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DETECTION OF ANTIBODIES AGAINST HOG CHOLERA VIRUS AND BOVINE VIRAL DIARRHEA VIRUS IN PORCINE SERUM

A COMPARATIVE EXAMINATION USING CF, PLA AND NPLA ASSAYS

By

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JENSEN, MERETHE HOLM: *Detection of antibodies against hog cholera virus and bovine viral diarrhea virus in porcine serum. A comparative examination using CF, PLA and NPLA assays.* Acta vet. scand. 1981, 22, 85—98. — The antibodies in serum samples from an outbreak of low-virulent hog cholera in Spielbach, West Germany, 1966, as well as serum samples from pigs inoculated with hog cholera (HC) virus and bovine viral diarrhea (BVD) virus, respectively, were examined by means of 3 different methods:

1. A modified direct complement fixation (CF) test,
2. A peroxidase-linked antibody (PLA) assay based on microplates with fixed, viral-antigen containing cells,
3. A neutralization assay carried out in microplates using the "chessboard" principle and read by means of the peroxidase-linked antibody (NPLA) assay.

A good correlation was found in their ability to detect the antibodies. Generally neutralizing antibodies could be found 2 weeks after inoculation. By CF and PLA antibodies could be detected at the same time or up to 2 weeks later. All sera were tested by the 3 methods against both HC viral antigen and BVD viral antigen. HC-antibodies could not be distinguished from BVD-antibodies by CF but to a certain degree by PLA. BVD-antibodies could to a certain degree be distinguished from HC-antibodies by CF but not by PLA. This means that CF and PLA together provide a good possibility for differentiation between the two types of antibodies. NPLA could to a high degree of reliability distinguish between HC-antibodies and BVD-antibodies.

hog cholera; bovine viral diarrhea; porcine serum; antibodies; peroxidase-linked antibody; microneutralization assay.

In the diagnosis of hog cholera (HC) serological examinations can give a valid answer regarding the presence or absence of the disease. Under certain conditions the demonstration of antibodies against HC-virus may be the only way of tracing the infection, e.g. in cases of subclinical HC (*Terpstra* 1977 b). Furthermore, in HC-free countries it can be of importance currently to document the absence of the disease by serological surveys of the pig population. Under such conditions crossreactions between HC-antibodies and antibodies against the virus of bovine viral diarrhea (BVD) may create problems.

Immunoelectro-osmophoresis (*Terpstra* 1977 a) as well as complement fixation (*Eskildsen & Overby* 1976, *Eskildsen* 1977) can be used in a screening for antibodies against HC-virus, but they do not differentiate between antibodies against HC-virus and BVD-virus. Sera giving positive reaction must be examined by neutralization tests, i.e. plaque reduction test (*Korn & Matthaeus* 1971) or neutralization immunofluorescent test (*Liess & Prager* 1976).

In 1977 *Saunders* described an enzyme-labeled antibody test (ELA) for the detection of antibodies against HC-virus. He used microplates containing fixed cells infected with HC- or BVD-virus, respectively, as well as fixed, non-infected, control-cells. In other words a technique analogous to indirect immunofluorescence as described by *Ressang & den Boer* in 1969. Based on the use of ELA technique for the detection of antigen-containing cells a "chessboard" neutralization assay for non-cytopathogenic viruses and their corresponding antibodies was developed similar to the method described by *Booth et al.* (1978) in work on Foot-and-Mouth Disease virus.

In the present study a number of porcine sera were examined for antibodies against HC-virus and BVD-virus by 3 different methods:

1. A modified direct complement fixation (CF) test.
2. A peroxidase-linked antibody (PLA) assay ad modum *Saunders* (1977).
3. A neutralization assay in microplates after the "chessboard" principle and read by peroxidase-linked antibody assay (NPLA).

The methods were compared with regard to sensitivity and ability to differentiate between HC-antibodies and BVD-antibodies.

MATERIALS AND METHODS

Sera

A. A number of 30 serum samples from an outbreak of low-virulent HC in Spielbach, West Germany, in 1966* and 11 negative control sera collected at Lindholm during the period 1963—1967 in connection with HC virus inoculation experiments.

