts. The difference in length between electropherotypes as determined by their migration in PAGE, if

any, could then be used as a valuable tool to study the epidemiology of EHDV from different continents (Mohammed and Taylor, 1987).

3- Nucleic Acid Extraction and RNA probes:

This requires extraction of ds RNA from cell culture or clinical samples. Details for total nucleic acid extraction was described previously (Aradaib et al., 1994). The extracted viral RNA is usually run in PAGE. The RNA segments will then be identified according to their electrophoretic migration on the gel. The RNA genome segment of interest will be eluded from the gel labeled with radioactive material and used as a probe. This EHDV probe will specifically hybridized with the corresponding segment of different EHDV serotypes (Huisman et al., 1979 and Wilson et al., 1990). After Northern blot of the RNA these RNA probes could be used to hybridized with the corresponding segment of EHDV serogroup. Because RNA probes are unstable, they are not commonly used in Northen blot hybridization assays.

4- cDNA probes:

Complementary DNA (cDNA) probes are stable and are more commonly used as probes in Northern blot hybridization of RNA. CDNA probes are used in southern blot hybridization. The cDNA probe of a particular segment could be used as a fragment of the segment or as a full-length genome. The cDNA could also be synthesized by cloning in a vector (Wilson et al., 1990). The cloning procedure is tedious laborious and time consuming. Recently PCR-generated probes has been described and used for detection of EHDV serogroup (Mohammed & Mellor, 1990 and Venter et al., 1991). A PCR-generated 224 bp cDNA probe derived from genome segment of EHDV-2 (Alberta strain), repreenting 15% of the full length genome, was used for chemiluminescent hybridization of the blots (Aradaib et al., 1994).

5- Nucleic-Acid Hybridization (NAH) Assay Using cDNA Probes:

For detection of EHDV RNA, dot blot, slot blot and Northern blot hybridization assays were used. However, for detection of cDNA southern blot is the most common procedure. The probe could be labeled with radioactive material such as ^{32}P or I^{125} (De Mattos et al., 2000). The nonradiolabeled procedure of the probe removes the hazardous and cumbersome laboratory procedure of working with radioactive materials. The enzyme labeling procedure of working with non-radiolabeled chemiluminescent hybridization assay has been decribed (Aradaib et al., 1994; Aradaib et al., 1994; Aaradaib & Osbum, 1994 and Aradaib et al., 1997).

Segment 6 and 8 probes of EHDV-2 (Alberta) did not hybridize with BLU dsRNA. A probe derived from genome segment 3 of EHDV-1 cross hybridized with the corresponding segment of EHDV-2 but not with BLU viruses isolated in the United States. The best hybridization signals with all EHDV isolates was obtained using a probe derived from genome segment 6 which codes for NS1. The greater sensitivity of the NS1 gene specific probe is ascribed to the fact that its target, the NS1 mRNA, is transcribed more frequently than other viral target mRNA. In addition, it required small number of infected cells to produce a positive hybridization signal than the other nucleic acid probes (Wilson et al., 1990 and Wison et al., 1992).

The gene coding for viral protein 2 (VP2) has been identified cloned and sequenced (Roy et al., 1992). Nucleic acid hybridization techniques using Complementary DNA (cDNA) probes have been developed for detection of nucleic acid sequence of EHDV genome segments (Mohammed & Taylor, 1987 and Wilson et al., 1990).

Using cDNA probe, the dot blot hybridization technique, showed a positive hybridization signal with dsRNA from EHDV serogroup (Huisman et al., 1979 and Wilson et al.1990). The use of the peroxidase-labeled cDNA probe removes the hazardous and cumbersome laboratory procedure of working with radioactive labeling techniques. The dot blot hybridizaton technique, using PCR-generated cDNA derived from genome segment 6 of EHDV-2 (Alberta strain), showed positive hybrdiation signals with RNA from EHDV-1, EHDV-2 and-318. No hybridization signal was detected from total nucleic acid of the BHK-21 cell control (Aradaib et al., 1996). Because of its safety and rapidity, this technique readily lends itself to use in developing countries, such as the Sudan, for the detection of EHDV in cell culture. However, one of the disadvantages of dot blot hybridization is that the technique is incapable of direct detection of EHDV RNA in clinical samples from infected animals Hence, a prior amplification step by PCR technology deemed necessary.

