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Comparative study of different immunoserological techniques for the detection of antibodies against transmissible gastroenteritis (TGE) coronavirus

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Summary — In order to carry out a sero-epidemiological survey of transmissible gastroenteritis (TGE) infection in a high-density pig-breeding area (the Murcia region, Spain), the sensitivity and the specificity of 5 different techniques were compared. Twenty-four sera from non-infected and infected pigs were analyzed by the following tests: *in vitro* neutralization, indirect ELISA with concentrated virus, indirect ELISA with purified virus, immunocapture ELISA and VELCIA (virus-enzyme linked cell immunoassay). All sera from infected pigs were detected as positive only by seroneutralization, ELISA with purified virus and VELCIA (sensitivity 100%). False-positive sera were detected with several tests (ELISA with purified virus and concentrated virus, ELISA immunocapture with a specificity of 88, 88 and 76% respectively). Only VELCIA provided a specificity as high as that obtained with seroneutralization (100%).

transmissible gastroenteritis (TGE) / coronavirus / enzyme-immuno assay (EIA) / antibody / seroneutralization

Résumé — Étude comparative de différentes techniques immuno-enzymologiques pour la détection des anticorps dirigés contre le virus de la gastro-entérite transmissible (GET). Pour détecter et suivre par sérologie les épidémies de gastro-entérite transmissible (GET), dans une région d'élevage intensif du porc (Murcia, Espagne), nous avons comparé la sensibilité et la spécificité de 5 techniques de séro-détection du virus. Vingt-quatre sérums d'animaux d'élevage ont été utilisés pour comparer les résultats entre la séroneutralisation *in vitro*, l'Elisa indirecte avec du virus concentré ou purifié, l'Elisa par immunocapture et la technique Velcia (virus enzyme linked cell immuno assay). En séroneutralisation, les sérums de porcs non infectés se sont révélés négatifs, alors que les sérums de porcs infectés étaient positifs. Cette technique a été prise comme référence. Seules les techniques Elisa avec du virus purifié et Velcia ont permis de détecter tous les sérums positifs (sensibilité : 100%). Des sérums faussement positifs ont été obtenus avec les 3 techniques immuno-enzymatiques utilisées (Elisa avec du virus purifié ou du virus concentré et Elisa par immunocapture avec des spécificités respectives de 88%, 88% et 76%) mais non avec Velcia (spécificité : 100%). Velcia a donné les résultats les mieux corrélés avec ceux obtenus par séroneutralisation.

gastro-entérite transmissible (GET) / coronavirus / test immuno-enzymologique / anticorps / séroneutralisation

* Correspondence and reprints

INTRODUCTION

Transmissible gastroenteritis (TGE) is a viral disease in swine which is characterized by a short incubation period, high infectivity, diarrhoea, vomiting and a high mortality in piglets < 2 wk old (Bohl, 1989).

Pigs which recover from the disease may become carriers of TGE virus (TGEV) and a source of infection for susceptible animals (Underdahl *et al*, 1975). Strict serological control should be undertaken to detect seropositive animals before their introduction into a herd, to reduce the risk of infection in a non-exposed herd, and before export to countries which require pigs to be free from TGEV infection (Bohl, 1989).

Several immunological methods for detection of TGEV antibodies have been developed, including seroneutralization (Young *et al*, 1953; Harada *et al*, 1967; Bohl *et al*, 1972), bentonite agglutination (Sibinovic *et al*, 1966), complement fixation (Stone *et al*, 1976), leukocyte aggregation (Woods, 1976), agar immunodiffusion (Bohac *et al*, 1975; Stone *et al*, 1976), indirect hemagglutination (Shimizu and Shimizu, 1977; Noda *et al*, 1987), immunoperoxidase (Kodama *et al*, 1980), indirect fluorescent antibody (Benfield *et al*, 1978).

The enzyme linked immunosorbent assay (ELISA) is utilized worldwide for the diagnosis of many viral infections (Winston *et al*, 1987). It has been shown to be a useful serological tool for TGE infection (Nelson and Keeling, 1984) and permits testing of a large number of samples, with a low detection limit (Hohdatsu *et al*, 1987).