B. Nine pigs of The Danish Landrace (weight 40—50 kg) were experimentally infected and blood samples collected as follows:

Four pigs (SP 1 — SP 4) were inoculated with a Hannover strain of HC-virus*. Each pig was given 1.5 ml virus-containing blood intravenously. Blood samples were collected before inoculation and ½, 1, 2, 3 and 5 weeks after; at this time the pig marked SP 1 was inoculated with a Tübingen strain of HC-virus*. Two ml of virus-containing blood were given intravenously. Blood samples were collected 1 and 3 weeks after this inoculation.

The pigs SP 2, SP 3 and SP 4 were in the same way reinoculated 7 weeks after 1st inoculation and blood samples collected 1 week later.

Four pigs (BVD 5 — BVD 8) were inoculated with BVD-virus (Danish strain Ug 59). Two ml of cell culture virus were given intravenously. Blood samples were collected before inoculation and ½, 1, 2, 4, 5 and 6 weeks after.

One pig (BVD 22 — 6) was inoculated similarly to pigs marked BVD 5 — BVD 8 and blood samples collected before and 1, 2, 7 and 13 weeks after inoculation. At this time the pig was reinoculated intravenously with 2 ml of the same virus preparation and blood samples were collected 2 and 4 weeks later.

Virus

The Japanese ALD strain of HC-virus* and The Danish Ug 59 strain of BVD-virus were used as viral antigens in the CF test as well as for PLA and NPLA assays in microplates. The HC-virus was propagated in primary pig kidney cell cultures. For the propagation of BVD-virus primary calf kidney and calf testis cell cultures were used.

BVD-virus for the inoculation of the pigs was propagated in the porcine cell line MVPK-1 clone 7** grown in medium supplemented with porcine serum.

CF test

The modified direct complement fixation test described by *Eskildsen & Overby* (1976) was used.

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** Kindly provided by Plum Island Animal Disease Center, Greenport, New York, U.S.A.

PLA and NPLA assays

Reagents. Porcine IgG was isolated by the method of *Metzger & Fougereau* (1967) and injected into rabbits using the immunization procedure described by *Harboe & Ingild* (1973). The immunoglobulin fraction of the rabbit antisera was isolated as also described (*Harboe & Ingild*). Conjugation of the immunoglobulins to horseradish peroxidase (Sigma Chemicals type VI) was accomplished by the two-step method described by *Avrameas & Ternynck* (1971). Ten mg of immunoglobulins were conjugated with 10 mg of peroxidase. The conjugate was used in dilutions from 1:100 to 1:600 depending upon the quality.

Fixation fluid, supplemented with 0.02 % serum albumin (bovine or human), and diluents for the peroxidase conjugate and for the antiserum as well as the wash fluid were prepared according to *Saunders* except that Na_2HPO_4 was used in stead of K_2HPO_4 for pH adjustments. Substrate for detection of peroxidase activity was 3-amino-9-ethylcarbazole as described by *Graham et al.* (1965) in connection with staining of histological slides.

Cell cultures. Primary pig kidney cell cultures were grown in Hanks' BSS with lactalbumin hydrolyzate (5 g per l) modified to a $NaHCO_3$ content of 0.75 g per l and supplemented with vitamins as Eagle's MEM, Glasgow modification, and antibiotics (Neomycin 50 mg per l and Streptomycin 100 mg per l). It was supplemented with 5 % fetal calf serum.

PK 15 cells as well as primary calf kidney and primary calf testis cells were grown in Eagle's medium based on Earle's BSS with 2 g glucose per l and with glutamine, aminoacids, inositol and vitamins as Eagle's MEM, Glasgow mod. and antibiotics as above and 5 % fetal calf serum.

Flat-bottom microplates for cell culture were used for the PLA and NPLA assays. Cells from primary pig kidney cell cultures and PK 15 cells were used in the work on HC-virus. The cells from primary calf kidney and primary calf testis cell cultures were used in the work on BVD-virus. Cell suspensions were prepared and the final cell concentration in the wells before incubation was 150,000 per ml for primary pig kidney and primary calf kidney cells. It was 100,000 per ml for PK 15 cells and primary calf testis cells. In all microplate cell cultures Eagle's medium with 2 % fetal calf serum was used for cell growth. The total volume per well was 0.15 ml. The plates were covered with a lid and incubated at 37°C in small boxes holding humidified air with an addition of 5 % carbon dioxide.

Microplates for PLA were inoculated with virus simultaneously with seeding of cells in the way that every second well was given virus and the rest were left as controls. Dose of virus was about 10^3 TCID50 per well. After incubation for 3 days the plates were fixed (see later).

