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Transmissible gastroenteritis coronavirus: surface antigens induced by virulent and attenuated strains

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SUMMARY

Three strains of the transmissible gastroenteritis virus (TGEV) possessing different degrees of pathogenicity for piglets were examined for their capacity to express M and S glycoproteins on the infected cell surface using a microwell immunoperoxidase test. These two viral glycoproteins were easily detected on the plasma membrane of 0.1 % paraformaldehyde-fixed swine testis (ST) or pig kidney (RP.D) cells which were infected with high-passaged Purdue-115 and low-passaged D-52 strains and a high-passaged attenuated (188-SG) mutant of TGEV. No significant differences were found between attenuated and virulent strains with regard to the viral antigen expression on the membrane of infected cells over a 14-h period.

Key-words: Coronavirus, Transmissible gastroenteritis, Virulence, Antigenicity; Expression, HP and LP strains, Immunoperoxidase, M and S glycoproteins.

INTRODUCTION

Transmissible gastroenteritis (TGE) is a highly contagious enteric infection of swine caused by a transmissible gastroenteritis coronavirus (TGEV) (Woode, 1969). The causative agent of TGE belongs to the *Coronaviridae,* a family of enveloped viruses possessing a single-stranded co-linear RNA genome of positive polarity (for review, see Sturman and Holmes, 1983). Three major structural proteins have been described for all coronaviruses: a high mol. wt. (220 kDa) glycoprotein (S) which forms the characteristic peplomers of the "corona", a small (29 kDa) transmembrane glycoprotein (M) and a phosphorylated protein (N, 47-50 kDa) associated with RNA (Garwes and Pocock, 1975; Garwes *et al.*, 1976; Horzinek *et al.*, 1982; Laude *et al.*, 1986). The peplomer glycoprotein is assumed to be involved in both virus adsorption to the cell and induction of virus-neutralizing antibody (Garwes *et al.*, 1978). The transmembrane glycoprotein is postulated to play a key role in alpha-interferon induction (Charley and Laude, 1988). TGEV infection is followed by a very high mortality rate of up to 100 % in piglets which are less than 2 weeks old (Haelterman, 1972). Sows that are naturally exposed to the virulent TGEV produce immune milk, which passively protects newborn pigs (Saif and Bohl, 1981; Bachman, 1982). In contrast, attenuated TGEV does not induce complete protection by lactogenic immunity (Saif and Bohl, 1981).

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Since the virulence of TGEV has been shown to decrease by serial passages in tissue culture, many authors have tried to differentiate the high-passaged (HP) attenuated strains from the low-passaged (LP) virulent strain by *in vitro* markers, such as the level of the thermosensitivity of replication (Furuuchi *et al.*, 1975; Hess and Bachman, 1976), the resistance to digestive enzymes, low pH and temperature (Laude *et al.*, 1981), and by comparing viral replication and synthesis of structural antigens (Nguyen *et al.*, 1987).

Using an HP attenuated mutant of TGEV (188-SG strain), which survives in the physicochemical environment of the digestive tract of adult pigs (Aynaud *et al.*, 1985), to study passive protection against TGEV infection in piglets, we found that this new TGEV mutant was capable of inducing protective lactogenic immunity and that it could be considered as candidate for an oral TGEV vaccine (Bernard *et al.*, 1990; Aynaud *et al.*, 1991). However, the exact mechanism leading to the induction of protective immunity following oral immunization of sows with this mutant is still unknown.

In mouse hepatitis virus (MHV), a well studied coronavirus, the M protein migrates to the Golgi apparatus, but is not transported to the plasma membrane as readily as the S protein (Sturman and Holmes, 1983). For porcine TGEV, the presence of the virus envelope S antigen on the surface of infected cells was demonstrated by immunofluorescence (Laude *et al.*, 1986), while the presence of the M antigen on the plasma membrane has only been suspected by unspecified monoclonal antibodies (mAb) (Welch and Saif, 1988). There has not been any published report concerning the presence of N antigen on the plasma membrane of infected cells. However, our group (To *et al.*, 1991) and others (Laviada *et al.*, 1990) have recently demonstrated the presence not only of S but also of M viral antigens on the membrane of ST cells infected with HP Purdue-115 strain of TGEV.