Before carrying out a sero-epidemiological survey to determine the prevalence of TGEV infection in a pig-breeding area with a high density of animals (Murcia region, Spain), the sensitivity

and specificity of several methods were compared. Five different techniques to detect anti-TGEV antibodies in sera of sows were used. The following tests were performed: *in vitro* neutralization (Toma and Benet, 1976), indirect ELISA with concentrated virus, indirect ELISA with purified virus (Nelson and Keeling, 1984), immunocapture ELISA with cell culture supernatants (Bernard *et al*, 1986) and indirect virus enzyme linked cell immunoassay (VELCIA) on infected cell monolayers (Grom and Bernard, 1985).

MATERIALS AND METHODS

Cell culture and medium

Swine testis (ST) cells were used to culture the Purdue-115 strain of TGEV (Laude *et al*, 1981). The ST cell cultures were maintained in minimal essential Eagle's medium (MEME) (Gibco, Paisley, Renfrewshire, UK) supplemented with 10% foetal calf serum (FCS) (Gibco), 100 IU/ml penicillin G and 100 µg/ml streptomycin sulfate. The TGEV-infected ST monolayers were used for the VELCIA test.

Viral antigen

Purdue-115 strain of TGEV was harvested when 90–95% of the ST cell monolayers showed a cytopathic effect (CPE). The ELISA antigens were prepared by 3 cycles of freezing and thawing and low-speed centrifugation (1 500 *g* for 20 min). This preparation was used for the immunocapture test.

Supernatant of infected and non-infected cells was concentrated by ultracentrifugation (Beckman R30, 55 000 *g* for 4 h at 4 °C). The pellet obtained was suspended in 1 ml of 0.02 M Tris-HCl, 0.001 M EDTA, 0.1 M NaCl, pH 7.4 (Merck, Darmstadt, Germany), and used as concentrated antigen (pellet). The control reaction was performed with supernatant of non-infected ST cells treated in the same manner.

The concentrated virus (pellet) was further purified on discontinuous 10–50% sucrose-density gradient centrifugation (Beckman SW 27, 25 000 g for 18 h at 4 °C). Bands formed in the density gradient were collected using a fraction collector (Gilson). The 1.18 g/ml fraction was used as purified antigen. The control reaction was performed with supernatant of non-infected ST cells treated in the same manner. Purity of the preparation was analyzed by silver staining on polyacrylamide gel (PAGE).

Monoclonal antibodies

Three monoclonal antibodies (mAb) against the envelope protein M (No 25–22), peplomer protein S (No 51-13) and nucleoprotein N (No 22-6) (Laude *et al.*, 1986) were used in the immunocapture ELISA test.

Sera

Twenty-four different sera from negative and positive breeding pigs were analyzed. A group of positive sera (Nos 18–24) was obtained from 7 sows immunized with the Nouzilly or Gep II strain (Aynaud *et al.*, 1985, 1991). A group of negative sera (Nos 1–17) was obtained from different farms which were negative for the TGEV and porcine respiratory coronavirus (PRCV) disease. All samples were heat-inactivated at 56 °C for 30 min. Sera were frozen at –20 °C until tested.

In vitro neutralizing test

The presence of neutralizing antibodies was examined by a microneutralization test in ST cells (Toma and Benet, 1976). Briefly, in 96-microwell plates (Falcon-3072, Beckton Dickinson), 2-fold dilutions of 50- μ l inactivated sera were incubated for 30 min at 37 °C with 200/plaque forming units (pfu)/well of Purdue-115 strain virus. Mixtures were then transferred onto plates containing ST cells (2–3.10⁵ cells/well) and incubated for 3–5 d under 0.5% CO₂ at 37 °C. The antibody titer was expressed as the reciprocal dilution of serum that neutralized the cytopathic effect.