Microplates for NPLA were also inoculated simultaneously with the seed of cells. All serum samples were heat inactivated (56°C

for 30 min). Virus was given in 10 fold dilutions and serum in 2 fold dilutions. Eagle's medium with 2 % fetal calf serum was used as diluent. The neutralization assay was carried out as a two-dimensional „chessboard" titration using 0.05 ml of serum dilution per well in one dimension and 0.05 ml of virus dilution in the other dimension. After mixing, the plates were incubated for 1 h at 37°C as described. Then 0.05 ml of cell suspension was added and the plates were incubated for 4 days. Plates with HC-virus were fixed on day 4. The BVD-virus used was cytopathogenic and allowed the plates to be read by ordinary light microscopy. Fixation and staining was here used occasionally merely as a control measure.

Fixation procedure. After a short rinse in saline solution and a short rinse in fixation fluid the fixation was carried out for 5 min at room temperature. Then the plates were drained thoroughly and allowed to dry at 25—30°C. They were stored in a plastic bag at —20°C.

Staining procedure. Initially all ingredients were heated to 25—30°C. The microplates were prewetted with saline solution for 5 min and then drained thoroughly. Antiserum (i.e. for the PLA assay the unknown serum diluted 1:10 and 1:20 and for the virus detection in the NPLA assay dilution 1:20 of a known positive serum) was added in an amount of 0.05 ml per well. The plates were shaken for about 10 s and then left at 25—30°C for 15 min.

The washing procedure was the same as used by *Saunders* except that saline solution was used in stead of tap water. The plates were drained carefully and 0.05 ml of the peroxidase-conjugate dilution was added per well. After a short shake (about 10 s) the plates were left at 25—30°C for 10 min. The washing procedure as above was repeated and the plates drained thoroughly. The substrate solution, freshly prepared according to *Graham et al.* was added using 0.05 ml per well and after a short shake the plates were left at 25—30°C for 15—30 min. The staining of the cells could be terminated by exchanging the substrate solution with wash fluid.

The staining reaction caused staining of the substrate as well as of the virus infected cells. Macroscopically all positive wells showed a dark red-brown colour, compared to the non stained negative wells. An ordinary light microscope as used for microplate reading revealed foci as small as single cells. It was a staining of the cytoplasm analogous to the finding by means of the immunofluorescence technique (*Mengeling et al.* 1963). The staining of the cells was quite stable for at least some days if plates were kept refrigerated.

In case of non-cytopathogenic virus the reading of the PLA test could be done macroscopically. If there was a difference between the cell-layer in virus-infected wells compared to the cell-layer in control wells, the reading was done by means of a microscope, so that reaction in single cells could be studied.

In the PLA test 2 dilutions of the serum samples (1:10 and 1:20) were initially examined against HC- as well as BVD-antigen. Sera

giving positive reactions were then titrated in two-fold dilutions against the 2 antigens.

Known positive and negative serum were included as controls.

Reading of the PLA test as positive or negative with regard to the presence of specific antibodies in a serum sample was based on comparison of the staining of the virus-containing cells in relation to the staining of the non-infected control cells. The PLA titer obtained was the reciprocal value of the highest serum dilution giving difference in colour.

Reading of the NPLA test. All wells with a staining were recorded as positive without regard to number of stained cells. The neutralization titer obtained was the reciprocal value of the highest serum dilution giving 2 $\log_{10}$ reduction of the virus titer, i.e. neutralizing 100 TCID50 per 0.05 ml. Calculations according to *Kärber* (1931).

RESULTS

Fig. 1 shows the results obtained from testing the field sera from the HC outbreak in Spielbach, West Germany, in 1966, including the negative control sera. Figs. 2 and 3 show results obtained from testing sera from animals experimentally infected with HC-virus and BVD-virus, respectively.

Sensitivity of the tests

Figs. 1, 2 and 3 demonstrate a good correlation between the 3 methods with regard to sensitivity for the homologous antigen.

When CF is compared with NPLA it can be seen that all HC-sera having CF-titer higher than 4 were neutralizing the homologous virus. Likewise all BVD-sera having CF-titer higher than 4—8 were able to neutralize BVD-virus. However, the results from the examination of the sera from Spielbach indicate that CF was less sensitive than NPLA, as 4 of these sera which were shown to contain virus-neutralizing antibodies were negative in CF.

