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Detection of African swine fever virus by a biotinylated DNA probe: assay on cell cultures and field samples

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Summary – African swine fever virus was detected in various samples using a molecular hybridization technique. A fragment located in a constant area of the viral genome was biotin-labelled. This probe, when present at a concentration of 100 ng/ml of the hybridization solution, could detect 10 pg of target DNA immobilized on nitrocellulose with cellular DNA and RNA. The virus was evidenced after being passaged on monkey kidney cells, either 8 h post-inoculation (pi) if the multiplicity of infection (MOI) was at least 1 hemadsorbing unit (HAD) per cell, or 24 h later if the inoculum was diluted up to 10^{-3} HAD per cell. When passaged on pig leukocytes with a MOI of 0.1 HAD per cell, the virus was evidenced 12 h pi, or 24 h pi with a MOI of 10^{-2} HAD per cell. The probe did not hybridize with another DNA virus passaged on cells, neither did it react with non-infected blood or ham, but did so if African swine fever virus was resuspended with the samples. The spleen from uninfected pig and the lymph nodes from a pig which had died from hog cholera were found to be negative, whereas the spleen from a pig which had died of African swine fever was positive. These samples were also tested with a ^{32}P -labelled probe whose sensitivity was 10-fold higher. A non-radioactive probe could be used both for the sensitive and specific diagnosis of African swine fever and the detection of the virus in an epidemiological survey.

African swine fever / molecular hybridization / non-radioactive probe

Résumé – Détection du virus de la peste porcine africaine par une sonde d'ADN marquée à la biotine: essais en cultures de cellules et sur prélèvements biologiques. Le virus de la peste porcine africaine a été détecté dans divers échantillons par hybridation moléculaire. Un fragment situé dans une région constante du génome a été marqué à la biotine. Cette sonde, à la concentration de 100 ng/ml, a détecté 10 pg d'ADN cible. Le virus a été mis en évidence après passage sur cellules MS, 8 h après inoculation avec une multiplicité d'infection (MI) d'au moins 1 unité hémadsorbante (HAD) par cellule, ou 24 h plus tard avec l'inoculum dilué jusqu'à 10^{-3} HAD par cellule. Le

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virus cultivé sur leucocytes de porc avec une MI de 0,1 HAd par cellule a été détecté 12 h après infection, ou 24 h après infection avec une MI de 10^{-2} HAd par cellule. La sonde n'a pas réagi avec un autre virus à ADN, ni avec un échantillon de sang ou de jambon non infecté, mais uniquement avec les prélèvements dans lesquels du virus de la peste porcine Africaine avait été rajouté. La rate de porc sain et les ganglions de porc mort de peste porcine classique étaient négatifs, alors que la rate du porc mort de peste porcine Africaine était positive. Ces échantillons ont également été testés avec la sonde marquée au ^{32}P , dont la sensibilité est 10 fois supérieure. Une sonde non-radioactive pourrait donc servir au diagnostic sensible et spécifique de la peste porcine Africaine, ainsi qu'à la mise en évidence du virus dans divers produits, dans le cadre de suivis épidémiologiques.

peste porcine Africaine / hybridation moléculaire / sonde froide

INTRODUCTION

African swine fever (ASF) is a viral disease which occurs in many countries in Africa, Europe and South America (Hess, 1981). Its varying clinical expressions range from peracute, acute to subacute, chronic and non apparent forms. In the absence of a vaccine, its eradication depends on quick detection and elimination of infected animals and their derivatives. These play a major part in the spread of the disease.

The routine techniques in use for the diagnosis of ASF are mainly hemadsorption in buffy coat cultures, direct and indirect immunofluorescence and the enzyme linked immunosorbant assay (ELISA) (Sanchez-Botija *et al*, 1977; Sanchez-Vizcaino, 1987). Although these are quite simple and useful techniques, they are inappropriate in some cases: the emergence of non-hemadsorbing strains of viruses (Pini and Wagenaar, 1974; Sanchez-Botija *et al*, 1977) renders the hemadsorption method ineffective; the sensitivity of direct immunofluorescence has decreased since 1970 (Sanchez-Botija *et al*, 1977). A reliable diagnosis was based

on well preserved samples with a low amount of contamination or damage, and still containing living viral particles or undegraded antibodies. This is not the case with cooked pork derivatives, which could be controlled for an epidemiological survey of infected herds.

