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for Diagnosis of
Foot and Mouth Disease virus (FMDV)

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3. Executive summary

Foot and mouth disease (FMDV) appears in different parts of the world and only a very careful vaccination program and constant surveillance for the virus can help in preventing epidemics. Since Israel is in an endemic region for FMD, development of molecular diagnostic techniques which will allow rapid and accurate diagnosis of the virus is essential for the protection against FMDV epidemics. The present research project was undertaken as a collaboration between the molecular virology team at the HU and two veterinary institutions: Dr. H. Yadin from the Kimron Veterinary Institute (KVI) in Israel and Dr. T.G. Nu from the Laboratorio Nacional De Investigacao Veterinaria in Lisbon, Portugal. The aims were to develop the molecular diagnostic technologies in collaboration with a FMDV research team working in a security laboratory (KVI). Since the European Economic Community (EEC) had decided to stop the FMDV vaccination programs, the participation of the Portugese researcher was designed to acquaint Dr. Nu with the new diagnaostic technologies and to transfer these technologies to the Lisbon laboratory. These aspects of the collaboration were successfully accomplished. The KVI laboratory is routinely using the polymerase chain reaction technique for FMDV diagnosis and Dr. Nu had studied the techniques during her two visits to HU and KVI. Moreover, two Egyptian scientists who participated in the binational (US-Egypt-Israel) A.I.D. program on FMD, were recently trained in the PCR technique of FMDV diagnosis by the HU and KVI teams.

The scientific approach was to clone cDNA of FMDV RNA from two Israeli isolates (01 Geshur and Asia 1) in plasmids to determine the nucleotide sequences of the VP1 gene of these viruses. This was accomplished by a collaboration with Dr. D. Moore at Plum Island FMDV laboratory. The plasmids were brought to the HU laboratory and was used in the studies. The development of molecular DNA and RNA probes to detect viral genomes in infected cultured cells using Southern and Northern blot techniques led to rapid detection of FMDV genomic RNA. Further studies on the application of the polymerase chain reaction (PCR) technique to FMDV diagnosis led to the development of a rapid diagnostic test which detects FMDV RNA in clinical material from suspected animals in the field. Another aspect of the research was the study on skin Langerhans cells (LC) and their role as antigen presenting cells in immunization against FMDV. It was found that certain FMDV vaccines damaged the skin of the immunized animals and the immune response to FMDV was less effective. We found that saponin (which is present in FMDV

vaccines) damaged skin LC and thus reduced antigen presentation and damaged the immune response. In summary, the research program had led to an active cooperation between molecular virologists and veterinarians in achieving new technologies for FMDV diagnosis and immunization which are available to other participants of the A.I.D. programs.

4. Research objectives

The main objective of the project was to develop molecular techniques to detect FMDV RNA in clinical materials to allow rapid and accurate diagnosis of the serotype of the field FMDV isolate. The second aim was to study the properties of skin Langerhans cells and their role as antigen presenting cells in the immunization of cattle with FMDV vaccines. The second aim was to study the properties of skin Langerhans cells and their role as antigen presenting cells in the immunization of cattle with FMDV vaccines. The third aim was to develop a herpesvirus recombinant carrying the FMDV VPI gene of Israeli isolates. The fourth aim was to train scientists from KVI in Israel and NLVR in Lisbon in the utilization of the molecular diagnostic techniques.

5. Methods and results.

The molecular approach to FMDV diagnosis was to a) use cloned FMDV VP1 gene for insertion in a transcription vector pGEM to synthesize riboprobes for the detection of positive and negative FMDV RNAs in infected cells. b) develop the polymerase chain reaction (PCR) on cloned FMDV VP1 gene, c) develop PCR tests on clinical materials from suspected animals in the field and d) cloning of cDNA from Israeli FMDV isolates into plasmids.

The FMDV riboprobes were found to be effective in the detection, by the Northern hybridization technique, of RNA genomes in cultured cells injected with Israeli FMDV isolates. However, the method requires relatively large amounts of viral RNA that can be obtained only after isolation of the wild type FMDV in cultured cells. This method requires several in-vitro passages, which lead to the accumulation of mutants in the FMDV isolate and to a result that does not allow detecting of the original mutations in the viral VP1 gene. Therefore, emphasis was put on the development of a PCR test which will allow direct detection of low quantities of FMDV RNA in clinical materials prior to virus isolation and propagation in cell cultures. The PCR test was developed and successfully applied to the detection of FMDV in clinical materials. The PCR method made possible the detection of FMDV RNA in a small number ($\sim 10^4$) skin Langerhans infected with FMDV under in-vitro conditions.

The role of skin LC in the immune response to FMDV vaccines. It was shown that saponin which was included in one of the commercial FMDV vaccines had damaged skin LC when injected into mouse skin. Since LC are potent antigen presenting cells, damage to these cells lead to a decline in the immune response.

Studies on Bovine Herpes virus-1 (BHV-1) were taken to investigate the feasibility of cloning and expressing FMDV VP1 gene cloned in BHV-1 DNA were initiated. It will be necessary to

eliminate the BHV-1 pathogenicity genes to reduce the virus pathogenicity in order to develop an apathogenic BHV-1 genome into which the FMDV VP1 gene will be cloned under the control of an immediate early promoter. To do so the VP1 genes of two Israeli FMDV isolates were cloned. The development of a BHV-1/FMDV VP1 recombinant virus is feasible, and will continue in the future.

The last objective, cooperation between laboratories and transfer of the technology was accomplished. The PCR diagnostic test is operational at KVI and available at the Laboratorio Nacional De Investigacao Veterinaria.

6. Impact, relevance and technology transfer.

The project had an impact not only on the Israel and Portugal Veterinary Institutes but on the Veterinary Institute in Cairo, Egypt. We were able to train two verinarians in the molecular approaches to rapid FMDV diagnosis. The two veterinarians studied the molecular technologies at HU and KVI and left with the detailed methodology og the PCR technology as well as reagents needed for PCR diagnosis of FMDV. The PCR method will be published in the scientific literature and will be available to all the laboratories around the world that deal with FMDV diagnosis.

7. Project activities.

The communication between the HU and KVI teams was very efficient. Numerous meetings of the two teams took place during the years. Prof. Becker visited Lisbon twice, at the start of the project and the end of the second year. Dr. Nu came twice to work with the HU adn KVI teams. The report on the second visit is attached. Prof. Becker visited Dr. D. Moore in Plum Island to discuss the cloning of FMDV cDNA. Five publications resulted from the research project.

8. Project productivity.

The major achievement of the project is the development of riboprobes to detect positive and negative viral RNA strands as well as the development of an effective PCR diagnostic test for FMDV. The need to train veterinarians in molecular virology caused a slow start of the project but time was eventually regained by using the sophisticated molecular technologies.

9. Future work.

Since FMDV threat in endemic areas requires not only a rapid diagnostic test but effective vaccines, the research at HU and KVI will continue and will be directed at a) improvements of the PCR diagnostic test to identify mutations in the VP1 gene b) studies on antigen presentation by skin Langerhans cells and how to augment their antigen presenting capacity to achieve a rapid immune response to FMDV antigens c) use of synthetic peptides according to FMDV VP1 antigenic domain for augmentation of the immune response and d) development of a live herpesvirus (BHV-1) apathogenic vaccine as a carrier of FMDV VP1 genes.

10. Publications resulting from the research program.

1. Sprecher E, DAvid D, Yadin H, Peleg B-A and Becker Y. Mouse footpad Langerhans cells as an indicator for sagety of foot and mouth disease virus vaccines. J Virol Meth 29, 189-196, 1990.
2. Laor O, Torgersen H, Yadin H and Becker Y. Detection of FMDV RNA amplified by the polymerase chain reaction (PCR). J Virol Meth, in press, 1992. (attached)
3. Laor O, Yadin H, Chai D and Becker Y. Detection of FMDV RNA in clinical material using the PCR reaction on viral genomic polyA-RNA isolated with oligo-dT on magnetic beads. Israel J Vet Med, December 1991 (attached).
4. Laor O, David D, Yadin H and Becker Y. PCR detection of Foot and Mouth Disease virus DNA isolated by oligo-dT magnetic beads from in vitro infected BHK and Bovine skin Langerhans cells. Israel J Vet Med, in press, 1992. (attached)
5. David D, Yadin H and Becker Y. Isolation of bovine skin Langerhans cells and maintenance of their viability in-vitro. Israel J vet Med, in press, 1992. (attached)

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Detection of FMDV RNA Amplified by the Polymerase Chain Reaction (PCR)

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SUMMARY

Molecular detection of Foot and Mouth Disease Virus (FMDV) using the Polymerase Chain Reaction (PCR) is a rapid and accurate method. In this study we present the PCR for the detection of FMDV RNA in infected BHK cells. Using the PCR and two primers selected from the RNA polymerase gene, a conserved sequence in all types and subtypes of FMDV, we were able to detect FMDV RNA present in RNA extracted from the FMDV-infected cells. RNA from uninfected BHK cells gave negative results. Another set of primers selected from the nucleotide sequence of the variable VP1 gene enabled us to show variations reaction among different FMDV Israeli isolates in the PCR. Two O1 type FMDV isolates out of a total of 6 FMDV field isolates (including O1 Geshur) gave a positive PCR while two other O1 isolates and two ASIA isolates were detected with the RNA polymerase gene primers but not using the VP1 primers. Serial dilutions of the RNA used in each reaction showed that a very small amount of RNA may be detected by PCR. The PCR products from the RNA polymerase and the VP1 genes were sequenced and the nucleotide sequences obtained were compared with a known nucleotide sequence of the FMDV O1 genome.

INTRODUCTION

Foot and Mouth Disease (FMD) has a worldwide distribution and is of cardinal importance for agriculture. Although the death rates of FMDV - infected animals may be low, losses due to reduction in milk and meat production should not be underestimated. In addition, FMDV zootics require extensive vaccination of livestock and result in reduced export. Due to the rapid spread of FMDV and rapid mutation rate leading to a multiplicity of known serotypes and subtypes, (Cheung et al., 1983), confusion of the clinical picture of FMD with other viral diseases is inevitable. The possibility of a carrier state

among infected animals (Wittmann, 1990) makes diagnosis, as well as control, quite difficult. The adoption of rapid and accurate diagnostic procedures to detect and identify FMDV in carrier animals is essential to an effective FMD control program.

The current availability of the Polymerase Chain Reaction (PCR) (Saiki et al., 1988) allowed the development of a rapid and accurate method to identify FMDV RNA in RNA from infected cells.

MATERIALS AND METHODS

Amplification of FMDV VP1 sequence by PCR

Total RNA from the Israeli FMDV isolate O1 Geshur (O1G) was used as a template for PCR amplification. The virus was grown in BHK cell culture and total RNA was isolated by the acid guanidinium thiocyanate method (Ghirgwin, et al, 1979). As a positive control we used a recombinant plasmid with an insert of 1034 bases from the VP1 area of the FMDV Type O1 Kaufbeurene (O1K) (Kurz et al., 1984). The insert, cloned in pBR 322, was provided by Prof. H. Schaller (Heidelberg University) and was transferred to pGEM 52 in our laboratory. In vitro transcription of the recombinant O1K 1034 in pGEM 52 was carried out in order to obtain virus-specific RNA as a positive control. The plasmid was cut with Pst1 (Boehringer Mannheim) and a Stratagene RNA transcription Kit was used.

Primers were selected from the VP1 sequence of FMDV O1K (Kunz et al, 1984).

The direct primer: AGTTCGACCTAACGTCGGA (Oligo # 2), a 19-mer oligonucleotide, is located in the P12 area adjacent to the 3' end of the VP1 (supplied at concentration of 100 pmol/µl).

The reverse primer: CAACTGCTTACCACAAAAGG (Oligo # 1), a 20-mer oligonucleotide, is located 424 bases upstream from the direct primer,

within the VP1 area (supplied at a concentration of 100pml/µl).

The direct primer (Oligo # 2) was also used as the reverse transcription primer.

1 µl of total RNA of 01G (at a concentration of 3.5 µg/µl) and

1 µl of the in vitro-transcribed (1034 01K) RNA was reverse transcribed to obtain cDNA for PCR.

The reaction volume was adjusted to 11 µl with ddH20 and heated for 5 minutes at 70°C.

To each sample the following were added:

1 µl RNasin (40 U/µl - Promega)

1 µl dNTPs (10 mM Pharmacia)

1 µl AMV Reverse transcriptase (16.3 U/µl, Life Science Inc.)

2 µl 10X buffer (according to the manufacturer's recommendation)

4 µl ddH_2O

The total reaction volume of 20 µl was heated for 1 h at 42°C.

dNTPs were supplied at 100 mM each and were mixed to obtain a final concentration of 10 mM each.

10 µl of each cDNA obtained was directly forwarded to PCR.

RNA samples used for the PCR:

1. 01G total RNA at a concentration of 3.5 µg/µl was first reverse transcribed (as described above) and used for the PCR.
2. RNA transcribed from the recombinant plasmid 1034 01K was reverse transcribed and used for PCR.
3. DNA recombinant plasmid 1034 01K (concentration 1 ng/µl) was used as a positive control for the PCR. 1 µl was taken for the reaction and the volume was brought to 10 µl with of dd H_2O.
4. Negative control (no RNA or DNA in the reaction) 10 µl dd H_2O was used.

For samples 1 and 2, where 10 µl of the reverse transcribed reaction were directly followed by PCR, the following reagents were added to

bring the reaction volume to 50 µl.