Some of the very early sera from the experimentally infected animals were also negative in CF but positive in NPLA (2 HC-sera and 3 BVD-sera).

When PLA is compared with NPLA it can be seen that all HC-sera having PLA titer of 10 or higher were positive in the homologous neutralization test. Likewise BVD-virus neutralizing antibodies were found in all BVD-sera having PLA

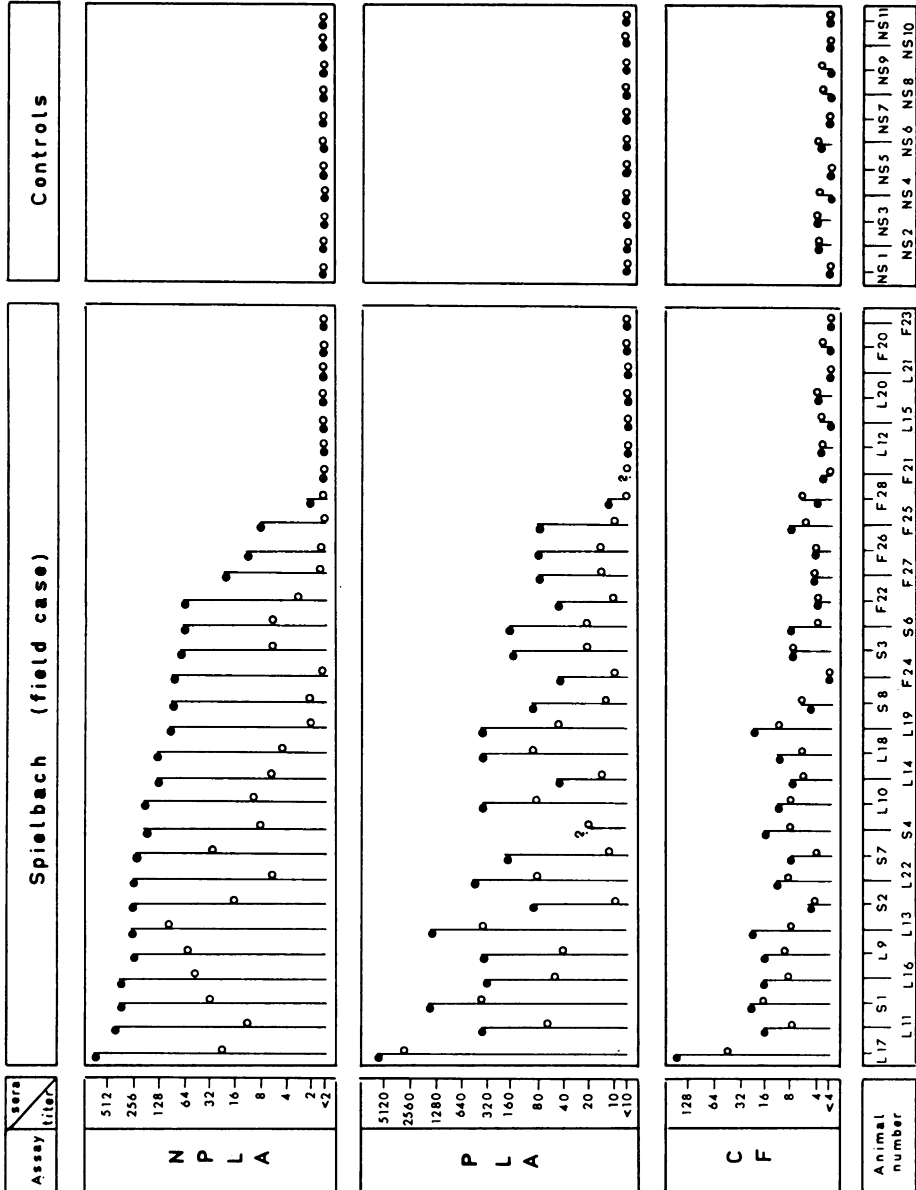
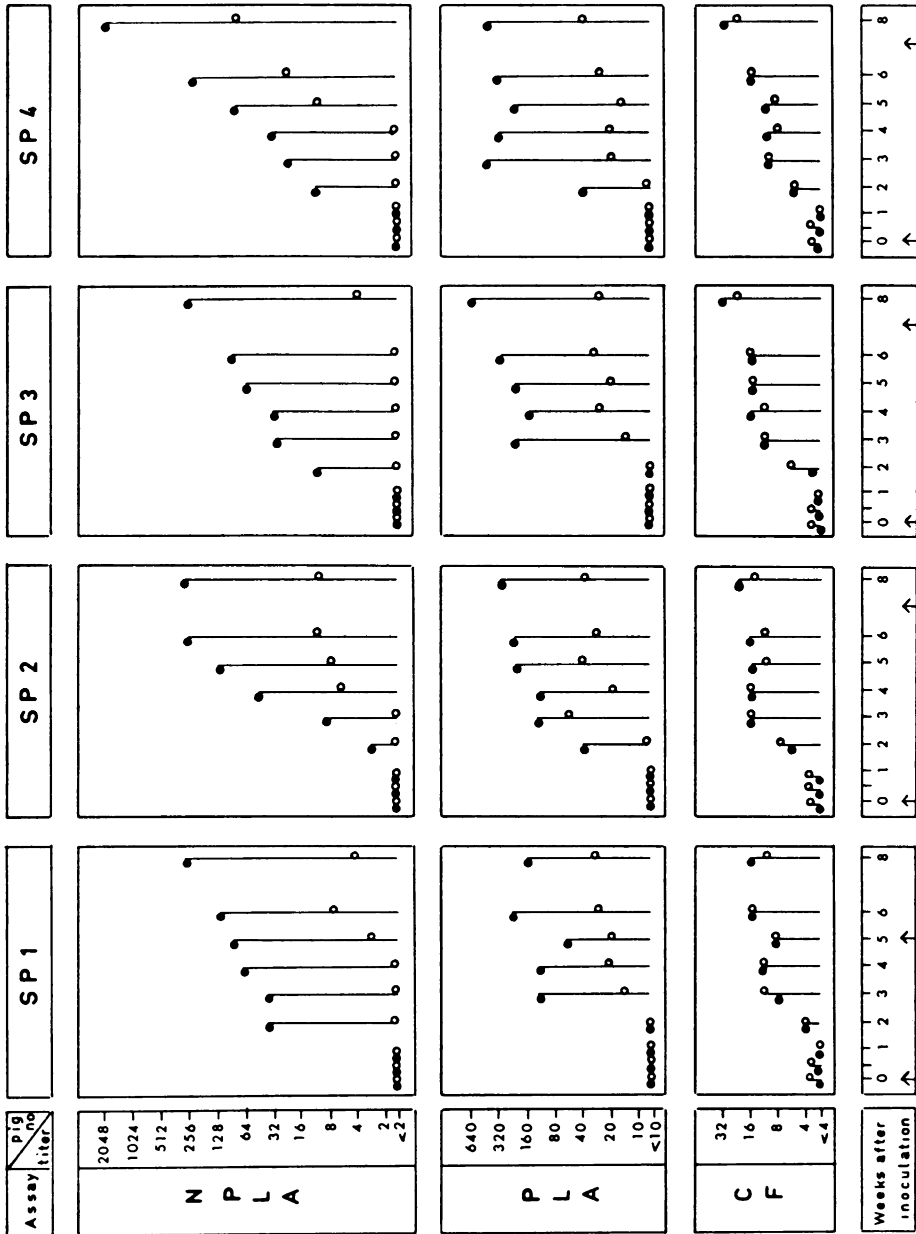