Indirect ELISA

Microtiter plates (Nunc immunoplates, 4-42404) were coated with 100 μ l antigen per well (10⁶ pfu/well for purified virus; 3.10⁶ pfu/well for pellet virus) or ST control (non-infected ST cell culture supernatant) in carbonate buffer, pH 9.6. After overnight incubation at 4 °C, the plates were saturated with skimmed milk (Regilait, France-Lait) in phosphate-saline buffer (PBS), for 15 min at 37 °C, and then washed 3 times with PBS, 0.05% Tween-20. Two-fold serial dilutions of serum (100 μ l/well, starting 1/100) were added to wells containing the TGEV antigens or control. The plates were incubated for 2 h at 37 °C with an optimal concentration (1/400 in PBS, 0.05% Tween, 1% milk) of rabbit IgG anti-swine Ig (H+L) peroxidase-labelled (Cappel). The plates were washed again and 100 μ l of hydrogen peroxide/2,2'-azino-bis (3-ethyl benzthiazoline-6 sulfonic acid) (ABTS) (Boehringer, Mannheim) substrate were added to each well. The reaction was stopped after a 60-min incubation at 37 °C with 20 μ l of 10% sodium lauryl sulfate (Serva). The optical density (OD) was measured on a Titertek spectrophotometer (Flow) at 405 nm.

Immunocapture ELISA

Microtiter plates (Nunc) were coated overnight at 4 °C with 50 μ l per well (4.6 μ g/ml) of a mAb mixture (Bernard *et al.*, 1986). The plates were saturated with 1% skimmed milk before incubation with 100 μ l of supernatant of Purdue-115 culture (5.10⁵ pfu/well) or non-infected ST cells culture. These plates were then used for the indirect ELISA.

Virus-enzyme linked cell immunoassay (VELCIA)

ST cells were grown to confluence in 96-well microplates (Falcon-3072) and infected overnight with Purdue-115 strain of TGEV (5.10⁵ pfu/well). Before any cytopathic effect occurred, the infected cell cultures were washed carefully 3 times with cold PBS and fixed with 80% (v/v) acetone-water solution. Then the plates were washed, saturated with skimmed milk and used as already described for the indirect ELISA.

Titre, sensitivity, specificity

The optical densities (OD) obtained in all the enzyme-immuno-assays were transformed in logit and plotted vs the dilution. The point of inflexion (logit = 0) was considered as the titre, and recalculated in \log_{10} of the initial dilution of serum.

We considered a serum positive for one test if the titre obtained in this test was greater than the control plus the SD of all the negative sera. A false negative sample was a positive serum found negative with this test, and a false positive sample a negative serum found positive.

The sensitivity of one test was assessed as the number of positive samples detected as positive in this test divided by the total of positive samples (true positive plus false negative). The specificity was the number of negative samples detected as negative in the test divided by the total of negative samples (true negative plus false positive).

RESULTS AND DISCUSSION

ELISA technology is now well known, and is utilized worldwide for many immune systems. The main problem is to find a suitable antigen preparation compatible with the sensitivity and cost of the test (Huang *et al*, 1987; Rukhazde *et al*, 1989). Different enzyme-immuno methods for detection of the TGE coronavirus should be com-

pared to find replacement technics for the seroneutralization test.

The titres of each serum obtained with the different techniques employed are shown in figure 1 and the means of titres given in table I. For all enzyme-immunoassays (EIA), we noticed the presence of antibodies raised against the non-infected ST cells (table I) but also other pig cells (kidney lines). The antibody titers were sometimes very high and gave a heterogeneous background that always necessitated a comparison of the titre obtained with infected and non-infected antigenic preparations. The difference between the titers obtained with and without virus (fig 1, shaded area and black area), showed that positive samples (Nos 18–24) were not detected by all the techniques, but false positives could be seen (shaded area), in the negative samples (Nos 1–17).