The purpose of the present study was to determine whether the LP virulent D-52 strain and HP attenuated 188-SG mutant were capable of expressing their M and S glycoproteins on the infected cell membrane in a similar way to the HP Purdue-115 strain. For the sake of comparison, the kinetics of expression of viral antigens on the plasma membrane and in the cytoplasm of ST and RP.D cells infected by these strains of TGEV was also studied with a view to discovering markers for differentiating HP attenuated strains from LP virulent strains.

MATERIALS AND METHODS

Cells, viruses and mAb

RP.D is a previously described pig kidney cell line (Laude *et al.*, 1981). The McClurkin swine testis (ST) cell line was supplied by E.H. Bohl (Wooster, OH, USA). Minimal essential medium (MEM) supplemented with 10 % foetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) was used for cell growth.

Purdue-115 is an HP TGEV strain (Bohl *et al.*, 1972), D-52 is a virulent strain which was isolated from an acute case of TGE (P. Vannier, CNEVA, Laboratory of Porcine Pathology, Ploufragan, France) and passaged 5 times in RP.TG cells (Aynaud *et al.*, 1985) and 188-SG is an HP attenuated mutant which was previously obtained in our laboratory through serial cycles of survivor selection in gastric juice (Aynaud *et al.*, 1985).

For the experiments with inactivated virus, a viral suspension of each of these 3 strains was exposed to ultraviolet light (120 s, 2 mW/cm²) (Charley *et al.*, 1983). Subsequent titration by plaque assay showed that the TGEV strains were fully inactivated following this treatment.

IPT = immunoperoxidase test.
mAb = monoclonal antibody.
MEM = minimal essential medium.
m.o.i. = multiplicity of infection.
OD = optical density.
PBS = phosphate-buffered saline.
PFA = paraformaldehyde.
PFU = plaque-forming unit.
p.i. = post-infection.
SDS = sodium dodecyl sulphate.
ST = swine testis.
TGE = transmissible gastroenteritis.
TGEV = TGE virus.

Three mAb, anti-M (25/22), anti-S (51/13) (Delmas *et al.*, 1986) and anti-N (22-6), were prepared and used as ascitic fluids following injection of BALB/c mice with the antibody-producing hybridomas (Laude *et al.*, 1986).

Monolayer infection

Confluent monolayers of 2.5×10^5 cells/cm^2 in 96-well, flat-bottomed plastic plates (Falcon 3072, Becton Dickinson) were incubated with a volume of 0.1 ml of virus suspension at a multiplicity of infection (m.o.i.) of 10. After a 30-min incubation at 37°C under 5.5 % CO_2, the inoculum in each well was removed and the cells were washed twice with phosphate-buffered saline (PBS). The monolayers were then overlaid with 0.1 ml of MEM containing 5 % heat-inactivated (56°C, 30 min) normal calf serum and the plate was incubated at 37°C under 5.5 % CO_2. The cell culture supernatant was harvested at the indicated time intervals and kept at −20°C until titration.

Quantification of viral antigen on the membrane or in the cytoplasm of TGEV-infected cells by an immunoperoxidase test (IPT)

An IPT which had been previously developed for the detection of surface viral antigens induced by Purdue-115 strain in infected ST cells (To *et al.*, 1991) was used. Briefly, the infected monolayers harvested at indicated times were washed twice with PBS and the cells fixed with 0.1 % paraformaldehyde (Prolabo-France) at 4°C for 30 min. After cell saturation with 5 % skimmed milk in PBS without calcium and magnesium for 15 min at room temperature, the monolayers were overlaid with 0.1 ml of each of 3 abovementioned mAb at working dilutions for 90 min at 4°C. The reagents were removed from the plates by rinsing twice with tap-water and twice with PBS containing 0.05 % Tween-20 (Serva) and were then replaced with 0.1 ml/well of an optimal dilution of peroxidase-labelled goat anti-mouse Fc serum (ICN Immunobiologicals, Israel). After a further 90 min of incubation at 4°C, the plates were washed as before and the enzymatic reaction was developed by incubation at 37°C for 1 h with 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS; Boehringer Mannheim)/H_2O_2 substrate solution. The supernatant was transferred to another plate containing 0.02 ml of sodium dodecyl sulphate (SDS) to stop the enzymatic reaction and to permit the reading of the plate. The peroxidase was quantified by measuring the optical density at 415 nm with "Titertek Multiscan" (Flow Laboratories, Irvine, Scotland, UK). Each antigen quantity, tested in quadruplicate, was expressed as the difference between the OD at 415 nm of virus- and mock-infected cells using the formula: OD at a given timepoint = [(OD of virus-infected cells — OD background of virus infected cells) — (OD of mock-infected cells — OD background of mock-infected cells)].