1 µl oligo 2

1 µl oligo 1

1 µl 2M KCl

1 µl Taq DNA polymerase (4 U/µl - IBI)

36 µl dd H_2O

In samples 3 and 4 the 50 µl reaction volume included:

10 µl DNA or dd H_2O

1 µl oligo 2

1 µl oligo 1

1 µl dNTPs 10 mM (Farmacia)

5 µl 10X amplification buffer (supplied with the enzyme)

1 µl Taq DNA polymerase (4 U/µl)

31 µl dd H_2O

Two drops of mineral oil were placed on top of each reaction to prevent evaporation.

All 4 samples were placed in a HYBAID Thermal Reactor for 35 cycles.

Denaturation at 93°C for 30 seconds

Annealing at 55°C for 30 seconds

Polymerization at 72°C for 1 minute

The third step was extended to 10 minutes at the end of the reaction.

10 µl of each sample were electrophoresed on a 1.6% agarose gel.

Amplification of 01 Geshur VP1 with modified primers

For this experiment a new primer was selected.

Oligo # 5 ; 5' TTCGAAACGGCACGGTCGGA 3'based on the known sequence of 01G. Oligo 5 replaced oligo 1.

The primer used to reverse transcribe the 01G RNA was oligo 2 as described above.

1 µl of the cDNA obtained was used for PCR using oligo 2 and oligo 5

as primers. 50 µl reaction volume contained 1µl cDNA, 100 pmol of each primer, 10 mM Tris HCl PH 8.3, 50 mM KCl, 1.5 mM MgCl2,dNTPs (Pharmacia)10 mM each and 4 units Taq DNA Polymerase (IBI)

Samples were placed in a Programmable Thermal Controller (MJ Research Inc) for 35 cycles composed of :

Denaturation at 93°C, 30 seconds

Annealing at 55°C, 30 seconds

Polymerization at 72°C 1 minute

The last step was extended to 10 minutes at the end of the reaction.

10 µl of each sample were electrophoresed on a 1.6% agarose gel.

(data not shown)

Amplification of VP1 sequence of six FMDV Israeli isolates

The same primers (oligo 2 and oligo 5) were used to test the variability in the VP1 sequence of six Israeli isolates : O1 GESHUR, O1 DALTON, O1 GAZIT, O1 SHCHEM, ASIA 1 and ASIA subtype SHAMIR. O1 GESHUR isolate was amplified also with the primers oligo 2 and oligo 1. All these FMDV isolates were grown in BHK cells and RNA was extracted by the Acid Guanidinium Thiocyanate method. PCR was carried out as above.

Amplification of FMDV RNA polymerase sequence in the RNA of six Israeli isolates.

The RNA polymerase gene is a conserved nucleotide sequence present in all reported FMDV genomes. Using the computer program of the University of Wisconsin Genetic Computer Group (UWGCG) (Devereux et al., 1984), we compared the published FMDV genomes of O1K (Forss et al.,1984), A12 (Robertson et al.,1985) and A10 (Boothroyd et al.,1981) and related primers that can be used to detect all FMD types and subtypes.

The oligonucleotides selected were:

Direct primer oligo # 7 : 5' GTAAAGTGATCTGTAGCTTGG 3'

Reverse primer oligo # 9 : 5'TTCGAGAACGGCACGGTCGGA 3'

Both primers amplify a 1 Kb segment within the RNA polymerase gene.

1 µl RNA of each of the six FMDV subtypes was heated with 100 pmol primer oligo 7 at 70°C for 5 minutes in 11 µl reaction volume.

The annealed RNA/primers were reverse transcribed with AMV reverse transcriptase as described above.

1 µl of the cDNA obtained was amplified by PCR as described above.

10 µl of the amplified sample were electrophoresed on 1.6% agarose gel.

Serial dilutions of FMDV RNA

Three dilutions, 1:10, 1:100 and 1:1000, were made from the total RNA extracted from BHK cells infected with FMDV isolate O1G. cDNA was synthesized using oligo 7 as primer from 1µl of each of the dilution.

1µl of each of the cDNA obtained was forwarded to PCR and 1Kb segment within the RNA polymerase gene was amplified using the primers oligo 7 and oligo 9 as described.

Purification and sequencing of the PCR products

The amplified segments of the VP1 gene (424 bases) and the RNA polymerase gene (1 Kb) were electrophoresed on a low gelling temperature agarose gel. Segments of appropriate size were removed and cDNA was extracted from the gel as described by Wieslander,(1979).

The purified segments were sequenced by the dideoxy-mediated chain termination method (Sanger et. al., 1977) using the "PUC sequencing kit" for DNA sequencing (Boehringer Mannheim).

Computer program

The "bestfit" computer program was used (Devereux et al., 1984).

RESULTS

The nucleotide sequence of FMDV RNA polymerase gene was chosen for amplification because it is conserved in all FMDV types. Comparison of the published nucleotide sequences of several FMDV isolates using the "Best fit" program of the University of Wisconsin Genetic Computer Group (UWGCG) software (Devereux et al., 1984) showed a marked homology among the sequences of all three FMDV types. We selected two oligonucleotide sequences limiting a 1 Kb segment,(designated oligos 7 and 9) to amplify RNA from cells infected with six Israeli isolates. The results of successful amplification by PCR using oligos 7 and 9 as primers are shown in Fig. 1.

PCR using the VP1 primers (oligos 1 and 2) is shown in Fig. 2. The oligonucleotides were selected according to the reported sequences of O1K isolate (Kurz et al.,1984)

The in vitro-transcribed RNA from the chosen VP1 gene of O1K and O1 Geshur RNA as well as cloned VP1 DNA present in the recombinant plasmid 1034 O1K (containing 1034 nucleotides) were amplified.

Although not measurable, a lower yield of amplified product, based on the amplified cDNA content in the bands, was observed in O1G VP1 sequence compared with the in vitro-transcribed O1K RNA.

Examination of data provided on the VP1 sequence of O1G (Pfaff et Al, 1988) revealed a difference, of two nucleotides relative to O1K, in the nucleotide sequence used for the synthesis of oligo 1 primer.

A new primer was synthesized based on the O1G nucleotide sequence (designated oligo 5) and O1G RNA was successfully amplified using oligos 2 and 5 as primers (data not shown).

PCR was carried out with total RNA purified from BHK cells infected with each of the six different Israeli FMDV isolates. Four isolates were identified serologically as FMDV type O1, and two others as FMDV ASIA. In this reaction the primers used were oligos 2 and 5. O1G

isolate was amplified also with the primers oligos 2 and 1.

A strongly positive result was obtained with O1 Geshur RNA, O1 Gazit was slightly positive and all the other samples, including O1G amplified with oligo 2 and oligo 1, were negative.(Fig. 3)

Serological data indicated a marked similarity between O1 Geshur and O1 Gazit compared to other O1 Israeli isolates. Molecular analysis (Samuel et al., 1990) showed that O1 Geshur and O1 Dalton were not closely related.

Serial dilutions, (1:10, 1:100 and 1:1000), of total RNA extracted from BHK cells infected with O1G isolate were performed. Undiluted RNA and 1:10 and 1:100 dilutions were successfully amplified with the PCR with respectively diminishing intensity. The 1:1000 dilution was negative (Fig. 4). This results suggests that PCR is at least 100 fold more sensitive than the Northern blot technique (unpublished).

The PCR amplified segments of the VP1 and the RNA polymerase genes were purified on low geling temperature agarose gel and directly sequenced. A segment of 177 bases was obtained from the RNA polymerase gene and of 159 bases from the amplified VP1 area. The sequences were compared to those of FMDV type O1K (Foss et.al., 1984) using the "bestfit" program in the UWGCG software (Devereux et. al., 1984). Homology of over 97% , was observed between the RNA polymerase sequences compared (Fig. 5 A). A lower percentage of homology yet relativly high, of more than 86%, was seen between the amplified O1G VP1 sequence and the O1K VP1 sequence (Fig. 5 B).

This result leave no doubt that the PCR amplified segments are sequences of the FMDV genome-specific.

DISCUSSION

By using PCR we have developed a rapid and accurate method to detect FMDV positive sense RNA extracted from cells infected with Israeli FMDVs and were able to detect FMDV RNA even when present in very small amounts.As shown in Fig. 4 it was possible to dilute the viral RNA preparation 100 folds and to obtein a positive detection. The PCR method is at least 100 times more sensitive than the northern blot technique. For the detection of FMDV RNA in infected cells, it was necessary to use conserved nucleotide sequences in all known FMDV types and subtypes. Using the "Best fit" program in the UWGCG software, it was possible to select two primers,designated oligo 7 and oligo 9. All six Israeli FMDV isolates propagated in BHK cells and their RNA examined by PCR using the primers from the RNA polymerase sequence were positive. However not all the Israeli FMDV O1 subtypes were detectable using the primers based on O1 VP1 sequence suggesting the presence of mutations in the VP1 nucleotide sequences of some Israeli isolates.

The use of VP1 sequence primers (oligos 2 and 5), which were synthesized according to the sequence of O1G (Pfaff et Al.,1988), indicated that differences exist in FMDV isolates in the nucleotide sequence of the VP1 gene that codes for antigenic domains in the virion capsid. Of the four FMDV isolates that were serologically identified as FMDV type O1, two were positive in PCR, though at different levels of cDNA synthesis. No amplification of the other two FMDV isolates was found by PCR. These results showed that the VP1 sequences of two isolates, O1G and O1 Gazit, resembled each other, while the other two did not. The remaining isolates not detected by PCR with the VP1 primers were serologically identified as subtype ASIA 1. These results suggest that primers oligos 1, 2 and 5 are useful in the detection of O1 FMDV isolates. Direct sequencing of the VP1 gene

of all the known Israeli isolates including the VP1 of ASIA isolates will allow the selection of specific amplimers from the VP1 gene coding for the antigenic loop in VP1 protein.The primers from the RNA polymerase sequence will serve to detect whether FMDV RNA is present in the suspected pathological material. The FMDV-specific VP1 primers will enable the rapid diagnosis of the type and subtype of the FMDV isolate prior to sequencing of the PCR cDNA product. Direct determination of the nucleotide sequence from the VP1 gene will provide information on the structure of the antigenic domain in the VP1 polypeptide using computer program (Becker, 1987; Becker, in preparation). The PCR was successfully applied as a diagnostic technique in suspected clinical material from cattle and sheep in the field. (Laor et. al.,1992 in press).

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LEGEND TO FIGURES:

Fig. 1 PCR of FMDV RNA using primers from the RNA polymerase gene.

1. 01 Geshur - 1Kb segment amplified from the polymerase area using oligos 7 and 9 as primers.
2. 01 Dalton
3. 01 Gazit
4. 01 Shchem
5. ASIA 1
6. ASIA Shamir
7. Negative control (no RNA or DNA in the reaction)
8. DNA molecular weight size Marker No. VI (Boehringer Mannheim)

Fig. 2 PCR analysis of FMDV using the amplimers oligos 1 and 2 within and near the VP1 gene sequence.

1. In vitro-transcribed RNA 1034 01K - as positive control for the reverse transcription reaction.
2. Total RNA 01 Geshur
3. DNA molecular weight size marker No. VI (Boehringer Mannheim)
4. DNA recombinant plasmid 1034 01K - as positive control for the PCR reaction.
5. Negative control - no DNA or RNA in the system.

Fig. 3 PCR analysis of local 01 and ASIA FMDV isolates using the amplimers for the VP1 gene sequence.

1. 01 Geshur - Positive result with the primer oligos 2 and 5 for VP1 area.
2. 01 Geshur - negative result with the primers oligos 2 and 1 for VP1 area.
3. 01 Dalton - negative result using oligos 2 and 5 as primers.
4. ASIA Shamir - negative result using oligo 2 and oligo 5 as primers.
5. 01 Gazit - Slight positive result with oligos 2 and 5 as primers.
6. 01 Schem - negative result with oligos 2 and 5 as primers.
7. ASIA 1 - negative result using oligos 2 and 5 as primers.
8. DNA molecular weight size marker No. VI (Boehringer Mannheim)
9. Negative control - no DNA or RNA in the system.

Fig. 4 Serial dilutions of total RNA extracted from BHK cells infected with 01G isolate, amplified with PCR using the primers oligos 7 and 9, within the RNA Polymerase gene.

1. Undiluted 01G total RNA - 1Kb segment amplified.
2. 1:10 dilution
3. 1:100 dilution
4. 1:1000 dilution
5. Negative control (no RNA or DNa in the reaction)
6. DNA molecular weight size marker No. VI (Boehringer Mannheim)

Fig.5 Comparison of the amplified RNA polymerase and the VP1 sequences of FMDV type 01G to those of FMDV type 01K, using the "bestfit" program of the UWGCG software.

A. Comparison of RNA polymerase gene of FMDV type 01K to a 177 base sequence of PCR amplified DNA from the RNA polymerase gene of FMDV type 01G.
B. Comparison of VP1 sequence of FMDV type 01K to a 159 base sequence of PCR amplified DNA from the VP1 of FMDV type 01G using primers oligos 2 and 5.