Indirect ELISA is the most frequently used method (Nelson and Keeling, 1984; Hohdatsu *et al*, 1987), but needs preparation of concentrated or purified antigens to avoid competition between the viral antigens and contamination on the plate coating. The use of concentrated virus is easy (ultracentrifugation pellet), but gave high variability in the negative sera, and low sensitivity (57%) (table II). On the contrary

Table I. Mean (SD) antibody titres (\log_{10} of serum dilution) of infected or non-infected pig sera tested by various EIA tests.

	Sera (7) from infected pigs tested against:		Sera (17) from non-infected pigs tested against:	
	Non-infected control	TGE-infected material	Non-infected control	TGE-infected material
ELISA purified virus	0.51 (0.06)	3.96 (0.48)	0.31 (0.13)	2.43 (1.01)
ELISA concentrated virus	2.34 (0.48)	3.52 (0.26)	1.58 (1.43)	1.85 (1.50)
ELISA immunocapture	1.45 (0.65)	3.73 (0.73)	0.81 (0.98)	1.42 (0.99)
VELCIA	2.69 (0.34)	4.17 (0.78)	2.60 (0.71)	2.65 (0.72)

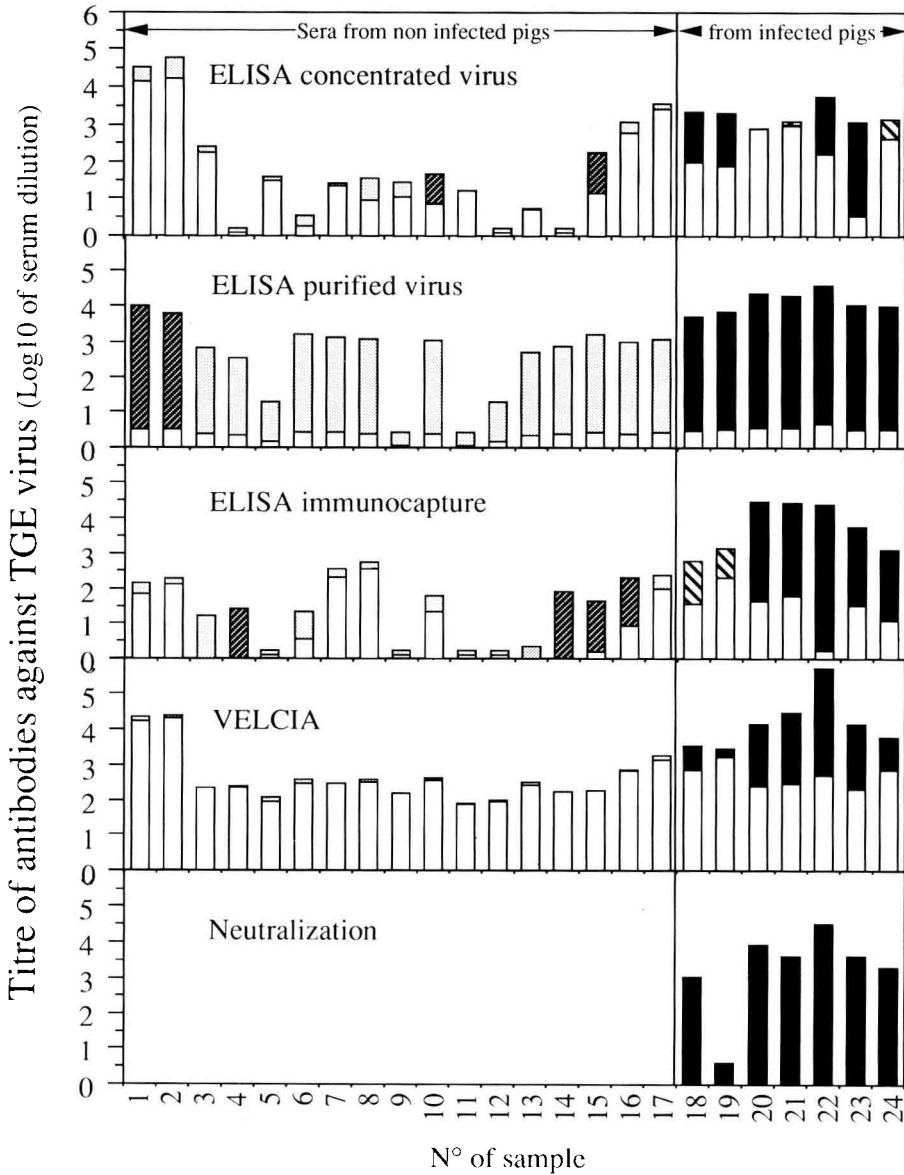


Fig 1. Titres of sera from non-infected (No 1–17) and infected (Nos 18–24) pigs, obtained with indirect ELISA with concentrated virus or purified virus, with ELISA immunocapture, with VELCIA and with neutralization test. Titres in the absence of TGEV AG (open boxes), and difference between assays with and without TGEV Ag (shaded and black boxes): true positive , false positive , false negative , true negative .

Table II. Sensitivity obtained with various tests to detect TGEV antigens.

	Sensitivity No (%)	Specificity No (%)
ELISA purified virus	7/7 (100) ^a	15/17 (88) ^b
ELISA concentrated virus	4/7 (57)	15/17 (88)
ELISA immunocapture	5/7 (71)	13/17 (76)
VELCIA	7/7 (100)	17/17 (100)
Neutralization	7/7 (100)	17/17 (100)

^a Number of infected pigs sera detected as positive in the used test/total number of positive sera (*ie* 7 = No of positive in seroneutralization). ^b Number of non infected pigs sera detected as negative in the used test/total number of negative (*ie* 17 = No of negative in seroneutralization).

use of purified virus allowed detection of all positive samples (sensitivity 100%), and only 2 false positives were seen (specificity 88%) (table II). The results obtained by Balsari (Balsari *et al*, 1981), using indirect ELISA with purified virus were comparable. However, indirect ELISA required precise control antigens which were difficult to prepare, because virus purification with sucrose gradient trapped a larger amount of contaminants with virus from infected samples than from non-infected samples (Nelson and Keeling, 1984; Bernard *et al*, 1986).