For the detection of virus-induced antigens in cytoplasm, the infected cells were fixed with 80 % acetone at −20°C for 30 min and the IPT was applied as for surface antigens.

Titration of infectious virus particles and detection of viral antigens in the cell culture supernatants

A plaque assay (Aynaud *et al.*, 1985) was used to titrate the infectious virus in the cell culture supernatants sampled. Briefly, 2 to 3-day-old monolayer cultures of ST cells were produced by seeding 5×10^5 cells per 30-mm container (6-well trays). The cultures were inoculated with an appropriate TGEV dilution, and 2 ml MEM supplemented with 2 % calf serum and 1 % agarose (Indubiose) were added. Plaques were counted by neutral red staining following incubation at 37 to 38°C in 5.5 % CO_2 for 48 h.

For the detection of M and S viral antigens in the culture supernatants, an ELISA immunocapture technique (Bernard *et al.*, 1986) was used. Briefly, 96-well microtitre plates (Nunc-immunoplates, 4-42404) precoated with anti-M, anti-S and anti-N mAb, were incubated for 2 h at 37°C in carbonate buffer (pH 9.6). After washing, the plates were blocked overnight at 4°C with 1 % skimmed milk in PBS. Viral antigens were bound onto the precoated plates by incubating wells for 2 h at 37°C with supernatants from ST and RP.D cell cultures infected with either Purdue-115, D-52 or 188-SG strain. The peroxidase-labelled pig IgG polyclonal antibodies (Bernard and Lantier, 1985) were added for the next 2 h at 37°C. The enzymatic reactions were developed as mentioned above.

RESULTS

Kinetics of M- and S-antigen expression on TGEV-infected cells

TGEV which was inactivated by ultraviolet irradiation failed to induce production of viral antigens while the infectious viruses did, as shown by IPT in infected ST cells (fig. 1a and b). Also, neither infectious virus particles nor structural viral antigens could be detected by plaque assay and ELISA immunocapture in the

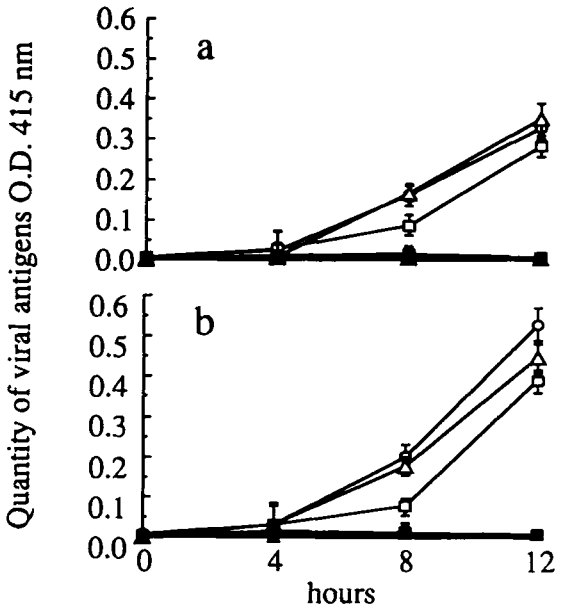

Fig. 1. Kinetics of surface expression of M (a) and S (b) viral antigens on ST cells infected with inactivated (full symbols) and infectious (open symbols) TGEV (m.o.i. = 10 PFU).

Purdue-115 strain (● ○), 188-SG strain (■ □) and D-52 strain (▲ △).

cell culture supernatants sampled at the indicated time intervals. This experiment showed clearly that LP virulent D-52 strain and HP attenuated 188-SG mutant were also capable of expressing their glycoproteins on the plasmic membrane of infected ST cells, as previously described for HP Purdue-115 strain (To *et al.*, 1991).