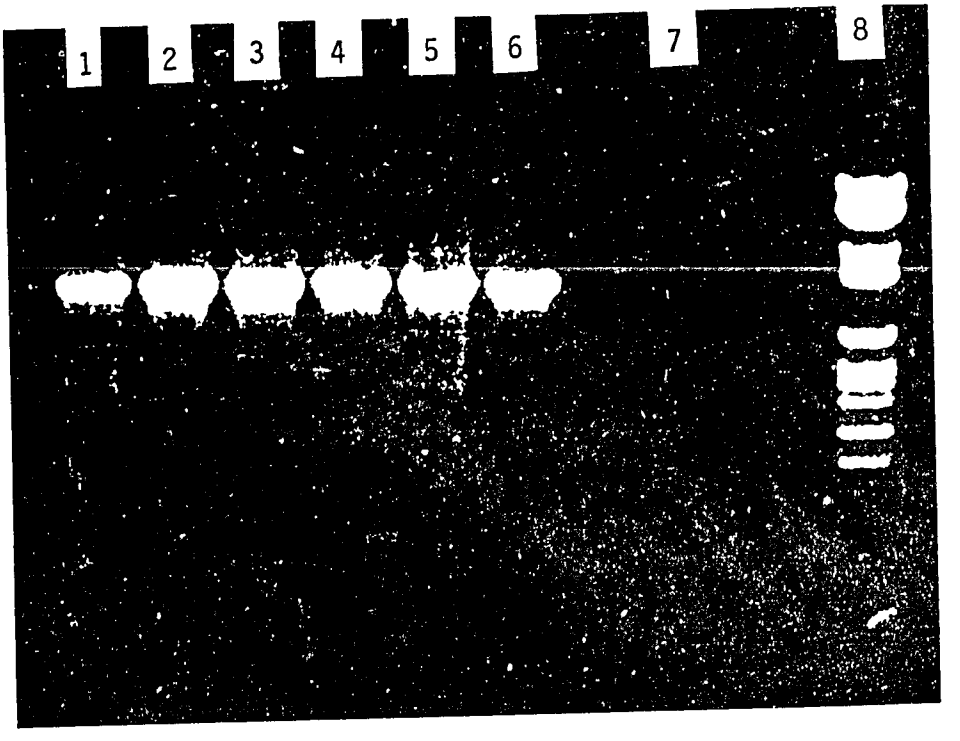


Fig. 1

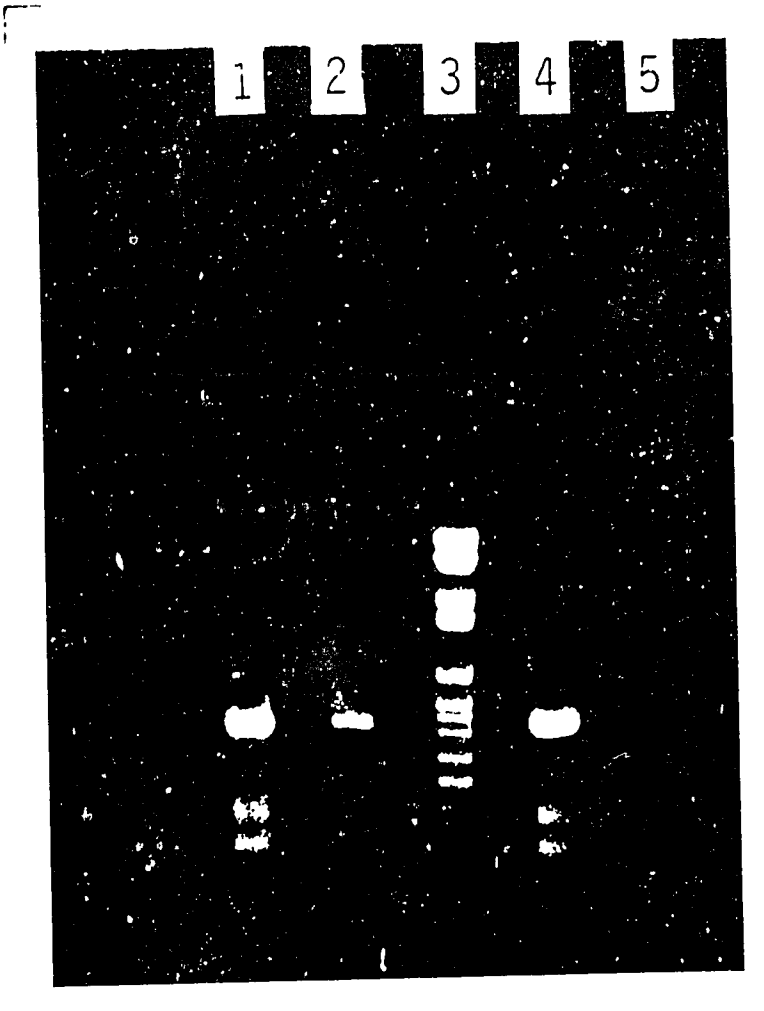
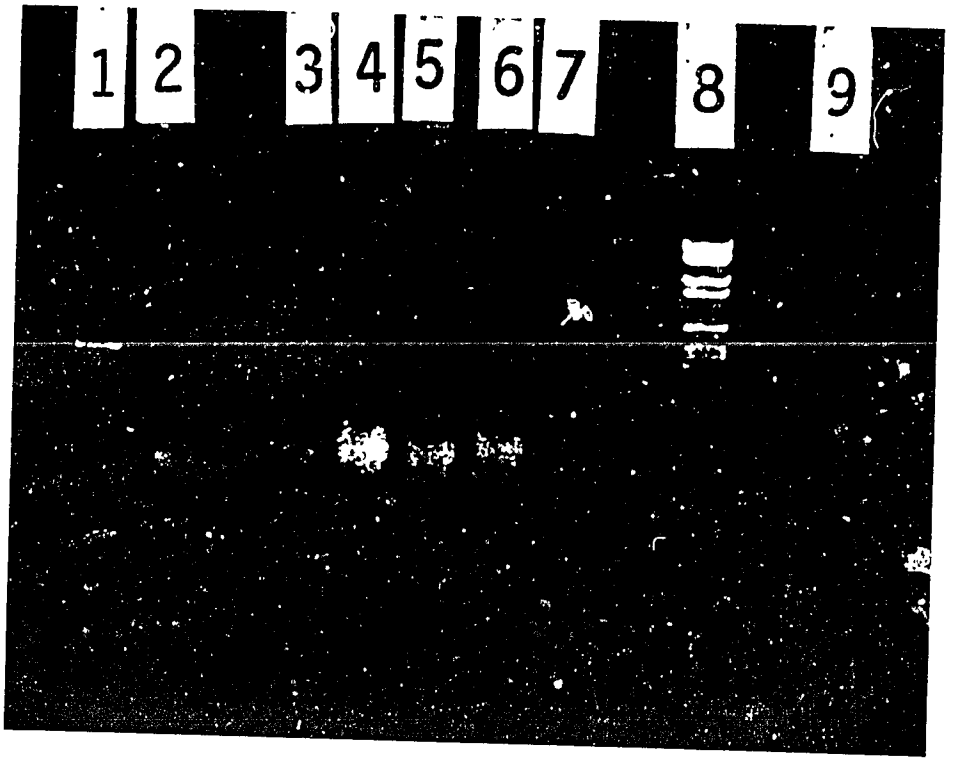


Fig. 2



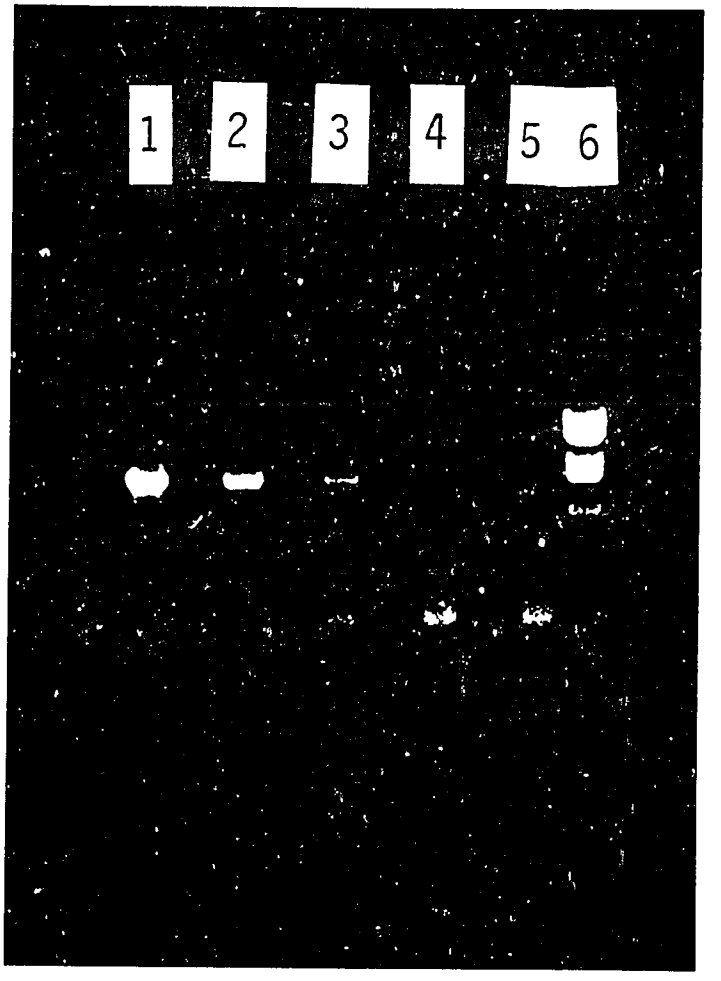


Fig. 4

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                 .         .         .         .         .
A 7408 TCCAGCTGACAAAAGCGACAAAGGTTTTGTTCTTGGTCACTCCATTACCG 7457
       ||||||||||||||||||||||||||||||||||||||||||||||||||
     1 TCCAGCTGACAAAAGCGACAAAGGTTTTGTTCTTGGTCACTCCATTACCG 50
                 .         .         .         .         .
  7458 ATGTCACT.TTCCTCAAAAGGCACTTCCACATGGACTATGGAACTGGGTT 7506
       |||||||| ||| ||||||| ||||||||||||||||||||||||||||
    51 ATGTCACTGTTCATCAAAAGACACTTCCACATGGACTATGGAACTGGGTT 100
                 .         .         .         .         .
  7507 TTACAAACCTGTGATGGCCTCAAAGACCCTTGAGG.CTATCCTCTCCTTT 7555
       |||||||||||||  |  ||| ||||||||||||| |||||||||||||
   101 TTACAAACCTGTG..GCTCTCGAAGACCCTTGAGGCCTATCCTCTCCTTT 148
                 .         .
  7556 GCACGCCGTGGGACCATACAGGAGAAGTT 7584
       |||||||||||||||||||||||||||||
   149 GCACGCCGTGGGACCATACAGGAGAAGTT 177
```

Gap Weight:	5.000	Average Match:	1.000
Length Weight:	0.300	Average Mismatch:	-0.900
Quality:	149.3	Length:	179
Ratio:	0.844	Gaps:	3
Percent Similarity:	97.143	Percent Identity:	97.143