With the immunocapture ELISA, numerous false positives were detected in the control, and 2 positive sera were not detected (sensitivity 71%; specificity 76%) (table II). With 254 sera collected from the slaughterhouse and 4 experimentally infected piglets, Lanza (Lanza *et al*, 1990) found better results with the immunocapture compared to the indirect ELISA method. However, to determine the cut-off value for identification of the negative samples, they used the mean absorbance of the negative sera plus 2 (indirect ELISA) or 3 (immunocapture) SD values. These results indicated that the variability of these 2 methods was not the same, and

numerous samples were required to determine with precision the cut-off value of negative samples.

With infected monolayer (VELCIA), all the positive samples were detected, but not with the non-infected cells (table II). The correlation between the titre obtained with neutralization and VELCIA ($R^2 = 0.87$) was better than the correlation between the titres obtained with seroneutralization and those obtained with ELISA purified virus ($R^2 = 0.77$), ELISA concentrated virus ($R^2 = 0.47$) and ELISA immunocapture ($R^2 = 0.67$). VELCIA test is as simple, rapid and useful as the other enzyme-immunoassays. Nevertheless, to obtain reproducible results, the cell monolayer must be regularly controlled with standard sera. With the fixed infected cells, we could not only detect antigens of the virion, but also other antigens not present on the structure of the virus. We suspect the presence of new antigens, or antigens in association with other protein, or lipid on the surface of infected cells, and absent or in a low amount on the virus itself (Bernard, unpublished results).

According to these results, the neutralization assay remains the reference technique for the detection of specific antibody

against TGEV. In the enzyme-immunoassays, the main problem is the reduction of the background values, and effort should be made to improve the relation between binding to control and positive antigens. However, VELCIA is well correlated with seroneutralization. More assays must be performed to confirm these results; but the sensitivity and specificity of this technique seemed sufficiently satisfactory to carry out an epidemiological investigation in a pig-breeding area in Murcia, Spain.

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