The time courses of the M and S antigen over a 14-h period were demonstrated on the membrane of ST (fig. 2a, c and e) and RP.D cells (fig. 2b, d and f), infected with LP virulent D-52 strain, HP attenuated 188-SG mutant and HP Purdue-115 strain of TGEV. The surface and cytoplasmic viral antigens were first detected at 4 h post-infection (p.i.) and showed a gradual increase in viral antigens on the cell membranes until 14 h p.i., while the cytoplasmic antigens began to decrease after 12 h p.i.

The expression kinetic profiles showed similarities for the 3 virus strains in the two cell lines whereas the quantity of viral antigens was significantly lower in RP.D cells than in ST cells. At 14 h p.i., Purdue-infected ST cells expressed a higher quantity of surface M and S antigens than those infected with the D-52 or 188-SG

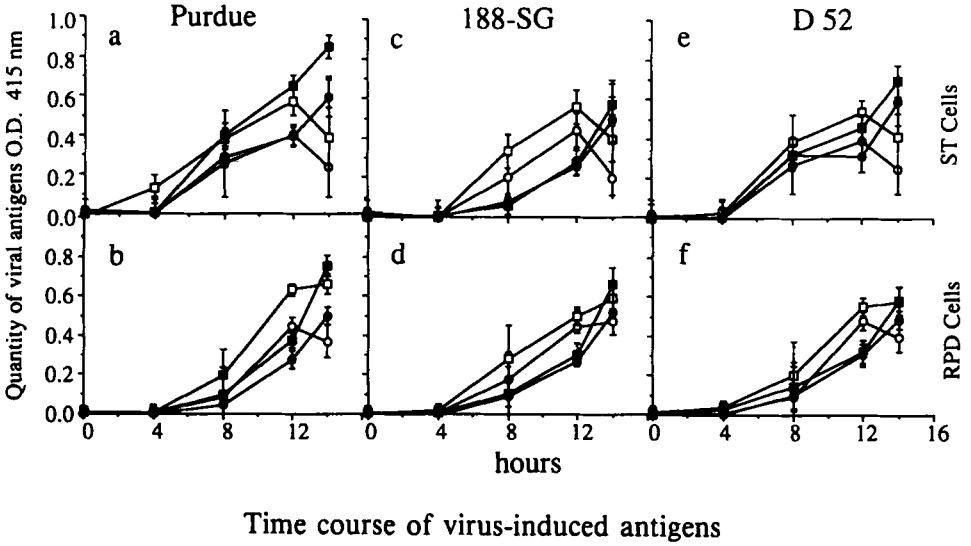

Fig. 2. Kinetics of viral M (● ○) and S (■ □) antigen expression on ST (a, c, e) and RP.D (b, d, f) cells infected with Purdue (a, b), 188-SG (c, d) and D-52 (e, f) TGEV strains (m.o.i. = 10 PFU).

Full symbols = surface antigens; open symbols = cytoplasmic antigens.

strain. In contrast, RP.D cells infected with each of these 3 TGEV strains showed the same OD values for M and S antigens at 14 h p.i.

Kinetics of production of M and S antigens and virus by TGEV-infected cells

The infectious virus particles and structural viral antigens of the Purdue-115, 188-SG and D-52 strain of TGEV could be detected in the cell culture supernatants at 8 h p.i. (fig. 3a, b and c). Infectious virus titre and viral antigen quantities in the supernatants of infected cells increased gradually and reached a plateau after about 12 h p.i. The infectious titre of Purdue-115 and D-52 strain was higher than that of the 188-SG strain, while the amounts of M and S antigens detected in the supernatant of the cells infected by these 3 viruses were similar.