```
                 .         .         .         .         .
B 3372 CGCTGCCTACCTCCTTCAACTACGGTGCCATCAAAGCGACCCGGGTCACC 3421
       ||||||| |||||||||||||||||||||||||||| || | ||| |||
     1 CGCTGCCCACCTCCTTCAACTACGGTGCCATCAAAGCTACTC.GGTGACC 49
                 .         .         .         .         .
  3422 GAGTTGCTTTACCGGATGAAGAGGGCCGAAACATACTGTCCAAGGCCCTT 3471
       ||| |||||||||| |||||||||||| || |||||||||||| | |||  |
    50 GAGCTGCTTTACCGCATGAAGAGGGCTGAGACATACTGTCCGACGCCTCT 99
                 .         .         .         .         .
  3472 GCTGGCAATCCACCCAACTGAAGCCAGACACAAACAGAAAATTGTGGCAC 3521
         |||| || ||||||| |||||| |||||||| |||||||| |||||||
   100 ATTGGCCATTCACCCAAGTGAAGCTAGACACAAGCAGAAAATAGTGGCAC 149
                 .
  3522 CGGTGAAACA 3531
       | ||||||||
   150 CTGTGAAACA 159
```

Gap Weight:	5.000	Average Match:	1.000
Length Weight:	0.300	Average Mismatch:	-0.900
Quality:	113.8	Length:	160
Ratio:	0.716	Gaps:	1
Percent Similarity:	86.792	Percent Identity:	86.792

Fig. 5

Israel J. Vet. Med. 1991

Detection of FMDV RNA in clinical material using the PCR reaction on viral genomic poly-A RNA isolated with oligo dT on magnetic beads

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Short title: PCR detection of FMDV RNA in clinical material

Key words: Foot and Mouth Disease virus (FMDV); Polymerase chain reaction PCR); FMD clinical material; Detection of FMDV RNA by PCR.

SUMMARY

Five samples from animals suspected for Foot and Mouth Disease (FMD) were received for FMDV diagnosis. In addition to the routine serological tests, injection into baby mice, inoculation of pig kidney (PK) cell culture and complement fixation test, the molecular diagnostic tool, the polymerase chain reaction (PCR) was used for the rapid detection of FMDV genomic RNA in the clinical sample. The RNA was extracted from the clinical material (tongue epithelium or skin epithelium) using DYNABEADS oligo (dT)25 which selectivly remove the polyadenylated RNA. The poly-A RNA thus extracted was released from the beads and was used in the PCR reaction after cDNA synthesis using primers selected to amplify a 1 Kb size segment present in the conserved gene of the viral RNA Polymerase. Another set of amplimers localized in the VP1 sequence, designed according to the known nucleotide sequence of the Israeli FMDV isolate O1 Geshur was synthesized and used to synthesize a 424 base pairs segment.

The presence of FMDV RNA in the clinical samples and its relation to the O1 type was determined in four samples in accordance with the positive results by all other tests. The fifth clinical sample which gave negative PCR results was negative by virus isolation and Complement Fixation tests. The FMDV RNA isolation technique coupled with the PCR technique is rapid and accurate and can be used for detection of FMDV RNA in clinical material.

INTRODUCTION

Foot and Mouth Disease (FMD) has a worldwide distribution and is of cardinal importance for agriculture. Due to the rapid spread of FMDV and the rapid mutation rate of the viral genome leading to multiplicity of serotypes and subtypes, (over 70 known subtypes, Cheung et al., 1983), the confusion of FMD's clinical picture with other diseases is eminent. The possibility for carrier state among infected animals (Wittmann, 1990) makes diagnosis as well as control quite difficult. Essential to an effective FMD control program would be the adoption of rapid and accurate molecular diagnostic procedures, based on the use of RNA extracted directly from clinical material obtained from suspected animals in the field, without the need of passaging the virus in cell cultures. The current availability of the Polymerase Chain Reaction (PCR) technique (Saiki et al., 1988) allowed us to develop a rapid and accurate method to identify FMDV RNA in RNA extracted from clinical material sent to the FMDV diagnostic laboratory.

Preparation of Viral FMDV RNA from virions requires their concentration with polyethyleneglycol (PEG) and purification of the virus on a sucrose or cesium chloride gradients. The RNA is then extracted with phenol/chloroform and precipitated with ethanol as already described (Beck and Strohmeir, 1987). Isolation of mRNA involves the isolation of total RNA followed by selection of poly A (+) RNA by affinity chromatography on dT cellulose column (Aviv and Leder, 1972). The genome of foot and mouth disease virus (FMDV) is a positive (+) sense polyadenylated RNA at its 3' end (Grubman et al., 1979). In order to isolate FMDV RNA we used a novel magnetic separation method designed for easy and rapid purification of poly A (+) sense RNA directly from plant tissues or cell cultures using DYNABEADS oligo (dT)25 (Jakobson et al., 1990). Dynabeads oligo (dT)25 are uniform, supermagnetic polystyrene beads 2.8 µm in diameter and contain a 25 nucleotide chain of deoxythymidylate covalently attached to the bead surface via a 5' linker group. The seperation of poly A (+) RNA relies on

base pairing between the poly A at the 3' end of the RNA with the oligo dT residues coupled to the surface of the beads which can be removed from the tissue homogenate by a magnet.

The poly A (+) RNA directly prepared by this method from FMDV infected tissues can be forwarded without further purification for cDNA synthesis and polymerase chain reaction (PCR) amplification (Saiki et al, 1988). In the present study the usefulness of this method for the detection of FMDV RNA in clinical samples from animals in the field was studied. It was found that the technique allowed detection of FMDV RNA in clinical samples which also were found to be positive by the conventional techniqes.

MATERIALS and METHODS

Clinical materials suspected for FMDV

Five samples from animals suspected to have symptoms of FMD were examined :

A. Tongue epithelial tissue from a cow showing clinical signs of FMD.

B. Tongue epithelial tissue from a cow showing clinical signs.

C. Foot epithelial tissue from a sheep showing clinical signs from the same outbreak as sample B.

D. Tongue epithelial tissue form a calf showing clinical signs from a third outbreak.

E. Tongue epithelial tissue from a one-week-old lamb from a flock where 20 out of 150 lambs died.

Purification of RNA from sample A

The tongue epithelial tissue was washed twice with ice cold PBS, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_4HPO_4 7H_2O$, 1.4 mM KH_2PO_4), and then ground with the ultra turrax type T-25 (Janke and Kunkel). A small amount of the ground tissue was transferred into two different Eppendorf tubes to pellet tissue fragments. The pellet was used for extraction of RNA.

Extraction of RNA using the Dynabeads oligo (dT)25.

Method 1. One ml of lysis/binding buffer (100 mM tris-HCl pH 8, 500 mM LiCl, 10 mM EDTA, pH 8, 1% SDS,5 mM DTT) and 80 units RNasin (Promega) were added to the tissue pellet. After mixing, the solution was left at

room temperature for 2 minutes. The lysate was centrifuged for 30 seconds in an Eppendorf centrifuge and the supernatant was transferred to an Eppendorf tube containing 1.5 mg Dynabeads oligo (dT)25 suspended in 100 µl lysis/ binding buffer. The mixture was put on ice for 5 minutes to allow the poly A RNA to anneal to the oligo dT on the beads. After annealing the tube was placed on MPC-E Magnetic Particle Concentrator for 30 seconds and the supernatant was removed. The beads were washed twice with 500 µl Washing buffer (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1mM EDTA, 0.1-0.3% SDS). The washed beads were incubated with 10 µl Elution buffer (2 mM EDTA pH 7.5) at 65°C for 2 minutes. The tube was placed on MPC-E and the eluted poly A RNA solution then removed.

Method 2. The tissue pellet was resuspended in 100 µl Lysis buffer (10 mM Tris-HCl pH 7.5, 0.14 M NaCl, 5 mM KCl, 1% Triton X 100) and 80 units of RNasin (Promega). The solution was placed on ice for 1 minute, centrifuged for 30 seconds and the supernatant was transferred to a new Eppendorf tube containing 450 µg Dynabeads oligo (dT)25 suspended in 100 µl 2X Binding buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA, 0.4-1% SDS). The Solution was left on ice for 5 minutes to allow annealing of the oligo dT to the poly A (+) RNA . After annealing, the tube was placed on MPC-E for 30 seconds and the supernatant removed. The beads were washed twice with 200 µl Washing buffer. The washed beads were incubated with 10 µl Elution buffer for 2 minutes at 65°C. The tube was replaced on the MPC-E for 30 seconds and the eluted poly A (+) RNA collected.

RNA purification from samples B, C and D

Each sample was treated separately. Approximately 1 gram of each of the

suspected tissues was washed a few times with PBS. The washed tissues were transferred to a mortar, 1 ml of Lysis/Binding buffer, 80 units of RNasin and powdered glass were added and the tissue was ground with a pestle. The ground tissue was centrifuged for 30 seconds in an Eppendorf centrifuge and the supernatant was transfered to an Eppendorf tube containing 1 mg Dynabeads oligo (dT)25 suspended in 100 µl Lysis/Binding buffer. The mixture was put on ice for 5 minutes to allow annealing of the beads to the polyadenylated RNA. The beads were separated on the MPE-C, washed twiced with 0.5 ml Washing buffer and then the poly A RNA was eluted in 10 ul Elution buffer for 2 minutes at 65°C. Beads were removed with the MPE-C magnet.

RNA purification from sample E

The tongue epithelial tissue was chopped and washed with a PBS solution. Two trypsinizations were carried out using 0.5% trypsin in a total volume of 50 ml for 30 minutes each. Solutions were collected by filtration through a gauze and centrifuged 2000 RPM for 5 minutes to pellet cells. The trypsinized cells were resuspended in 0.5 ml PBS and counted. A total amount of 1.6 X 10^6 cells was obtained and and the cells were used for RNA extraction with Dynabeads oligo (dT)25. Poly A RNA was extracted as described for the second extraction method in sample A.

Synthesis of cDNA and PCR

Each of the samples of purified poly A (+) RNA were reverse transcribed using AMV reverse transcriptase (Life Sciences Inc.) (Sambrook et al, 1989). A 19 mer synthetic oligonucleotide, located in the P12 area

adjacent to the 3' end of VP1, (oligo # 2 ; 5' AGTTCGACCTAACGTCGGA 3') was used as a primer for the VP1 region. A 21 mer (oligo # 7 5' GTAAAGTGATCTGTAGCTTGG 3') located within the RNA Polymerase area was used to amplify this region.

One hundred pmol primer were annealed to 2 µl purified poly A (+) RNA at 70°C for 5 minutes in a reaction volume of 11 µl.The annealed RNA/Primer was incubated with 18 units of AMV reverse transcriptase and dNTPs (Pharmacia), 10 mM each, at 42°C for one hour in 50 mM Tris-HCl pH 8.3, 8 mM MgCl2, 40 mM KCl and 40 units of RNasin (Promega) in a total reaction volume of 20 µl.