Among the recent techniques developed, hybridization with biotinylated probes has been used in the detection of DNA viruses (Dorman *et al*, 1985). Previous publications (Caballero and Tabares, 1986; Tabares, 1987) have discussed the possibility of using a DNA probe for the detection of the ASF virus. The aim of this study was to determine the limits of use of a biotin-labelled probe under various conditions. The biotin-labelled probe was tested for the detection of the virus passaged on cell cultures or extracted from field samples.

MATERIALS AND METHODS

Virus and cells

The Spain E-70 strain of ASF virus was used after adaptation to MS green mon-

key kidney cells (Tabares *et al*, 1987), cultured in 175-cm² plastic flasks, in Dulbecco's modification of Eagle's minimum essential medium (DME) (Dulbecco and Freeman, 1959) with 5% calf serum. Virus infectivity was measured by the hemadsorption method (Malmquist and Hay, 1960). The pseudorabies virus (Aujeszky's disease, Toulouse strain) was passaged on MS cells in DME containing 5% calf serum. Pig buffy coat cells were prepared from total blood as described previously (Malmquist and Hay, 1960) and diluted in 30% serum in Earle's medium to give $\approx 2 \times 10^6$ cells per ml. Two ml of the cell suspension were placed in plastic tubes held in a stationary rack at 37°C.

Field samples

Lymph nodes were taken from a pig which had died from naturally contracted hog cholera disease, and were subsequently maintained at -20°C.

Spleen was taken from a pig which had died from a natural ASF infection. Half of the spleen was lyophilized prior to congelation and the other half kept frozen at -20°C. Viral titer measured by hemadsorption was 2.5×10^6 hemadsorbing units (HAD) per 50 mg lyophilized spleen. Control spleen, total defibrinated blood and cooked ham were taken from uninfected pigs.

Infection of cell cultures

Infection of MS cells

When MS cells were confluent (2-d-old cultures), the medium was removed and replaced by a viral suspension of ASF virus. 175-cm² plastic flasks were infected with 8 ml of 10-fold dilutions of the viral inoculum, beginning with a multiplicity of infection of 1 HAD/cell. There was a 2-h contact between the virus and the cells, after which the cells were washed once with PBS and covered with 50 ml DME. The cells were harvested at different times post-infection.

One 175-cm² flask of MS cells was inoculated with a 8-ml suspension of pseudorabies virus, at an MOI of 1 plaque forming unit/cell. The cells were harvested 24 h post-infection, when the cytopathic effect was pronounced.

Infection of buffy coat cells

Three tubes of buffy coat cells cultures were used for each serial experiment. The nutrient medium was removed on the 3rd day of incubation. Buffy coat cells were inoculated with an MOI of 0.1 HAD per cell (4 series), 10^{-2} HAD/cell, 10^{-3} and 10^{-4} HAD/cell, with an inoculum of 200 μ l per tube. After a 2-h contact, the leukocytes were covered with 2 ml of nutrient medium. Three uninfected tubes were kept as controls.

Preparation of the DNA probe

The viral DNA fragment H was a gift from E Tabares (Facultad de Medicina, Universidad Autonoma, Madrid, Spain). It was generated by digestion of the ASF virus DNA with the *Cla*I enzyme (Caballero and Tabares, 1986) was 5.8 kb long and located in the central and constant area of the genome (Blasco *et al*, 1989). The viral fragment H was then inserted into the blue scribe plasmid (a derivative of pUC 19) (Maniatis *et al*, 1982), produced in host bacterium *E coli* HB101 (RecA⁻). The viral H fragment was then purified using the Nal-glass powder method (Vogelstein and Gillespie, 1979).

Part of the purified viral H fragment was biotin or ³²P-labelled by nick-translation following the instructions supplied with the BRL kit (Bethesda Research Laboratories, Gaithersburg, USA). The recombinant plasmid was also biotin-labelled and used for the detection of virus in naturally infected organs, in comparison with the purified fragment.

Preparation of nucleic acids from samples

Extraction from cell cultures

The plastic flasks and tubes containing infected and uninfected cells were rinsed twice with PBS at 37°C. Then the cells were incubated for 1 h at 37°C with 4 ml (175-cm² flasks) or 500 μ l (tubes) of 50 mM Tris-HCl pH:8.8, 30 mM EDTA pH:8, 0.5 M LiCl, 0.5% SDS, 0.1% β -mercaptoethanol and 100 μ g/ml proteinase K

(Boehringer Mannheim Biochemicals). The viscous lysate was extracted twice with phenol. The aqueous phase was precipitated in 2.5 vol cold ethanol. The DNA precipitated in long threads that could be rolled up around a Pasteur pipette and dissolved in 2 ml (175-cm² flasks) or 100- μ l (tubes) of Tris-EDTA pH 7.5 (TE). The RNA was obtained by further precipitation in ethanol and centrifugation at 4 000 *g* for 30 min. Both RNA and DNAs were quantified by agarose gel electrophoresis.