6- Polymerase Chain Reaction (PCR):

Application of the polymerase chain reaction (PCR) has proliferated because of its simplicity, rapidity, reliability, reproducibility, sensitivity and specificity for monitoring of microorganisms (Aradaib et al., 1994; Aradaib et al., 1994; Aradaib & Osbum, 1994; Aradaib et al., 1995 and Aradaib et al., 1995) and for detection of animal-derived products in processed human food or in commercial animal feed concentrate (Aradaib et al., 1997). PCR proved

satisfactory to replace the immunological and DNA hybridization methods using cDNA probes.

A. Reverse Transcriptase (RT-PCR) For Detection of EHDV Serogroup:

EHDV RT-PCR has been successfully applied for detection of RNA viruses by addition of a complementary DNA (cDNA) synthesis step using reverse transcripatse (RT) enzyme, before cyclic amplification by PCR (Aradaib et al., 1994). This Rt- PCR-based assay for detection of EHDV serogroup in cell culture and clinical samples, using primers derived from NS1 genome sequence analysis of EHDV-2 Alerta strain (Aradaib & Osburn, 1994 and Aradaib et al., 1997).

This EHDV RT-PCR was compared with different virus isolation (VI) procedures from a variety of tissue samples. The results indicated that the sensitivity of the previously described EHDV-PCR assay is comparable or more sensitive than VI method in BHK-21 cell culture or ECE. In addition, the EHDV RT-PCR assay could provide a superior diagnostic alternative to replace the current cumbersome and time-consuming virus isolation procedures (Aradaib et al., 1995). Serogrouping and topotyping of United States and Sudanese isolates of EHDV were also compared using EHDV RT-PCR (Aradaib et al., 1996).

The recently reported EHDV serogroup-specific PCR-based assay using well-characterized serogroup-specific primers derived from segment 6 of EHDV-2, which codes for NS1, reproducibly and specifically detected RNA from EHDV-1 and EHDV-2 in cell culture and a variety of biological specimens (Aradaib, 1994; Aradaib et al., 1997 and Aradaib et al., 1998). The serogroup-specific 387 bp PCR products, visualized on ethidium bromide-stained agarose gel or detected with chemilumiescent hybridization, were obtained from the EHDV RNA sample used in this study. This confirms the ability of the PCR assay to detect new serotypes of EHDV from different continents using the same serogroup primers. The EHDV PCR assay provides an attractive diagnostic alternative to the lengthy and cumbersome conventional virus isolation procedures.

Studies on experimental EHDV infection demonstrated that cattle could amplify the virus, as determined by conventional virus isolation (VI) and PCR technology, and will become seropositive as determined by the serotyp-specific EHDV cHLISA (Aradaib et al., 1994). Using VI and PCR assay, simlar results were obtained during evaluation of EHDV infection in sentinel cattle from the San Joaquin 1

valley of California (Aradaib et al., 2000). Thus, cattle can provide virus for insect transmission to more susceptible wild ruminants, such as the white-tailed deer populations, where a fatal disease and high mortality may occur. Further studies are needed to determine the complete nucleotide sequence of L2 of EHDV-318 using PCR-based detection assay. Widespread application of the molecular biological techniques described in this study should facilitate rapid detection and epidemiological investigation of EHDV outbreaks among susceptible ruminants in the North America and African continents.

B. Reverse Transcriptase (RT-PCR) For Serotype-EHDV:

Specific identification of EHDV serotype 1 (EHDV-1), in cell culture or tissue samples, was desribed using RT-PCR-based assay (Aradiab et al., 1995). Similar study was conducted to demonstrate specific identification of EHDV-2 in cell culture and clinical samples (Aradaib et al., 1995). This RT-PCR was based on nucleotide sequences of genome segment 2, which codes for viral structural protein 2(VP2). This nucleotide sequence of this genome was found to be variable among cognate genes of different serotypes of EHDV serogroup. No cross amplification product or hybridization signals were detected between serotype-specific EHDV-1 and EHDV-2 PCR-based detection assays. In addition, using RNA from EHDV-318, the described serotype-specific (EDDV-1) or (EHDV-2) assays failed to produce the specific PCR products. This result confirms the variability of nucleotide sequences of genome segment 2 (L2) among cognates of different EHDV serotypes (Mohammed et al., 1996).