Five µl of the cDNA were used in the PCR reaction. For the VP1 sequence the direct primer used was oligo # 2 (the one used for reverse transcription) and the reverse primer was a 20 mer oligonucleotide (oligo # 5: 5' AGTTCGACCTAACGTCGGA 3') located 424 nucleotides upstream from the direct primer. For the RNA polymerase gene amplification the direct primer used was oligo # 7 (used for reverse transcription) and the reverse primer was a 21 mer (oligo # 9: 5' TTCGAGAACGGCACGGTCGGA 3') located approximatly 1 Kb upstream from the direct primer within the RNA Polymerase gene.

A fifty µl reaction volume contained 5 µl cDNA, 100 pmol of each of the primers, 10 mM Tris-HCl pH 8.3, 50 mM KCL, 1.5 mM MgCl2, dNTPs (Pharmacia) 10 mM each and 4 units Taq DNA Polymerase (International Biotechnology Incorporation, IBI).

Samples were placed on a programmable thermal controller (M J Research Corporation) for 35 cycles consisting of :

Denaturation	93°C 30 seconds
Annealing	55°C 30 seconds
Polymerazation	72°C 1 minute.

The last step was increased to 10 minutes at the end of the reaction. 10 µl of each of the reaction products was electrophoresed on an 1.6% agarose gel.

Serological tests

Each clinical sample was washed with PBS and ground with powdered glass in a mortar with a pestle in presence of Earle's Medium. The homogenate was centrifuged and the supernatant was taken for injection of 10 mice, inoculation of pig kidney (PK) cell cultures and for complement fixation test (CF)(Lombard et al., 1982). The CF test was carried out against the following antisera:

A. O1 BFS - U.K., 1967

B. O1 GESHUR - Israel, 1985

C. O1 MANISA - Turkey, 1969

D. O1 DALTON - Israel, 1988

E. A22 - Iraq, 1964

F. ASIA 1 - Lebanon, 1983

The supernatant recovered from sample C was too small for all tests. Therefore, a PK cell culture was inoculated and CF test was carried out with the cell culture medium following the appearance of a cytopatogenic effect (CPE) in the cell culture.

Sample B was anti-complementary in the complement fixation test with the field clinical material therefore CF test was carried out with cell culture medium following CPE in the cells.

RESULTS

Five field samples of epithelial tissue from FMD suspected animals from the field were examined using the combined novel methods for RNA extraction (DYNABEADS oligo (dT)25) and the PCR technique.

Two sets of primers were used. Oligo#7 and oligo #9 located in the conserved RNA polymerase area of the FMDV genome indicate whether or not FMDV RNA is present in the examined material. The other set, oligo #2 and oligo #5 are located in the variable VP1 gene. These primers were synthesized based on the 01 Geshur sequence and indicate the relation of the FMDV RNA present in the biological material in question to 01 type.

The presence of FMDV RNA was detected in four out of the five clinical samples. Sample A was obtained from a cow showing clinical signs (Fig. 1). The RNA was extracted using two slightly different methods. In the PCR reaction on the isolated poly A + RNA with amplimers designed for the detection of the RNA polymerase gene (oligo # 7 and oligo # 9) gave a positive amplification reaction although with different intensities (Fig.1 lanes 1 & 2 showing a 1 Kbp amplified DNA). The specific amplimers for the VP1 nucleotide sequence gave positive results though with a reduced intensity (Fig. 1 lanes 3 & 4 showing a 424 bp amplified DNA). This result indicated that the examined FMDV RNA could be related to the FMDV type 01.

Samples B (Fig. 2) and sample C (Fig. 3) obtained from a cow and a sheep, respectivly, both showing clinical signs from the same outbreak, gave positive results using the amplimers for the RNA polymerse gene (Fig.2 lane 1 & Fig.3 lane 1). The amplimers for the VP1 region gave also positive results (Fig. 2 lane 2 & Fig. 3 lane 2 the latter with a reduced intensity). Sample D (Fig. 4) obtained from a calf showing clinical signs, gave a positive result using both pairs of amplimers, the one for

the RNA polymerase (#7 & #9) and that of the VP1 (#2 & #5) genes (Fig.4 lanes 1 & 2). A lower intensity was observed in the amplified VP1 segment. A negative PCR amplification with FMDV amplimers both for the RNA Polymerase gene and the VP1 sequence was found with the RNA from sample E (Fig. 5) obtained from a one week old lamb.

RNA from FMDV type O1 Geshur, extracted as described by Laor et al., 1991 was used as a positive control in these tests.

The results were confirmed by serological (CF) tests, inoculation to baby mice and infection of PK cell cultures. All 10 baby mice inoculated with the suspected material from each of the clinical samples A, B, C and D died within 48 hours, none of the mice inoculated with the material of sample E died. A complete CPE was observed within 24 houres in PK cell culture infected with the suspected material of samples A, B, C and D. No CPE was observed in sample E. Complement Fixation test carried out with the clinical material of sample A indicated relation of the suspected FMDV to the isolate O1 Geshur. Samples B and C obtained from different animals in the same outbreak were found by CF test to be related to the isolate O1 Dalton carried out following one passage on PK cell culture. Sample D showed similar reaction,in the CF test, with all O1 subtypes tested. Sample E was negative in the CF test.

DISCUSSION

The availability of the PCR technique for the amplification of FMDV RNA (Laor et al, 1991) and the novel and rapid method for poly A (+) RNA extraction using Dynabeads oligo (dT)25 made possible the detection of FMDV RNA in clinical material received from FMD suspected animals in the field. The combination of the two techniques for RNA isolation and RNA amplification allows the detection of FMDV in small epithelial tissue samples, shortly after arrival in the FMDV Diagnostic Laboratory and

without the need of passaging the virus in cell cultures. The use of RNA Polymerase gene amplimers made possible the decision whether or not FMDV is present in the suspected clinical material. Using specific amplimers located in the VP1 gene it might be possible to determine the relation of FMDV RNA to FMDV type O1 as can be confirmed by the serological test. CF was carried out on the suspected material in 3 out of the 5 samples immediatly as they arrived. In one case the sample was too small to be checked directly with the complement fixation test, and in the other case the sample was anti complementary. A passage on PK cell culture before the CF test was inevitable in these cases. Use of the RNA isolation technique followed by PCR made possible an immediate diagnosis of FMDV RNA in the sample.

The present method of molecular diagnosis of FMDV RNA in field clinical materials from animals in the field is rapid, accurate and overcomes difficulties with the CF test and the need to passage the virus in cell cultures. Further studies will study the feasibility of direct sequencing of the VP1 gene of the FMDV isolate in the clinical material to assess possible changes in the antigenic domains with the help of computer programs (Becker Y., to be published).

ACKNOWLEDEGMENTS

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Legend to Figures:

Fig 1. RNA extracted by Dynabeads oligo (dT)25 from tongue epithelial tissue of a FMD suspected cow from the field (sample A). The RNA was amplified using the PCR technique, after cDNA synthesis, with specific primers for the RNA Polymerase gene and the VP1 area.

1. RNA extracted with 1.5 mg Dynabeads using Lysis/Binding buffer (as recommended by the manufacturer for solid tissue) and amplified with the PCR technique using oligonucleotides (#7 & #9) selected for the RNA polymerase gene.
2. RNA extracted with 450 µg Dynabeads using Lysis buffer (as recommended by the manufacturer for tissue culture cells) and amplified with the RNA polymerase gene amplimers (#7 & #9).
3. RNA from the sample used for lane 1 was amplified using specific amplimers for the VP1 area (#2 & #5) selected on the basis of the VP1 nucleotide sequence of 01 Geshur isolate.
4. RNA from the sample in lane 2 was amplified with amplimers #2 & #5.
5. Positive control: RNA from BHK cells infected with FMDV 01 Geshur isolate.The amplimers for the RNA polymerase gene (#7 & #9) were used.
6. Negative control : no RNA or DNA present in the system.
7. DNA molecular weight size marker No. VI.

Fig. 2. RNA extracted with 1 mg Dynabeads oligo (dT)25 from tongue epithelial tissue of an FMD suspected cow in the field (sample B). The RNA was amplified with the PCR technique after cDNA synthesis using amplimers designated to amplify the RNA polymerase and the VP1 genes.

1. RNA extracted with 1 mg Dynabeads and amplified with the RNA polymerse gene amplimers (#7 & #9).

2. RNA amplified with the amplimers designated for amplification of the VP1 gene (#2 &# 5).

Lanes 3, 4 and 5 as in Fig. 1.

Fig. 3. RNA extracted by Dynabeads oligo (dT)25 from foot skin epithelial tissue of an FMDV suspected sheep in the field (sample C). The extracted RNA was amplified, via cDNA synthesis, with the PCR technique using amplimers designed to amplify the RNA polymerase and the VP1 genes.

Lanes 1-5 as in Fig. 2.

Fig. 4. RNA extracted with 1 mg Dynabeads oligo (dT)25 from tongue epithelial tissue of an FMD suspected calf in the field (sample D). The RNA was amplified with the PCR technique after cDNA synthesis using amplimers designated to amplify the RNA polymerase and the VP1 genes.

Lanes 1-5 as in Fig. 2 except for the positive control (lane 3) where the amplimers for the VP1 gene (#2 & #5) were used.

Fig. 5. RNA extracted by Dynabeads oligo (dT)25 from tongue epithelial tissue from an FMD suspected lamb (sample E). The RNA was amplified using the PCR technique after cDNA synthesis, with specific amplimers for the VP1 and the RNA polymerase genes.

Lanes 1-5 as in Fig. 2.

Fig. 1

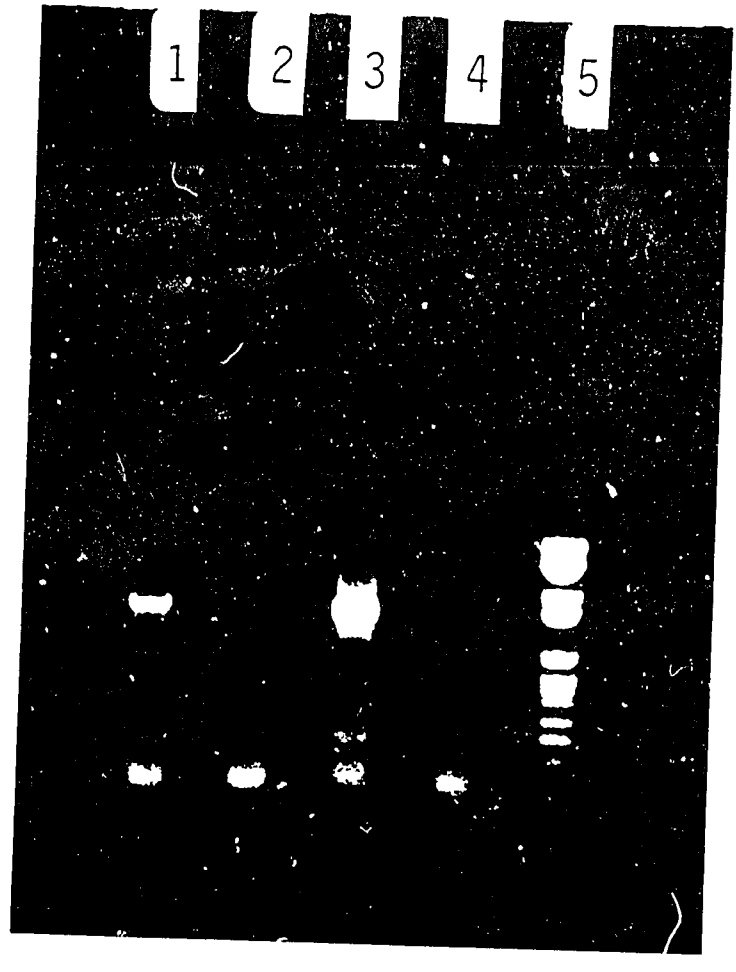

Fig. 2

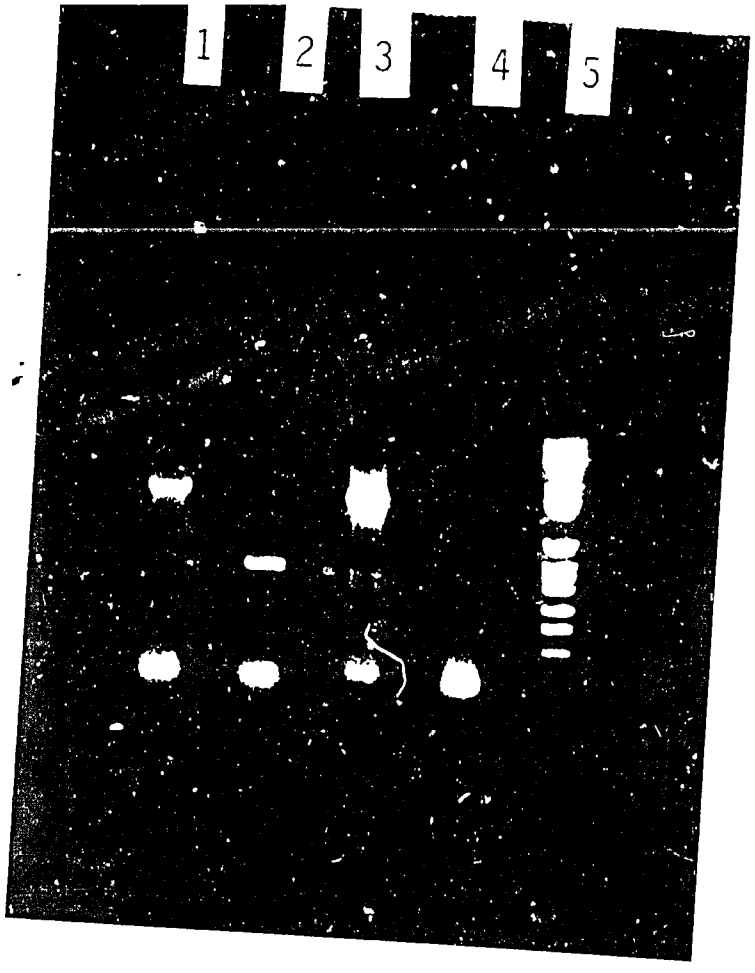

Fig. 3

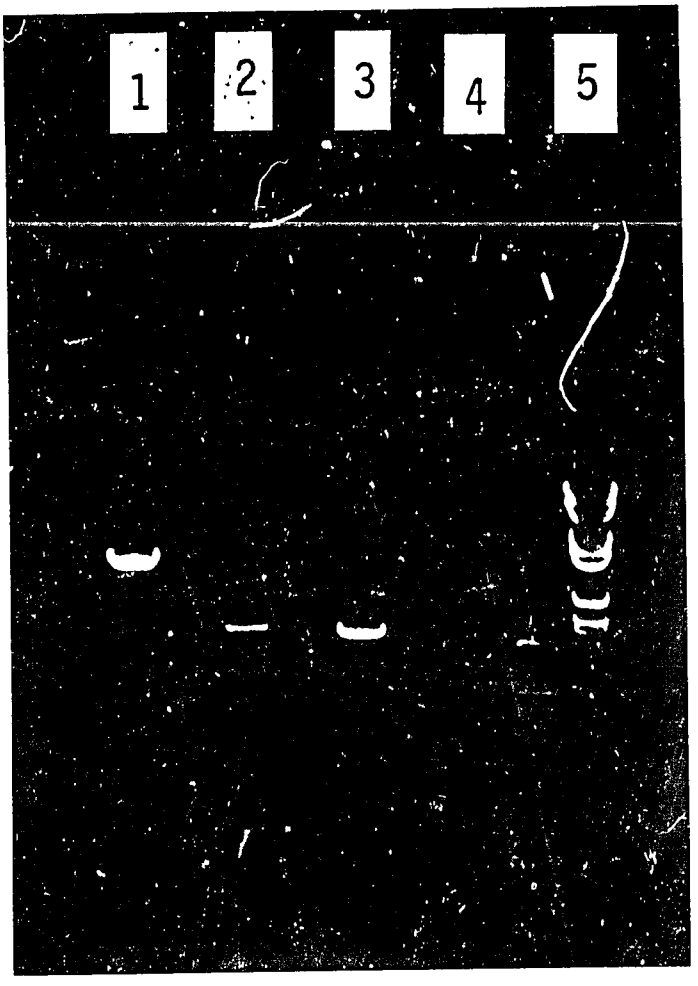

Fig. 4

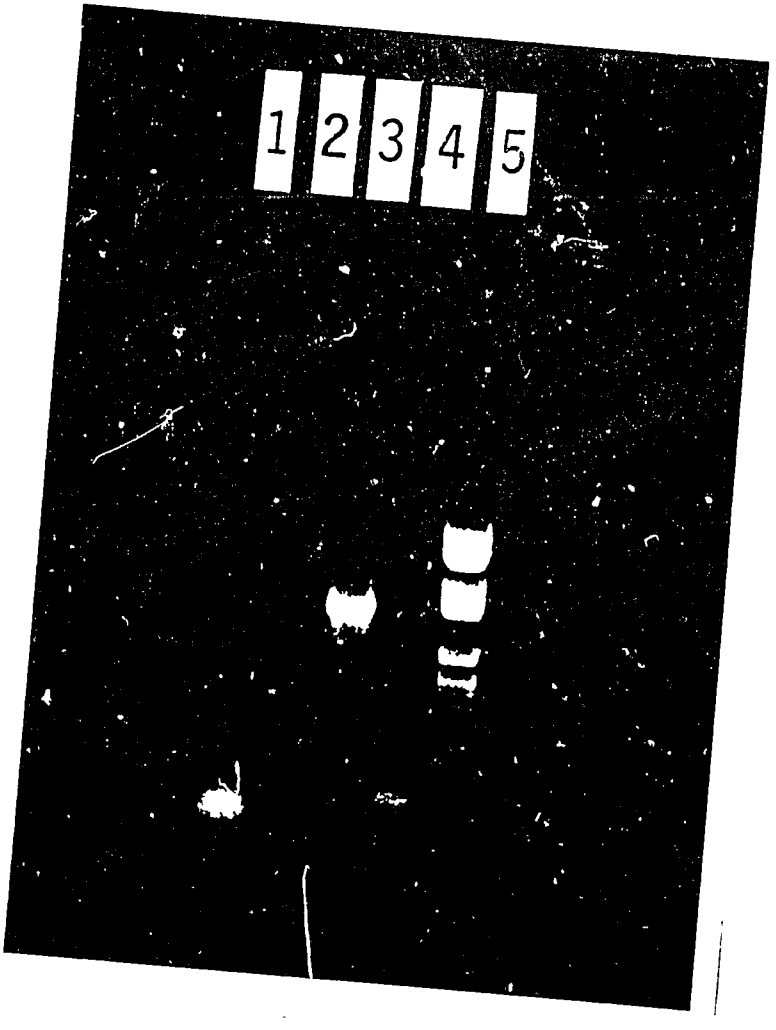

Fig. 5

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PCR Detection of Foot and Mouth Disease Virus RNA Isolated by Oligo-dT magnetic beads from *in vitro* infected BHK and Bovine Skin Langerhans Cells

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Short title: Detection of FMDV by PCR

Key words: Polymerase chain reaction; Foot and Mouth Disease virus; Oligo-dT magnetic beads; Bovine Langerhans cells.

Summary