Extraction from field samples

The samples were prepared as described elsewhere (Caballero and Tabares, 1986). Briefly, 1-g samples were cut into 2–3-mm³ pieces. Lyophilized spleen (50 mg) and total defibrinated blood (100 μ l) were dissolved in 100 μ l of TE. The cells were disrupted in a Dounce homogenizer. The final volume was adjusted to 1 ml with TE buffer. The samples were treated for 10 min with 1.5 ml NaOH 0.2 N, 2% SDS. After 10 min sonication, they were neutralized by 0.1 N HCl and extracted twice with phenol. The DNA was then precipitated in ethanol and dissolved in 200 μ l of TE.

Hybridization and visualization of hybrids

The samples were applied to nitrocellulose and hybridized to the probe as previously described (Tabares, 1987). The hybrids were visualized by treatment with streptavidin alkaline phosphatase conjugate and straining with the dyes nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP), according to a previous publication (Leary *et al*, 1983). When the ³²P-labelled probe was used, it had a specific activity of $\approx 2 \cdot 10^9$ dpm/ μ g. The hybrids were visualized by autoradiography.

Indirect immunofluorescence test

This test was applied on a smear of infected spleen: after binding to a coverslip, the smear was incubated with serum anti-ASF virus (from a pig which had died of

African swine fever, diluted 1/80) for 30 min at 37°C, rinsed twice in phosphate buffered saline pH 7.5 (PBS) and incubated for 30 min at 37°C with FITC labelled goat immunoglobulins anti-swine IgG (BioSys, Compiègne, France). Finally, the smear was rinsed twice in PBS.

RESULTS

Sensitivity of the method

The sensitivity of ASF virus DNA detection was determined after the application of serial 10-fold dilutions of purified and denatured viral H fragment (1 pg–10 ng) to nitrocellulose membranes. Using the H fragment as a biotin-labelled probe, 10 pg of target DNA were detectable. As the size of the viral DNA is ≈ 170 kb (Santurde *et al*, 1988), and the size of the viral fragment H is 5.8 kb, the biotinylated probe at a concentration of 100 ng/ml detects 300 pg of genomic viral DNA.

Detection of ASF virus in cell cultures

Detection in MS cells

Viral and cellular DNA and RNA were collected at different stages after inoculation in cell cultures. There was no hybridization with the DNA from uninfected cells. At 8 h post-infection the virus was detected, although no cytopathic effect was yet evident. From 18–72 h post-infection it was not possible to differentiate target DNA levels. When the RNA was applied to nitrocellulose with the DNA there was no significant improvement in the earliest point at which the cell cultures might be detected as positive.

The ASF virus DNA could be detected in the extract from the cells infected with 1 , 10^{-1} and 10^{-2} HAd per cell. When the MOI was 10^{-3} HAd per cell the signal became too weak for the virus to be detected in $< 10^6$ cells. It was not possible to show the presence of the virus in cells infected with an MOI of 10^{-4} HAd/cell. Hybridization with both DNA and RNA, as compared to hybridization with DNA only, did not significantly increase the sensitivity.

Detection in buffy coat cell cultures

With a MOI of 0.1 HAd/cell, the probe detected the viral DNA extracted from 3 tubes 12 h post-infection. The signal was too faint at 8 h post-inoculation to be considered positive. With a MOI of 10^{-2} HAd/cell, the probe detected the virus 24 h post-infection, but not with a lower MOI.

Specificity of the virus detection

The pseudorabies virus was passaged on MS cells, and the DNA was then extracted and assayed with the biotinylated probe. No hybrids could be visualized, even when the DNA from 2×10^6 cells was deposited on the paper (fig 1).

Detection of virus in pork derivatives

No hybridization was observed between non-infected blood or non-infected cooked ham and the probe (fig 2); when ASF virus (10^4 HAd) was added to the samples before extraction of the DNA, there was an intense colored reaction in the

corresponding spots on nitrocellulose (fig 2).