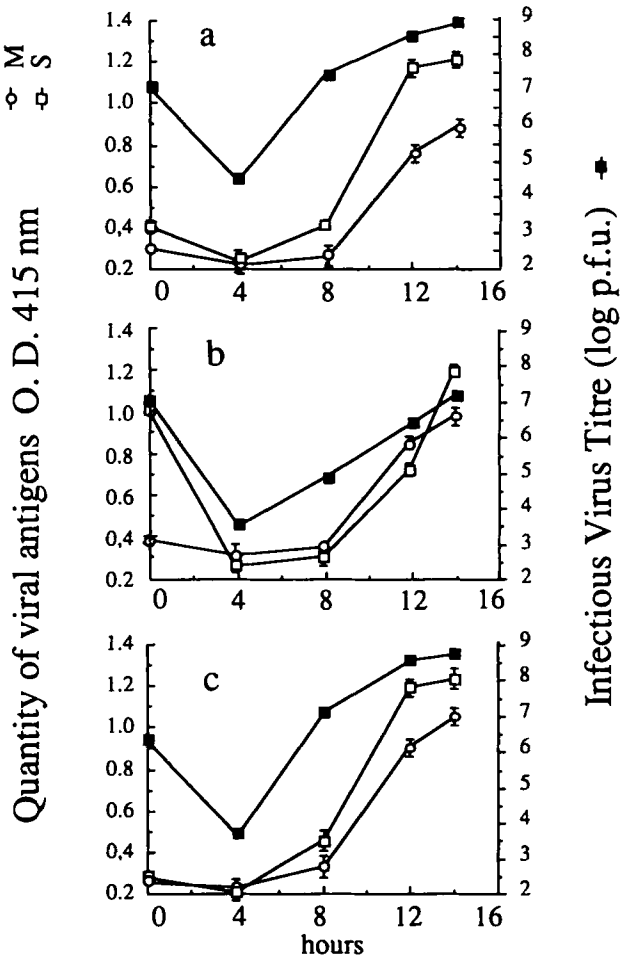

Fig. 3. Kinetics of infectious virus particles, titre in PFU (■) by plaque assay, and of M (○) and S (□) structural viral antigen expression by ELISA immunocapture in supernatants of cultured cells infected with Purdue-115 (a), 188-SG (b) and D-52 (c) strain of TGEV.

DISCUSSION

We have recently developed a microwell IPT for detecting and quantifying the expression of viral S and M glycoproteins on the plasma membrane of ST cells infected with Purdue-115 strain of TGEV (To *et al.*, 1991). In the present study, this technique was used to demonstrate and compare the expression of surface viral antigens in ST and RP.D cells infected with LP virulent D-52, HP Purdue-115 strains and HP attenuated 188-SG mutant. With this approach, we tried to find markers which would enable the differentiation of HP attenuated strains and LP virulent strains with regard to antigen expression on infected cell surface.

Of the 3 mutant viruses tested, the Purdue-115 is an HP attenuated strain (115 passages in ST-cell culture). However, under our experimental conditions, this strain was weakly virulent for newborn piglets (Shiraï *et al.*, 1988). The 188-SG is an attenuated mutant previously obtained in our laboratory from the virulent Gep-II strain by 188 serial cycles of survivor selection in gastric juice of adult pigs (Aynaud *et al.*, 1985). This mutant survives in the physico-chemical environment of the digestive tract of adult pigs, is non-pathogenic for piglets (Aynaud *et al.*, 1985) and is capable of inducing lactogenic immunity in sows following oral immunization (Bernard *et al.*, 1990; Aynaud *et al.*, 1991). The original virulent D-52 strain is a mutant obtained from the virulent Gep-II strain by 5 passages in RP.TG cells (Aynaud *et al.*, 1985) and is pathogenic for newborn piglets (Bernard, unpublished data). Unlike the Gep-II strain, the virulent D-52 strain could be grown in *in vitro* cell culture.

No differences in the capacity to express surface viral glycoproteins (fig. 1) were found between the 3 TGEV strains, as the presence of M

and S glycoproteins was determined easily in infected ST and RP.D cells, while the presence of N antigen was not (data not shown). In contrast, the N (data not shown), M and S (fig. 2) antigens were easily detected by IPT in the cytoplasm of TGEV-infected cells which were fixed with 80 % acetone. For the purpose of comparing the expression of viral antigens on the surface of infected cells, the anti-N mAb was used as a marker to ensure that after PFA fixation, the cell membrane would remain intact and only the viral antigens expressed on the plasmic membrane of infected cells would be detected. Experiments using inactivated virus have demonstrated that protein synthesis is a prerequisite for antigen expression on the cell membrane.