BHK 21 cell line and bovine skin Langerhans cells were infected in vitro with Foot and Mouth Disease Virus (FMDV) Serotype 01, isolate Geshur (Isr/85). In order to detect the presence of the virus in the cells, we isolated poly A positive, genomic viral RNA with DYNABEADS oligo (dT)25. This method is based on magnetic separation technique using superparamagnetic Polystyrene beads coupled to oligo dT. A 25 nucleotide long deoxythymidylate chain is covalently attached to the surface of the bead. FMDV RNA isolated by this method was directly used for cDNA synthesis and Polymerase Chain Reaction (PCR) amplification. Using known and specific FMDV oligonucleotide primers a 424 nucleotide long segment from within the VP1 region of FMDV was amplified. This method made possible the detection of FMDV RNA in FMDV infected BHK 21 cells and bovine skin Langerhans cells after isolation with magnetic beads.

Introduction

Viral FMDV RNA isolated from virions requires concentration with polyethylenglycol (PEG) and purification of the virus on a sucrose or cesium chloride gradient. The RNA is then extracted with phenol/ chloroform and precipitated with ethanol as described (1). Isolation of mRNA from cells involves isolation of total RNA followed by selection of poly A (+) RNA by affinity chromatography on oligo dT cellulose columns (2).

The genome of Foot and Mouth Disease Virus (FMDV) is a positive (+) sense polyadenylated RNA at its 3' end (3). In order to isolate FMDV RNA we used a novel method designed for easy and rapid purification of poly A (+) sense RNA directly from solid tissues or cell cultures, using Dyanbeads oligo (dT)25 (4). This method is based on the magnetic separation technology. Dynabeads oligo (dT) 25 are uniform,

superparamagnetic polystyrene beads 2.8μm in diameter and contain a 25 nucleotides long chain of deoxythymidylate covalently attached to the beads surface via a 5' linker group. It relies on base pairing between the poly A at the 3' end of the RNA which forms hydrogen bonds with the oligo dT residues coupled to the surface of the beads.This method enabled us to isolate viral RNA from a relativly small amount of infected cells very rapidly, within 15 minutes.Langerhans cells were used in this study being the first cellular element in the epidermis were viral antigen can be detected. It is thought that the virus replicates in these cells (5). Langerhans cells function in the epidermis as very potent accessory cells. Lagerhans cells are bone marrow derived, bear class II antigen, possess C3 and Fc receptors, and play an essential role in the defense against the HSV-1 (6) and Vaccinia viruses (7). Di Girolamo et al. (8) demonstrated that in the infection of the epidermis of guinea pigs by injection of Foot and Mouth Disease Virus (FMDV) during the viral dissemination stage, LC played a significant role since they are the first cellular element in the epidermis where viral antigens can be detected.

The RNA prepared by this magnetic beads method can be directly used, without further purification,for cDNA synthesis followed by PCR amplification (6).

Materials and Methods

BHK21 Cells and FMDV virus

The virus used in this experiment was of Serotype 01 strain Geshur, isolated in 1985 from gazelles in Israel. A monolayer of baby hamster kidney (BHK-21) cell line in a Roux flask (about 10^8 cells) were infected with FMDV. Twelve hours after infection the cells were scraped with a rubber policeman and counted.

Studies with Langerhans cells

Animals

Skin samples were obtained from ventral flanks of femal frisi cows, 2.5 - 5 years of age, immediately after slaughter.

FMDV Viruses and titrations

O1Geshur strain virus (Kimron Veterinary Institute, Israel) was used. Virus titrations were performed to plaque on pig kidney (PK) cells virus titer (1×10^6 PFU/Ml).

Anti bovine Ia Monoclonal antibody

Monoclonal antibody (mab) anti bovine Ia were used (VMRD Inc.Pullman, Washington,USA) The mab are designated TH14B (0.5mg/0.5ml) which is a murine Ig2a specific for a highly conserved monomorphic antigenic determinant present in one of three detectable class II antigens in cattle (10).

Dynabeads M-450 with Sheep anti mouse IgG (SHAM)

2ml dynabeads (Dyna, Oslo, Norway) are uniform magnetic 4.5µm diameter polystrene beads with affinity purified SHAM covalently bound on the

surface (1 ml Suspension contain $4X10^8$ beads).

Preparation of Skin epidermal cells (EC) suspension:

Fifty fresh bovine skin specimens (0.5 cm2) were obtained from the sample that had been shaved and cleaned as described (11). The specimens were washed twice with phosphate buffered saline (PBS) at 4°C, then blotted, dermal side down, on the surface of 0.5% solution of trypsin (2.5% trypsin, Kimron Vet. Inst,Isreal) in carbonate buffer, pH 7.3 (0.68% NaCl, 0.04% KCl, 0.1% glucose and 0.22% $NaHCO_3$) for 105 minutes at 37°C, 5% CO_2). Epidermal sheets were separated from the dermis by fine forceps and were immediately transferred to a solution containing RPMI 1640 medium supplemented with L-glutamine; 10% heat inactivated fetal calf serum (Bet Haemek, Israel); streptomycin, 100 µg/ml; penicillin 100 u/ml and fungison, 1.25 µg/ml. After 3 min the epidermal sheets were pippetted with a 10 ml pipette and the suspension passed through a sterile nylon filter (Monodur PA 100 K nylon gauses,Stokvis and smit,Haarlem, Netherland). The EC were washed three times by centrifugation (Sorvall RT 6000)for 30 minutes at 1000 rpm and 4°C. The EC were resuspended in 30 ml medium with 10% FCS and kept at 4°C. The average yeild of EC was 30.106/ml with a viability > 80% as estimated by trypan blue staining.

Isolation of LC

As described (11) suspensions of 30 x 10^6 EC were used in each experiment.To the cells which were pelletted by centrifugation (1200 rpm for 10 min) and kept on ice.for 30 min with agitation every 10 min.together with 200 µL of monoclonal antibody TH14B anti-MHC class II IgG2a (0.5mg/0.5ml) diluted 1:500 with PBS. The EC suspension was washed three times at 4°C with cold RPMI1640 medium supplemented with L-glutamine; streptomycin, 100 µg/ml; penicillin, 100 u/ml; fungison, 1.25 µg/ml by centrifugation at 1000 rpm for 30 min.

Before using the magnetic beads, the latter were washed three times with cold PBS and then added to 0.5 ml suspension of EC in PBS. The suspension was gently agitated and kept on ice for 30 min at 4°C. Ice cold RPMI medium without serum was added to a volume of 2 ml. Rosetted beads and free beads were separated from non-rosetted cells by placing the tube on a Dynal Magnetic Particle Concentrator (Dynal Co., Oslo, Norway). After 2 min the non-rosetted cells were removed by washing the magnetic beads which adhered to the magnet.The nonadhering cells were peletted and collected. The rosetted positive cell fraction was washed at least three times with cold medium using the same technique. The rosetted cells were stained with trypan blue,and the number of trypan blue negative cells (living cells) and trypan blue stained cells (dead cells) were counted in a hemocytometer (Camera Dauble Hanover, West Germany).The EC that attached to the magnetic beads are LC and the nonadhering cell fraction are the kerarinocytes.

FMDV LC-Cells Interaction in-vitro

FMDV O1Geshur (1.10^6 Pfu virus per 10^5 LCs) in 1 ml RPMI 1640 medium (supplemented with L-glutamine and antibiotics) was added to the LC or Keratinocytes suspensions. The cell preparations were incubated at 37°C for 1 h at 4°C, 5% CO2. The cells were washed twice with the same medium and further incubated.Samples of infected cells were removed at 0, 4 and 24 hours p.i, and the cells were separated by centrifugation (1000 RPM for 10 min.at 4). The medium and cells of each sample were collected and stored at -20°C until used for virus titration.

Virus titrations

The virus contentsin the keratinocytes and LC suspension and the culture medium of each sample were titrated on PK2 cells maintained in Earl's

Medium supplemented with 10% calf serum and antibiotics. The titration was based on the determination of virus infectivity by the plaque assay andby determination of the TCID50.

RNA purification

One million infected BHK-21 cells were centrifuged, the supernatants were removed and the cells were washed with ice-cold PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 7H_2O$, 1.4 mM KH_4PO_4), transferred to Eppendorf tubes and pelleted.

The cell pellet was resuspended with 100 µl lysis buffer (10 mM Tris, HCl, pH 7.5, 0.14 M NaCl, 5 mM KCl, 1% Triton X-100),80 units of RNasin (Promega) were added and the solution was placed on ice for 1 minute. After centifugation for 30 seconds the supernatant was transferred to a new Eppendorf tube containing 450 µg Dynabeads oligo (dT)25 in 100 µl of 2 X Binding buffer (20 mM Tris, HCl, pH 7.5, 1 M LiCl, 2 mM EDTA, 0.4-1.0% SDS). The solution was kept at room temperature for 5 minutes to allow the poly-A RNA to anneal to the oligo dT on the beads. After hybridization, the tube was placed on MPC-E Magnetic Particle Concentrator (Dynal Co.,Oslo,Norway) for 30 seconds and the supernatnant was removed. The beads were washed twice with 200 µl Washing buffer (10 mM Tris, HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA 0.1-0.3% SDS) The washed beads were incubated with 10 µl Elution buffer (2 mM EDTA pH 7.5) at 65°C for 2 minutes. The tube was placed on MPC-E magnetic particle concentrator and the eluted poly A RNA,including the viral RNA was removed. Beads were washed three times with Elution buffer and reused for the same procedure with another sample of 1 X 10^7 infected BHK-21 cells. Viral and cellular mRNAs were purified the same way from 8.4 X 10^5 FMDV infected bovine Langerhans cells.

Synthesis of cDNA and PCR

Each of the three samples of purified poly A RNA were reverse transcribed using AMV reverse transcriptase (Life Science Inc.) (2). A 19 mer oligonucleotide, located on P 12 area adjacent to the 3' end of VP1, (oligo # 2) (5' AGTTCGACCTAACGTCGGA 3') was used as a primer.100 pmol primer were annealed to 1 µl purified RNA at 70°C for 5 minutes in a reaction volume of 11 µl. The annealed RNA/primer was incubated with 18 units of AMV Reverse transcriptase and the 4 deoxyriboonucleosde triphosphates (dNTPs)(Pharmacia) 10 mM each at 42°C for 1 hour, in 50 mM Tris, HCl, pH 8.3, 8 mM $MgCl_2$, 40 mM KCl and 40 units RNasin (Promega) in a total reaction volume of 20 µl. 1 µl of the obtained cDNA was used for the PCR reaction. The direct primer used was oligo # 2 (the one used for the reverse transcription) and the reverse primer was a 20-mer oligonucleotide (oligo # 5) (5' AGTTCGACCTAACGTCGGA 3') located 424 nucleotides upstream from the direct primer.

A 50 µl reaction volume contained 1 µl cDNA, 100 pmol of each of the primers, 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, dNTPs (Pharmacia) 10 mM each and 4 units Taq DNA Polymerase (IBI).