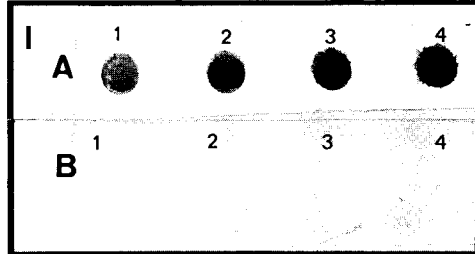


Fig 1. Detection of virus at 24 h post-infection A. MS cells were infected with ASF virus with a MOI of 1 HAd/cell. B. MS cells were infected with pseudorabies virus with a MOI of 1 plaque forming unit/cell. 1: 10^5 cells. 2: $5 \cdot 10^5$ cells. 3: 10^6 cells. 4: $2 \cdot 10^6$ cells.

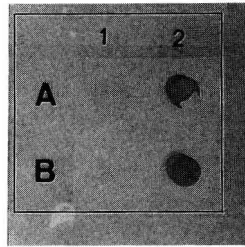


Fig 2. Detection of virus in pork derivatives. The DNAs from total defibrinated blood (A) and cooked ham (B) were deposited on nitrocellulose and hybridized with the biotin-labelled probe. 1: Uninfected products. 2: $100\mu\text{l}$ of an ASF virus suspension containing 10^5 HAd/ml were added to the uninfected extract.

Detection of virus in naturally infected organs

The biotinylated probe did not hybridize with the spleen from a non-infected pig (fig 3), neither did it show a non-specific reaction with the lymph nodes from an animal which had died of hog cholera. When

tested on the spleen of a pig which had died of ASF, the probe detected the virus in 1/200 of the extract prepared from 1 g of samples (or 50 mg if the tissue was lyophilized). As the viral titer of the lyophilized spleen was 2.5×10^6 HAd/ 50 mg, this limit represented 1.25×10^4 HAd (fig 3).

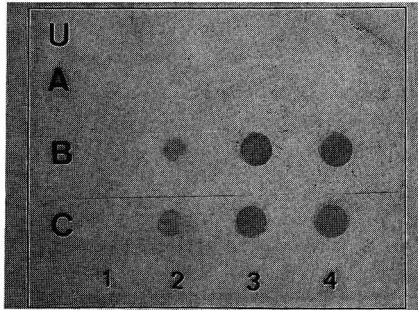


Fig 3. Detection of virus in naturally infected organs. Of the 200 μ l of DNA dissolved in TE buffer, 0.1 μ l (1), 1 μ l (2), 5 μ l (3) and 10 μ l (4) were deposited on nitrocellulose paper. They were hybridized with the biotin-labelled probe. U: Uninfected pig spleen. A: Lymph nodes from pig infected with hog cholera B: Lyophilized spleen from an ASF infected pig. C: Spleen from an ASF-infected pig.

The biotin-labelled recombinant plasmid detected the positive specimen, but the results were not significant due to a high background level with the non-infected samples.

When compared with the biotin-ylated probe, the ^{32}P -labelled probe presented the same specificity, *ie* no hybridization with samples from non-infected or hog cholera-infected animals (fig 4). The sensitivity was greater, since as little as 1/2 000 of the extract from the lyophilized spleen of the ASF-infected pig was sufficient to show the presence of the virus. This corresponded to 1.25×10^3 HAd.

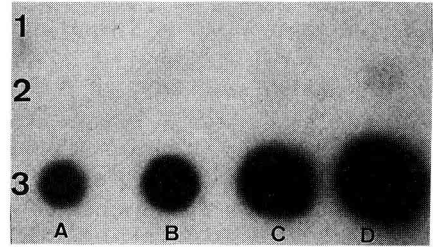


Fig 4. Hybridization with a ^{32}P -labelled probe. 1: Uninfected spleen. 2: Lymph nodes of a pig infected with hog cholera. 3: Lyophilized spleen of a pig infected with ASF. Volume of the samples deposited on the paper A: 0.1 μ l. B: 1 μ l. C: 5 μ l. D: 10 μ l. The hybridization solution had a specific activity of 2.3×10^6 dpm/ml. The autoradiographs were developed after a 30-h exposure.

No virus could be displayed by direct immunofluorescence: a diffused fluorescence was observed all over the smear, masking the specific reaction due to the viral antigens.