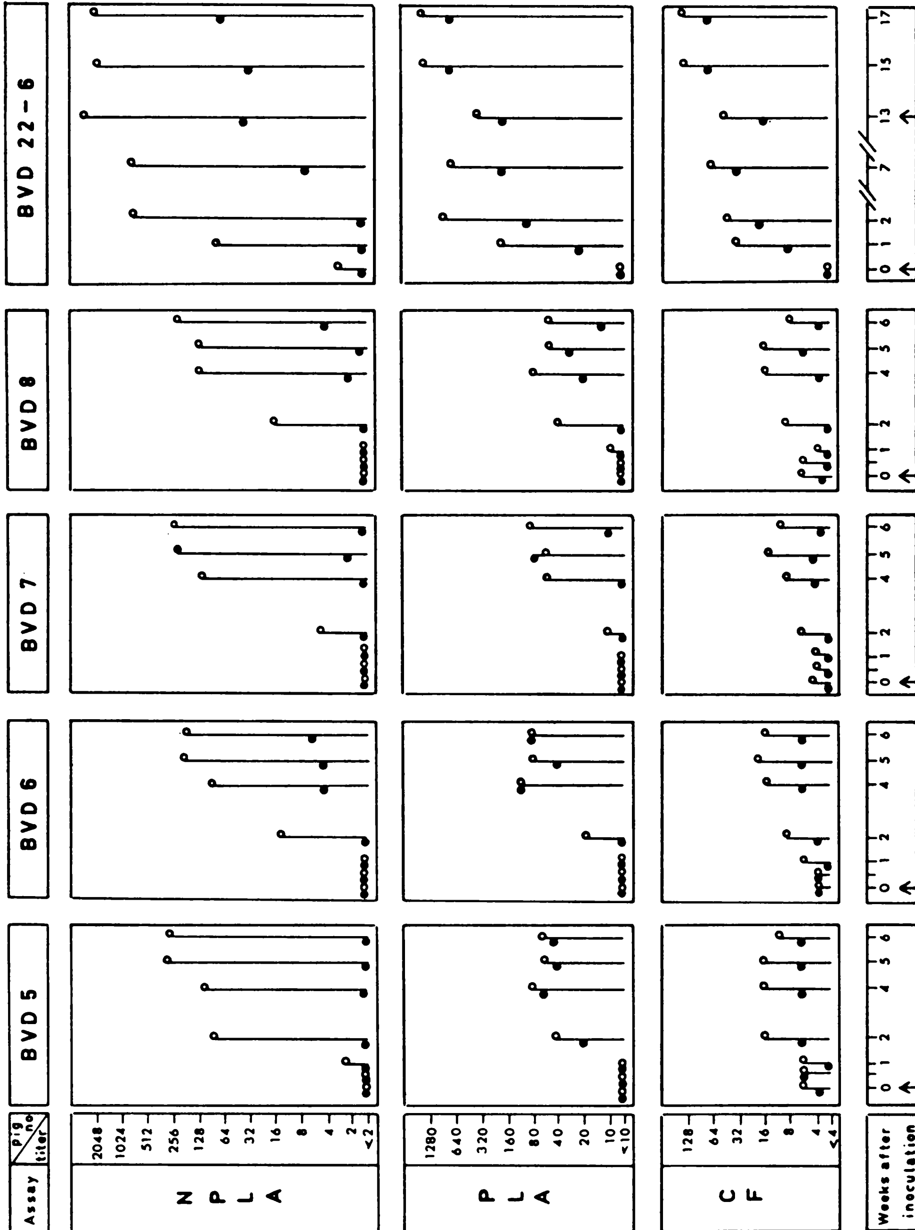
Figure 1. Results obtained from testing the field sera from the HC outbreak in Spielbach, West Germany in 1966.