Samples were placed in a programmable thermal controller (M J Research Inc.) for 35 cycles consisting of:

Denaturation 93°C 30 seconds

Annealing 55°C 30 seconds

Polymerazation 72°C 1 minute

The last step was increased to 10 minutes at the end of the reaction.

10 µl of each sample were electrophoresed on a 1.6% agarose gel.

Results

RNA was extracted from in-vitro infected cell cultures by Dynabeads oligo (dT)25 and amplified with the PCR technique, using primers for the VP1 gene, after cDNA synthesis.All infected cells examined gave positive results after PCR amplification of the viral RNA isolated from infected cells with the Dynabeads oligo (dT)25 method (Fig. 1) The positive band intensity was different with relation to the amount and type of cells used. All bands obtained in all lanes were the expected size segment of ~400 nucleotides within the VP1 gene.The amplified viral DNA in Fig 1 (Lane 1)was done on Viral RNA which was extracted from 1 X 10^6 infected BHK cells.The viral cDNA Lane 2, shows the amplification of RNA extracted from 1 X 10^7 infected BHK cells. The same beadswhich were used in the first experiments were washed three times with the Elution buffer and were reused.The result in Lane 3, shows detectable cDNA at a lower yield of the PCR productwith the RNA extracted from infected LC. This lane represents the amplification of poly-A FMDV RNA isolated from the in-vitro infected (8.4 X 10^5) bovine skin Langerhans cells.

A negative control Lane 4 and a DNA molecular weight size marker Lane 5 are persented.

Discussion

The use of the Dynabeads oligo (dT)25 method enabled us to rapidly purify the poly A+ viral RNA from infected cells without the need to concentrate and purify the RNA from virions. This method was described for extraction of RNA fron plant tissue (4) and the present study is the first report on the isolation of poly A+ mRNA fron eucaryotic cells or viral RNA with the magnetic beads.

Preliminary experiments to amplify FMDV RNA was done on RNA extracted from a relatively small FMDV infected cell preparation (1 X 10^6 BHK cells) gave a positive result (data not shown).

In this study the use of Dynabeads oligo (dT)25 for RNA purification

combined with the PCR amplification technique made possible the detection of the presence of FMDV genomic RNA in small amounts of two different cell types. The results of this study lead us to sugggest the combination of the two techniques as a possible diagnostic tool for FMDV. The possibility of detection of low yields of virus fron a small biological sample might allow the diagnosis of FMDV while avoiding multiple passages in cell cultures which can lead to mutations in the VP1 sequences that codes for the antigenic domains. Indeed, the method suggested by the present study made possible the PCR diagnosis of FMDV RNA in samples from cattle suspected for FMD (13).

The detection of FMDV RNA in in-vitro infected bovine skin LC can be taken to indicate that FMDV can interact with LC under in-vitro conditions.Further experiments in progress will provide evidence on the fate of FMDV in Langerhans cells.

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Legend to figure:

Fig. 1. RNA extracted from in-vitro infected cell cultures by Dynabeads oligo (dT)25 and amplified with the PCR technique, using primers for the VP1 gene, after cDNA synthesis.

Lanes: 1. RNA purified by Dynabeads oligo (dT)25 from 1 X 10^6 infected BHK-21 cell culture.

2. RNA purified from 1 X 10^7 infected BHK-21 cell culture.

3. RNA purified from 8.4 X 10^5 bovine skin Langerhanse cells.

4. Negative control - no RNA or DNA in the systhem.

5. DNA molecular weight size marker No. VI.

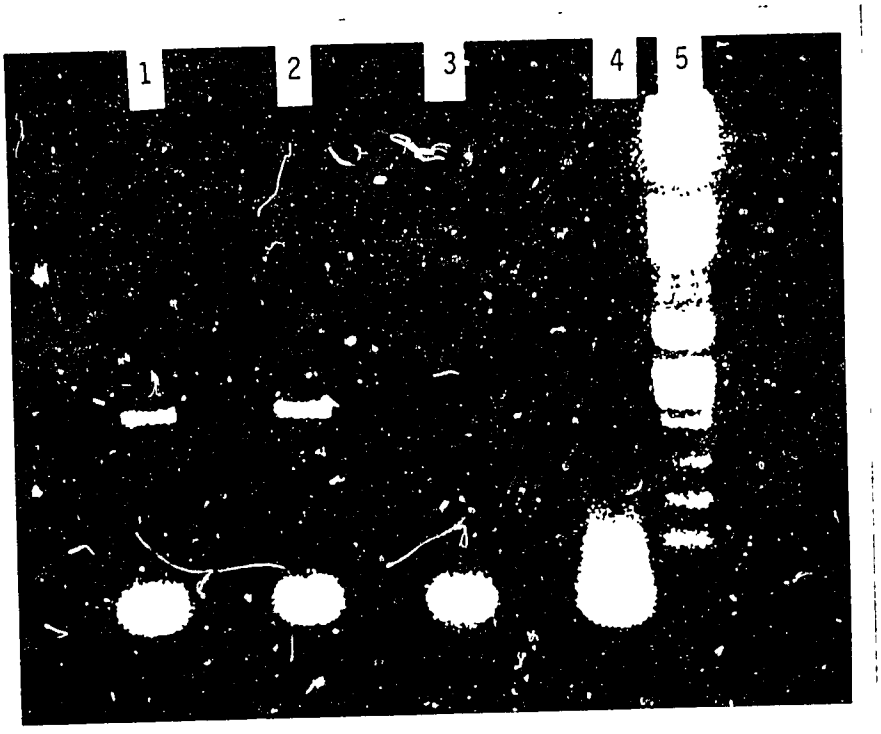

Fig. 1

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ISOLATION OF BOVINE SKIN LANGERHANS CELLS AND MAINTENANCE OF THEIR VIABILITY IN VITRO

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ABSTRACT

A method for the isolation and purification of bovine skin Langerhans cells (LC) is described. LC were isolated with magnetic beads covered with anti bodies to mouse IgG and reacted with monoclonal antibodies (mab) specific for Bovine Ia antigen. The purified LC were incubated in conditioned medium from Hodgkin's Disease (HD) cell line L-428. This conditioned medium retained LC viability during 125 hrs in vitro and allowed part of the LC to divide. LC in regular RPMI 1640 medium were able to retain viability for a shorter period in vitro.The present study describes a rapid method to isolate bovine skin LC and provides cell culture conditions for improved LC viability under in vitro conditions.

INTRODUCTION

The mammalian skin epidermis is composed of four different cell types: keratinocytes, melanocytes, Merkel cells and Langerhans cells (LC).

Bone marrow derived LC (1) represent 2-4% of the skin epidermal cells (EC). LC play an important role in the skin immune response and function as antigen presenting cells, capable of inducing allogeneic antigen presentation and cytotoxic T-cell proliferation. LC are responsible for induction of contact hypersensitivity in the skin and take part in skin graft rejection.

Bovine LC express two specific surface markers that can be used to distinguish them from other EC: the major histocompatability complex, Class II antigen (MHC, Class II)(2) and the CD1 receptor (3) which was suggested to have a role in antigen recognition by T cells. LC interact with adjecent keratinocytes which secrete a variety of immunoregulatory cytokines important for the viability of LC:

- Granulocyte/macrophage colony stimulating Factor (GM-CSF) (4)

- Epidermal cell-driven thymocyte activating factor (ETAF) equivalent to interleukin 1 cytokine necessary for antigen presentation (5).
- Tumor necrosis factor (TNF) α (6).

To study the biological, biochemical, immunological and characteristics of bovine LC it is necessary to isolate LC from the EC.

There are several techniques (7) for isolation of LC from EC: The cell sorting method (8),The density gradient centrifugation technique (9),

The solid phase immuno adsorption technique (10),The magnetic beads technique (11). The latter technique was adopted (12,13) for the isolation of LC from human skin epidermis. LC form rosettes with immunomagnetic microspheres carrying antimouse IgG.The microspheres interact with a monoclonal antibody to anti MHC class II surface marker which was attached to the LC. The rosetted LC can be isolated from the remaining EC by attachment of the magnetic beads to a magnet. In the present study we have adopted the magnetic beads technique for the isolation of bovine skin Langerhans cells and have studied the optimal conditions for maintenance of their viability in vitro.

MATERIALS AND METHODS

A. Animals

Skin samples were obtained from ventral flank of female Frisi cows, 2.5 to 5 years of age, immediately after slaughter.

The skin samples were placed in physiological saline (0.8% NaCl) and held at 4°C until processed in the laboratory.

B. Preparation of epidermal sheets

The skin was shaved and cleaned with physiological saline and ethanol 70%. Epidermal sheets were prepared as described (14): Skin biopsies

from ventral flank female Frisi cows were immersed in PBS containing 0.82% ethylenediamine tetraacetic acid (pH 7.4) (EDTA,Sigma Chemicals) for 2.5 h at 37°C in 5% $C0_2$. The epidermal sheets were separated from the underlying dermis and placed in PBS (pH 7.4) at room temperature until stained with immunoflorescence method.

C. **Antibodies:**

Monoclonal antibody (mAb) (VMRD Inc.Pullman,Washington,USA) Bovine Ia antigen designated TH14B (0.5mg/0.5 ml) which is a murine IgG_{2a} specific for a highly conserved monomorphic antigenic determinant present on one of three detachable class II antigens in cattle (2). This antibody stains bovine LC (dendritic cells) (15). It was used in association with affinity purified fluorescein isothiocynate (FITC) conjugated goat and rabbit anti mouse IgG (Biomakor, Israel).

D. **Magnetic beads:**

Dynabeads M-450 coated with Sheep anti mouse IgG (SHAM).

2ml dynabeads M-450 (Dynal Co., Oslo, Norway) uniform magnetic (4.5µm diameter) polystyrene beads with affinity purified SHAM covalently bound on the surface (1ml contains 4 x 108 beads) were used.

E. **Preparation of epidermal cells (EC) suspension**

Fifty fresh bovine skin specimens (0.5cm2 each) were obtained from the sample that had been shaved and cleaned. The specimens were washed twice with phosphate buffer saline (PBS) at 4°C, then blotted, dermal side down, on the surface of 0.5% solution of trypsin (2.5%) in carbonate buffer, pH 7.3 (0.68% NaCl, 0.04% KCl, 0.1% glucose and 0.22% $NaHCO_3$) for 105 minutes at 37°C, 5% CO_2.

Epidermal sheets were separated from the dermis by fine forceps and were immediately transferred to a solution containing RPMI 1640 medium supplemented with L-glutamine 300µg/ml; 10% heat inactivated fetal calf erum (Bet Haemek, Israel); streptomycin, 100µg/ml; penicillin 100 u/ml and fungison, 1.25 µg/ml. After 3 min the epidermal sheets were pippetted with a 10ml pipette and the suspension was passed through a sterile nylon filter (Monodur PA 100 N nylon gause,Stokvis en Smit, Haarlem, Netherland). The EC were washed three times for 10 minutes each in a cold (4°C) centrifuge (Sorvall RT-6000) at 1000 rpm. The EC were resuspended in 30 ml medium supplemented with 10% fetal calf serum (FCS) at 4°C. The average yield was 3.10^7/ml EC with a viability > 80% as estimated by trypan blue staining.

F. <u>Isolation of LC with the magnetic beads</u>

Suspensions of 3 x 10^7 EC were used as described (12,13).The cells were pelletted by centrifugation (1000 rpm for 10 min) and kept on ice for 30 min with agitation every 10 min with 200 µl of monoclonal antibody TH14B anti MHC class II diluted 1:500 with PBS. The EC suspension was centrifuged three times at 4°C (Sorvall RT-6000, 1000 rpm for 10 min each) in cold RPMI 1640 medium supplemented with L-glutamine 300 µg/ml; streptomycin, 100 µg/ml; penicillin, 100 u/ml; fungison, 1.25 µg/ml.

Before using the beads,(5 beads for one LC) the cells were washed three times with cold PBS and then the magnetic beads were added to 0.5ml suspension of the mab treated EC in PBS. The mixture was gently agitated and was further incubated on ice for 30 min at 4°C. Ice cold medium was added to the cells to a volume of 2ml Rosetted and free beads were separated from non-rosetted cells by placing the tube on a Dynal, Magnetic Particle Consectrator (Dynal Co., MPC-1 Oslo, Norway). After 2 min the non-rosetted cells were removed by washing. These cells were peletted and