DISCUSSION

When used at a concentration of 100 ng/ml, the probe can detect 10 pg of target DNA. Our intention was to work as closely as possible to field conditions, when the virus is present in infected cells. Its DNA was mixed with comparatively large amounts of cellular DNA and RNA from uninfected cells for this purpose. Our results are consistent with those of other authors (Sanchez-Vizcaino, 1987), although the latter used the probe at 1 $\mu\text{g/ml}$, a concentration at which too many background problems were met. Contrary to what had been described, using a probe concentration not exceeding 100 ng/ml and the purified viral fragment in-

stead of the recombinant plasmid gave the best results: some non-specific signal appeared in the latter case, possibly due to the hybridization of the plasmid part of the probe with related DNA sequences present in the biological sample extracts.

As the length of the viral genome of the ASF virus is 170 kb, 10 pg of target DNA corresponds to an equivalent of $\approx 2 \times 10^6$ viral particles. This is the theoretical limit of this biotin-labelled probe. Our results indicate that the probe can show the presence of virus in blood or ham extract loaded with only 10^4 HAd. This apparent discordance reflects the ability of the probe to hybridize with killed or defective as well as with non-hemadsorbing viruses, and to generate a positive test with samples containing less infectious or hemadsorbing units than it is theoretically possible to detect.

The probe can also detect the ASF virus as early as 8 h post-inoculation in MS cell cultures infected with a MOI of 1 HAd/cell. Previous results (Pan *et al*, 1980) indicate that the first progeny virus associated with cells appears between 7–8 h post-inoculation. The first progeny virus therefore detected by the probe is at 8 h post-inoculation. The increase in intensity of the reaction reflects the increase in cell infectivity.

As the culture of ASF virus on MS cells requires previous adaptation, a similar experiment was held with buffy coat cells, which are spontaneously susceptible cells (Malmquist and Hay, 1960). The results indicate that the probe can detect the virus 24 h post-infection in leukocytes infected with 4×10^4 HAd per tube.

This means that low-infective samples, for example from carrier pigs and chronic or subclinical cases, could be positive if the virus multiplies in susceptible cells previous to DNA extraction.

The sensitivity threshold is not improved when both viral RNA and DNA are spotted on the membrane. This could be due to the low level, the absence or an incomplete transcription of the genomic area corresponding to the probe.

Previous authors (Heuschele *et al*, 1966) have investigated the immunofluorescence test in porcine kidney cell cultures infected with ASF virus, and have observed specific staining of the infected cells 10 h post-infection. This delay is comparable to the point at which the probe can detect the virus in cell cultures.

When the biotinylated probe was tested on samples from a pig which had died during the ASF outbreak in 1964 in Perpignan (France), the hybrids could be easily visualized, indicating that as a probe the H fragment can detect different strains of viruses.

The ASF virus was not evidenced by the indirect immunofluorescence test on spleen smear. Two reasons may account for the high background level: first, the spleen contains IgG which give a non-specific reaction with the FITC conjugate (an alternate method would be to prepare FITC labelled anti-ASF virus pig IgG, which implies more handling). Secondly, the smear acts like a sponge that absorbs the conjugate, resulting in a diffused fluorescence. A non-specific fluorescence has been reported previously by authors who prepared smears from various specimens

(Heuschele *et al*, 1966), although fluorescent anti-ASF antibodies were used.

The hybridization reaction was specific: the probe did not hybridize with non-infected cells, blood, spleen or ham.

There was no reaction with hog cholera virus. It is a RNA virus, and is radically different from ASF virus (Maurer *et al*, 1958). However, as hog cholera is clinically indistinguishable from ASF, laboratories are often asked to make a differential diagnosis between the 2 diseases.

Neither did the probe hybridize with the pseudorabies virus. The ASF sometimes has a nervous expression that can be mistaken for pseudorabies (Ben-Porat and Kaplan, 1980). This disease is caused by a double-stranded DNA virus, which produces a cytopathic effect on leukocytes, as does the ASF virus. For these reasons it was important that the probe should not hybridize with this virus.

We have confirmed that the presence of the ASF virus can be detected by a biotinylated probe, and that no non-specific reactions occurred with biological samples. Comparing this technique with other diagnostic methods, it offers the advantages of being very sensitive, specific and of detecting non-hemadsorbing strains of viruses, small amounts of virus and killed or defective viruses, which is particularly evident when one compares the positive hybridization limit with infective viral units. This probe should therefore prove to be of interest within the framework of an epidemiological survey, mainly for pork derivatives imported from infected countries. Being non-radioactive, this probe is non-

hazardous and of great stability: it could be used for a routine diagnosis in veterinary department laboratories, either alone or together with the polymerase chain reaction technique.

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