●——: titers obtained using HC viral antigen
○——: titers obtained using BVD viral antigen



F i g u r e 2. Results obtained from testing sera from animals experimentally infected with HC virus. Arrows indicate time of inoculation.

●——: titers obtained using HC viral antigen
○——: titers obtained using BVD viral antigen



F i g u r e 3. Results obtained from testing sera from animals experimentally infected with BVD virus. Arrows indicate time of inoculation.

●——: titers obtained using HC viral antigen
○——: titers obtained using BVD viral antigen

titer higher than 10. Two of the serum samples from Spielbach could not be read in PLA with HC viral antigen. One did contain HC-virus neutralizing antibodies whereas the other did not.

It can also be seen that PLA was not as sensitive as NPLA regarding detection of antibodies in the very early sera (2 HC- and 3 BVD-sera).

When CF is compared with PLA it appears that PLA was more sensitive than CF because the four Spielbach sera which were negative in CF but positive in NPLA, were all positive in the homologous PLA test. However, the two Spielbach sera which gave problems in reading the PLA, gave no such problems in CF.

Neither CF nor PLA resulted in false positive reactions when compared to the neutralization test.

Crossreaction

Crossreaction can also be studied in the Figs. 1, 2 and 3. The HC-sera all crossreacted to a high degree in the CF, where they gave positive reaction at the same time (in relation to the virus inoculation) and same level for BVD-antigen as for HC-antigen. The BVD-sera also give crossreactions for HC-antigen but the crossreactions came to a lower level and appeared later than did the reaction against the homologous antigen. The HC-sera also gave crossreactions when examined by PLA. However, the reaction against BVD-antigen came a little later than the reaction against HC-antigen, and it persisted at a slightly lower level. BVD-sera exhibited strong crossreaction in PLA. The reaction against HC-antigen came at the same time and practically to the same level as the homologous reaction against BVD-antigen.