collected. The rosetted positive fraction was washed at least three times with cold medium using the same technique. The number of rosetted cells was estimated by staining with trypan blue (Camera Dauble, Neubaver, West Germany).

G. Conditioned medium from L-428 Hodgkin Disease (HD) cell-line

The conditioned medium from L-428 HD cells (16) was centrifuged (Sorvall RT 6000) for 10 min at 1500 rpm. The supernatant was removed and passed through a 0.45 µm and 0.20 µm sterile filters (Schleicher and Schull Einmol - filterhalter FP 030/2, 030/3 West Germany) and stored at -20°C.

H. Langerhans cell cultures

3 x 10^5 cells of the rosetted positive fraction (obtained by the addition of 5 beads per one LC) were pelletted in a round bottomed test tube. 2 ml of conditioned medium supplemented with 10% heat inactivated FCS (Bet Haemek, Israel) was added. The control consisted of 2.77 x 10^5 LC of the rosetted positive fraction pelletted in a round test tube with 2 ml RPMI 1640 medium supplemented with 10% FCS;L-glutamine 300µg/ml streptomycin, 100 µg/ml; penicillin, 100 u/ml; and fungison, 1.25 µg/ml. The two test tubes were incubated for 5 days in 37°C, 5% CO_2. Each day 50 µL of LC suspension was removed for determination of the viability and for immunofluorescent staining.

IMMUNOFLUORESCENCE STAINING

1. Epidermal sheets

Epidermal sheets were fixed in acetone for 20 min at room temperature.Specimens were washed three times with PBS containing 1% heat inactivated FCS (Bet Haemek, Israel) for 60 min at 4°C. The sheets wee incubated with mAb TH14B at a 1:500 dilution in a total volume of 0.2 ml for 16 hours at 4°C. Sheets were then washed three times at 23°C for 2 h, and were incubated for 105 min in FITC goat anti mouse IgG (Biomakor, Israel) diluted 1:16 in PBS. (17).

The specimens were finally washed three times in PBS on a Vari Shaker (Dynatech) for 24 hours at 23°C, then mounted in 9:1 glycerol:PBS solution and examined in a NIKON epifluorescent microscope. The control consisted of specimens incubated without primary monoclonal antibody.

2. <u>Preparation of LC for immunofluorescence staining</u>

25µL of LC suspensions were incubated in L-438 HD conditioned medium or in RPMI medium and placed on a slide (18).

The cells were left for 30 min to dry at room temperature and then fixed for 10 min in acetone at room temp. One drop of mAb antibody TH14B at 1:500 dilution was added to the fixed cell preparation.

The slides were incubated in a humidified chamber for 1 hour at 37°C. After washing twice with PBS and distilled water for 10 min, the slides were dried in an incubator.

One drop of fluorescein isothiocyanate (FITC) conjugated rabbit anti mouse IgG (Biomakor, Israel) at 1:30 diluton in PBS was added. The cells were incubated for 1 hour at 37°C and again washed twice with PBS for 20 min and twice with distilled water for 10 min. The slides were dried, a small drop of glycerin-PBS (1:1) was placed with a Pasteur pipete and the cells were covered with a coverslip (18 x 24 mm) and examined with a NIKON epifluorescent microscope.

3. **Langerhans cells Suspension**

30 x 10^6 EC were incubated for 30 min on ice at 4°C with the mab TH14B diluted 1:500 in PBS. After three washes with cold RPMI medium supplemented with L-glutamine, 300µg/ul 100 ug streptomycin, 100 U/ml penicillin, and 1.25 µg/ml fungison at 4°C. After three washes with cold medium, Dynal beads M-450 SHAM IgG was added.The mixture was gently agitated and further incubated with agitation for 30 min. at 4°C after which 2 ml medium was added. Rosetted and free beads were than separated from non-rosetted cells by placing the tube on a magnetic particle concentretor (MPC-1, Dynal Co., Oslo, Norway). After 2 min. the non- rosetted cells were removed by pipetting and collected. The rosetted positive fraction was incubated for 30 min at 4°C with 0.2 ml of fluorescein isothiocyante conjugated goat anti mouse IgG (Biomakor, Israel) diluted 1:16 with PBS after 2 additional washes with cold medium, The cells were examined with a NIKON epifluorescent microscope.

Preparation of the rosette positive fraction for scanning electron microscopy

The morphological characteristics of the isolated cells were examined using scanning electron microscopy. The rossette positive fraction was transferred to a coverslip and fixed in 1% gluteraldehyde in 0.1M Phosphate Buffer Saline (PBS), and fixed for 1 h in 1% osmium tetraoxide at 4°C, washed with PBS (pH 7.2) and critically dried, then dehydrated through graded alcohol concentrations. The coverslip containing the cells were then coated with gold and examined and photographed in the scanning electron microscope (Geol 35 C,Faculty of Agriculture Hebrew University of Jerusalem).

RESULTS AND DISCUSSION

The isolation of bovine skin LC from EC suspensions by immunomagnetic beads technique proved to be efficient. This method is rapid, makes it easy to work in sterile conditions, an gives a good yield with a high degree of purity (>95%). In addition, it does not require expensive equipment. Despite the fact that the beads remained attached to the isolated Langerhans cells, the rosetted LC remained viable and functional.

This study presents the results of a typical experiment on the morphology of bovine skin LC. LC in the skin have a dendrite shape (Fig.1) Scanning electron microscoy and light microscopy depicted LC with short dendrites, but with attached magnetic beads revealing their property of having Ia antigen on the cell surface (Figs. 2 and 3).LC preparation which were incubated in HD conditioned medium (Table 1) increased in number (from 3 x 10^5 LC/ml to 4.5 x 10 LC/ml) within 83 hrs of incubation in vitro. The viability of LC in the HD conditioned medium was 90% (Table 1). In the HD conditioned medium, cells which resemble dividing cells were noted (Fig. 4). Such cells were not seen in LC preparations incubated in RPMI 1640 with 10% FCS medium. The viability of LC in this non-conditioned medium was only ~50% at 83 hrs of in vitro incubation. The experiment in which LC were incubated in HD conditioned medium was repeated 3 times with identical results. The results of this study provide a new insight on the method for purification of LC and especially on the viability of LC in vitro. It was noted that the viability of the LC which were incubated in HD conditioned medium was maintained for 5 days. This could be due to the presence of cytokines like GM-CSF, IL-1 and TGF-beta in the conditioned medium (19). Indeed it was reported (20,21) that TNF alfa maintains viability of murine LC in vitro and GM-CGF and IL-1 mediate the maturation of skin LC into

potent immunostimulatory dendritic cells. The effect of the HD conditioned medium on the ability of the LC to divide is of interest and requires further elucidation. Since the transformed HD cells are thought to be of dendritic cell origin (22) it is possible that the above mentioned cytokine, and possibly unknown cytokines, allow the maturation of the bovine skin LC and their ability to function under in vitro conditions. Further studies are in progress to define the properties of cytokine stimulated bovine skin LC.

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Legend to Figures

1. Bovine skin Langerhans cells (400)
2. Scanning electron microscopy of a skin Langerhans cell isolated by magnetic beads (4800)
3. IF of rosetted skin bovine Langerhans cells after incubation with monoclonal antibody TH14B anti MCH class II followed by incubation with magnetic particle coated with sheep anti mouse IgG and FITC goat anti mouse IgG.
4. IF of skin bovine Langerhans cells after 66 hours incubation with conditioned medium L 428 HD.

Table 1

Viability of isolated bovine LC incubated in conditioned medium from HD L-428 cells

TABLE 1

Viability of isolated bovine LC

incubated in conditioned medium from HD L-428 cells

Hrs	0 % HD conditioned medium			1 0 0 % HD conditioned medium			Volume
	Total Cells	# dead cells	% dead cells	Total Cells	# dead cells	% dead cells	
0	$2.77 \cdot 10^5$	$1.5 \cdot 10^4$	5.41 %	$3.00 \cdot 10^5$	$0.50 \cdot 10^4$	1.6 %	2 ml
18	$2.82 \cdot 10^5$	$8.53 \cdot 10^4$	30.[illegible] %	$4.24 \cdot 10^5$	$3.41 \cdot 10^4$	8.04 %	1.95ml
83	$2.99 \cdot 10^5$	$13.40 \cdot 10^4$	44.8 %	$4.46 \cdot 10^5$	$4.75 \cdot 10^4$	10.65 %	1.90ml
125	$3.01 \cdot 10^5$	$1.40 \cdot 10^5$	46.5 %	$4.50 \cdot 10^5$	$1.08 \cdot 10^5$	22.2 %	1.85ml

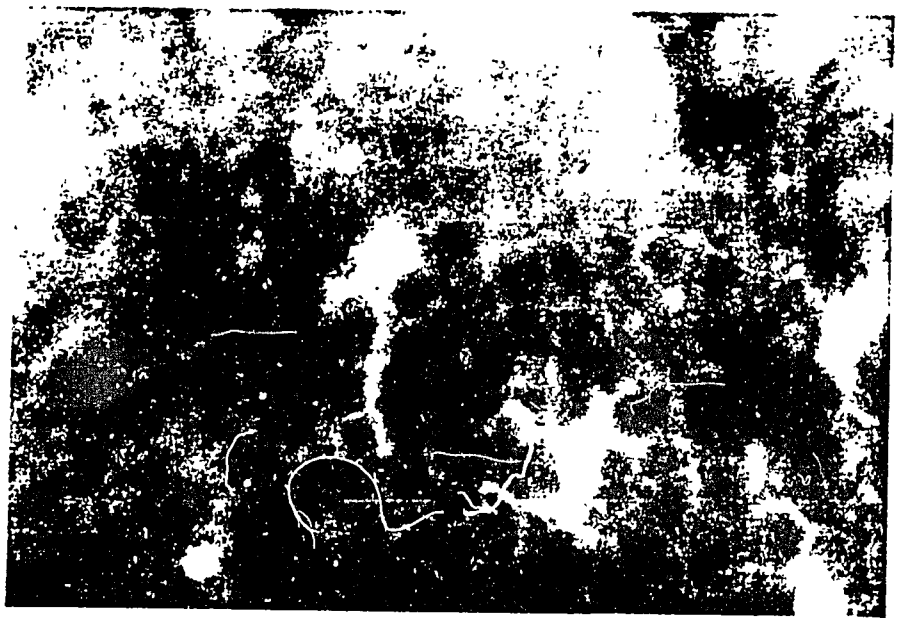

Fig. 1

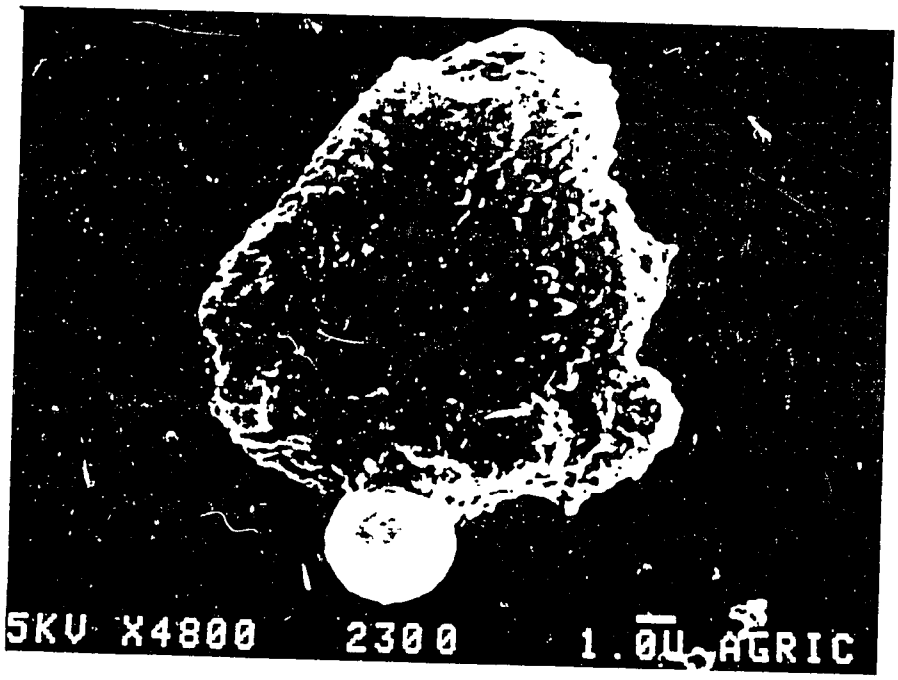

Fig. 2

Fig. 3

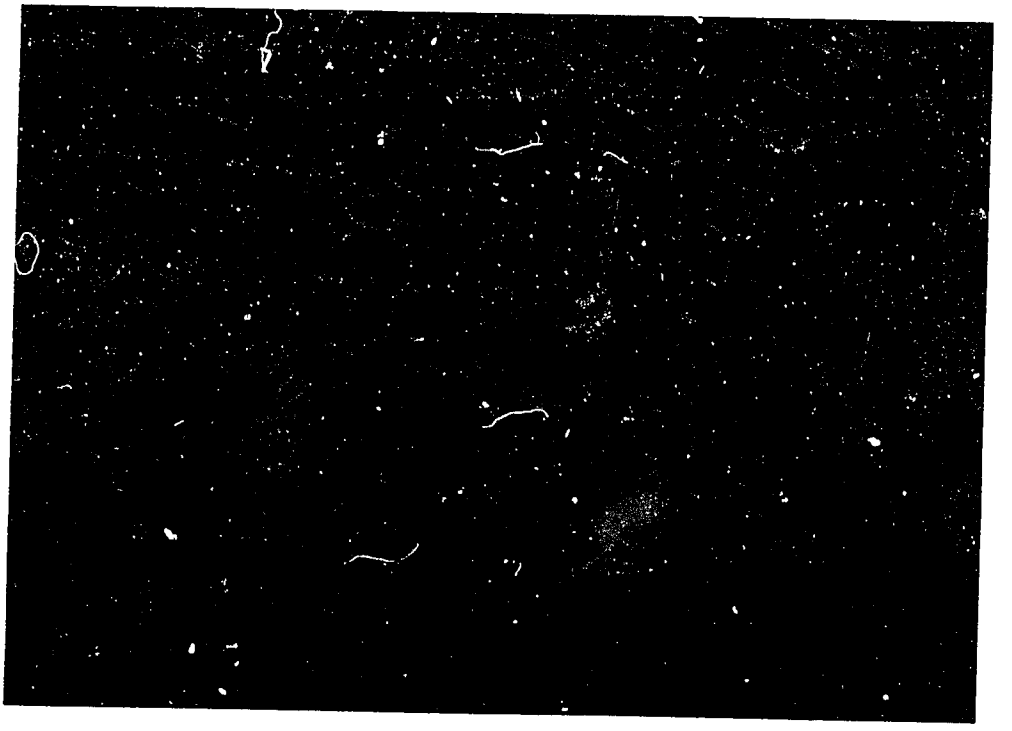

Fig. 4

11. Report by Dr. Teresa Nu

S. R.

MINISTÉRIO DA AGRICULTURA, PESCAS E ALIMENTAÇÃO

DIRECÇÃO-GERAL DA PECUÁRIA

LABORATÓRIO NACIONAL DE INVESTIGAÇÃO VETERINÁRIA

1

REPORT

on the visit to Israel of Teresa N. Gonçalves Nú, to study the PCR technology for FMDV diagnosis, as part of the collaborative

US-Israel-Portugal CDR Project C7-152

The visit took place between April 28th and May 25th 1991 and included studies in:

1 - The Department of Molecular Virology, in the Hebrew University of Jerusalem, with Prof. Yechiel Becker and collaborators

2 - The Kimrom Veterinary Institute, FMDV laboratory and safety unit, with Dr. Hagai Yadin and collaborators.

1 - In the Department of Molecular Virology:

The polymerase chain reaction (PCR) was used as a molecular diagnostic tool for the detection of FMDV RNA, which had previously been extracted at Kimrom safety lab from infected cell cultures, bovine skin Langherhans cells (LC) and clinical material.

Furthermore it was possible to get aquainted with a new technique for FMDV RNA extraction, based on magnetic separation technology, using Dynabeads oligo (DT) 25, which selectively remove polyadenylated RNA from FMDV infected material and forward it without further purification for cDNA synthesis followed by PCR amplification.

The advantages obtained from the combination of the two techniques for RNA isolation and amplification enable a very rapid and accurate diagnosis of field cases of FMD.

It is possible to detect very low yields of virus from small biological samples, avoiding the need for multiple passages in cell cultures which can lead to mutations in the VP1 sequence coding for the antigenic domain.

2 - At Kimrom Veterinary Institute, FMDV Laboratory and safety unit:

Collection of samples from animals suspected of FMD; treatment of samples and routine diagnostic tests (inoculation into baby mice and cell cultures, complement fixation and ELISA tests).

RNA extraction and purification; treatment of bovine skin with proteolitic enzymes in order to release epidermal Keratinocytes and Langerhans cells (LC).

Immunomagnetic technology, usind Dynabeads coated with monoclonal antibodies to separate and purify LC.

Lisbon, August 14,1991

Maria Teresa Neves Gonçalves Nó