C. Nested Polymerase Chain Reaction (nPCR):

In the nested PCR, two pairs of oligonucleotides primers are required. The PCR reaction is carried out in 2 amplification steps. The first pair of primers (outer primers) is used to amplify specific PCR product. This second pair of nested primers (internal primers) is designed internal to the annealing sites of the outer primers to amplify specific PCR product, which is shorter than that produced by the first pair of primers.

The second amplification step using the nested primers is necessary to confirm the specificity of the first amplified product and to increase the sensitivity of the PCR-based assay.

The use of nested PCR removes the hazardous and cumbersome radioactive laboratory procedures of working with ^{32}P or ^{33}P (Aradaib et al., 1994; Aradaib et al., 1994 and Aradaib et al., 1998). Details for the nested PCR protecol was previously described (Aradaib et al., 1998).

D. Multiplex Polymerase Chain Reaction (Mpcr):

In this type of PCR different primer pairs derived from different EHDV serotypes are used in a single-tube PCR amplification. The multiplex PCR-based assay, using primers derived from NS3 genome of BTV-10 and NS1 genome of EHDV-2, reproducibly and specifically detected BTV and EHDV RNAs in infected cell cultures. The multiplex PCR assay was a simple procedure that utilized a single amplification in which EHDV and BIV primers were used simultaneously.

The level of sensitivity could be increased using a more sensitive chemiluminescent hybridization techniques (Aradaib et al., 1994; Aradaib et al., 1994; Aradaib et al., 1998; Aradaib et al., 1998 and Aradaib, 1999).

Simultaneous detection and differentiation of orbivirus serogroups will simplify the assay, save time and above all save on cost, because each clinical sample will be tested once instead of individual testing each clinical sample will be tested once instead of individual testing each specific viral nucleic acids. Because of its rapidity, sensitivity and specificity, the multiplex PCR assay would be advantageous in epidemiological investigations where field isolates can be tested to determine the prevalence and frequency of orbivirus infections in susceptible animal populations, and can also be used for evidence of viral incursion in a particular geographical region.

The multiplex PCR assay could be used for export regulation to certify animals free of Orbivirus serogroup should be carried out in separate rooms. Aerosol resistant tips should be used to minimize contamination. Negative and positive controls should be included in each PCR amplification to estimate the lower limit of specificity and the higher limit of sensitivity.

CONCLUSION

Nucleic acid amplification technology, using PCR or RT-PCR, will serve as the bases for future diagnostic investigation PCR-based detection assay is an extremely sensitive procedure and hence PCR-positive and virus isolation-negative results from the same clinical sample is not suprising. This is because PCR positive results could be obtained from clinical samples containing incomplete virons or viral nucleic acid as well as infectious virus. Therefore, the biological significance of a PCR-positive result has to be interpreted with caution in light of presence of viral nucleic acid and absence of infectious virus.

Cross contamination in PCR reactions is also a problem. In experimental BLU virus infection of calves, PCR-positive and virus isolation-negative blood samples were not infectious either to the insect vector or to calves suggesting that only intact infectious viral particles may play a role in the infection and epidemiology of bluetongue (Aradaib et al., 199). Additional reearch is necessary to confirm this assumption with other orbiviruses including EHDV.

The PCR-based detection assays, in spite of the problems associated with their use, will continue to be important diagnostic techniques for detection and differentiation of orbivirus infections during disease outbreaks, where at least 2-4 weeks are required for isolation and identification of the virus in a susceptible cell line.

A definitive diagnosis of infected clinical sample using the EHDV PCR-based detection assay with chemiluninescent hybridization could be obtained within 2-3 days (Aradaib et al., 1994a) and within the same working day using a nested PCR-based assay (reference). It is worth mentioning that conventional virus isolation procedures will remain important for recovery of an infectious virus and for understanding the biology, genetic diversity and the epidemiology of their disease.

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