Crossneutralization (NPLA) was demonstrated for HC-sera as well as for BVD-sera, but as it can be seen in the experimentally infected animals, the crossreactions appeared far later as compared to the reactions against the homologous virus. The crossreaction persisted in all cases at a considerably lower level than did the reaction against the homologous virus.

DISCUSSION AND CONCLUSIONS

Saunders (1977) demonstrated presence or absence of HC antibodies using ELA technique and found a good correlation

between his method and the serum neutralization test. In the present work it was found, that CF and PLA are sensitive but not quite as sensitive as NPLA in detecting the antibodies in the very early infection. However, by examination of a number of sera from the same herd/area, CF as well as PLA will detect HC/BVD antibodies.

Saunders mentioned the interference caused by BVD-antibodies and also the possibility that the method after further development could be useful for the differential diagnosis. The present work confirmed the possibility to distinguish HC-antibodies from BVD-antibodies using PLA. Comparing PLA and CF the latter seemed to be more useful for distinguishing BVD-antibodies from HC-antibodies. On a herd level a parallel titration against HC and BVD viral antigen in both CF and PLA will provide a possibility for a distinction between HC- and BVD-antibodies. The distinction so obtained seems to be nearly as reliable as by NPLA. Compared to NPLA the use of CF and PLA for differentiation will save time and labour.

It was shown earlier (*Eskildsen & Overby* 1976), that CF can detect HC-antibodies 2 weeks after inoculation. In the present work neutralizing antibodies against HC (NPLA) were detected at the same time or earlier than complement fixing and PLA antibodies. The peroxidase-linked antibody reading was used here for virus detection in a neutralization assay after the "chess-board" principle. The same reading of the microplate can be used in connection with the usual neutralization assay where varying serum dilutions are tested against a fixed amount of virus in analogy to the microimmunofluorescence neutralization test described by *Witte* (1979).

The PLA test was found to be very useful and simple to establish because of the way the antigenic component is fixed in the wells of the microplates. This is in contrast to the ELISA test where the antigen or antibody must be available in concentrated and purified form before adsorption onto the bottom and walls of the plastic wells.

It was concluded, that CF and PLA may both be useful as preliminary methods in screening a large number of pig sera for HC/BVD antibodies. Both methods are sensitive and reliable, and both are rapid (few hours). The materials can be stored ready for use and at least partial automatization can take place.

The procedure in a screening could be as follows:

1. CF or PLA is used with one or two dilutions per serum sample against both antigens.
2. Sera giving positive reaction in step 1 can then be titrated in parallel against the 2 antigens in both CF and PLA in order to establish the differential diagnosis.
3. Doubtfully reacting sera can be examined by NPLA.

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SAMMENDRAG

Påvisning af antistoffer mod svinepestvirus og bovin virus diarrhea virus i porcint serum.

Til påvisning af antistofindhold i sera fra et udbrud af lavvirulent klassisk svinepest i Spielbach, Vesttyskland i 1966, samt i sera fra grise, podet med henholdsvis svinepest (SP) virus og bovin virus diarrhea (BVD) virus blev anvendt 3 forskellige metoder:

1. Modificeret direkte komplementbinding (CF)
2. Peroxidasemærket antistof i forbindelse med fixerede virus-antigenholdige celler i mikroplader (PLA)
3. Neutralisationstitrering udført i mikroplader efter skakbrætsprincippet og aflæst ved hjælp af peroxidasemærket antistof (NPLA).

Der blev fundet god overensstemmelse i antistofpåvisningen ved de 3 metoder. Gennemgående kunne neutraliserende antistoffer påvises 2 uger efter podning, og samtidig hermed eller senest 2 uger derefter kunne antistoffer påvises ved CF og PLA. Samtlige sera blev undersøgt overfor både SP antigen og BVD antigen. SP antistoffer kunne ikke skelnes fra BVD antistoffer ved CF men med en vis sikkerhed ved hjælp af PLA. BVD antistoffer kunne med en vis sikkerhed skelnes fra SP antistoffer ved CF, men ikke ved PLA. Det vil sige, at såfremt både CF og PLA anvendes fås en mulighed for at skelne mellem SP og BVD antistoffer. NPLA kan med stor sikkerhed skelne imellem de 2 typer af antistoffer.

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