Our previous results indicated that the expression of M, S and N antigens appeared in multimodal patterns which peaks at 14, 16 and 18 h p.i. when ST cells were infected with 2 m.o.i. of Purdue-115 virus (To *et al.*, 1991). Using the same m.o.i. of D-52 strain and 188-SG mutant, the patterns of expression of viral antigens in infected cells were also multimodal (data not shown). This phenomenon was due to incomplete infection of cell monolayers, which led to multi-cycle multiplication of virus. Laude *et al.* (1986) found that about 20 % of ST cells expressed S antigen at 20 h p.i. when cells were infected at a m.o.i. of 2.5×10^{-2}PFU/cell of Purdue-115 virus. In order to have glycoproteins appearing at the cell surface under single-cycle conditions of viral multiplication, a high m.o.i. (10 PFU/cell) was chosen to ensure that all cells were infected. It is interesting to note that the levels of expression of surface M and S antigens of the 3 virus strains were not significantly different when cells were infected with high m.o.i. (10 PFU/cell) (fig. 2). This observation implies that the capacity to express glycoproteins on the cell membrane was not a marker for differentiating HP and LP TGEV strains. For all 3 mutant viruses used, surface virus antigen quantity was significantly lower with the RP.D cells than with the ST cells. This could be explained by the influence of cell culture systems on virus replication and synthesis of viral antigens (Nguyen *et al.*, 1987). Furthermore, the appearance of the M and S antigens on the outer membrane of the cells could depend on an antigen-processing system, as previously described for other viruses (Long and Jacobson, 1989; Yewdell *et al.*, 1981).

With all our different combinations of viruses and cells, a lag was seen between the cytoplasmic antigens which had decreased in quantity 12 h p.i., while the surface antigens were still increasing. The decrease in cytoplasmic antigen expression can be explained since 12 h p.i. is the moment at which the virus progeny begin to be released from the cytoplasm of infected cells.

Concerning the production of infectious viruses and synthesis of structural antigens (fig. 3) the infectious titres of HP Purdue-115 and LP virulent D-52 (about 10^9 PFU/ml) were higher than that of HP attenuated 188-SG mutant (about 10^7 PFU/ml) whereas the quantities of viral M and S antigens in the culture supernatants and cytoplasm of cells infected with these 3 viruses were similar. This experiment indicated clearly that the 188-SG mutant was characterized by a high structural antigen content and low infectivity in comparison with the 2 other viruses, since a high quantity of S antigen was detected at time 0. These observations are consistent with our previous results (Nguyen *et al.*, 1987) of the comparison of viral replication and synthesis of structural antigens of these 3 strains of TGEV. The 188-SG mutant was characterized by low infectivity, delayed and restricted growth associated with low and delayed RNA synthesis and a high structural antigen content. In contrast, Purdue-115 and D-52 strains were characterized by high infectivity and a normal pattern of virus replication RNA and structural antigen synthesis.

In conclusion, no significant differences in *in vitro* expression of TGE viral antigens on plasma membranes were observed between the 3 virus strains and the 2 cell lines used which could explain the major differences existing between the virulence and the immunogenicity conferred by the different virus strains (Saif and Bohl 1981; Bernard *et al.*, 1990; Aynaud *et al.*, 1991). Research on *in vivo* expression of TGEV antigens on the surface of intestinal cells of infected sows, especially in those undergoing oral

immunization by HP attenuated 188-SG mutant, should be carried out in order to answer this question.

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Antigènes de surface induits par des souches virulentes et atténuées du coronavirus de la gastroentérite transmissible

Trois souches du virus de la gastroentérite transmissible (GET) possédant une pathogénicité différente pour les porcelets ont été examinés, à l'aide d'une technique d'immunopéroxidase en microplaque, pour leur capacité d'expression des glycoprotéines M et S à la surface des cellules infectées. Ces deux glycoprotéines sont facilement détectées sur la membrane plasmique des cellules ST (testicule du porc) et des cellules RP.D (rein de porc) infectées par trois souches différentes de virus de la GET, et fixées à la paraformaldéhyde. Aucune différence d'expression des antigènes viraux sur la membrane des cellules infectées sont observables en fonction des souches virales et des lignées cellulaires utilisées.

Mots-clés: Coronavirus, gastroentérite transmissible, Virulence, Antigénicité; Expression, Souches HP et LP, immunoperoxidase, glycoprotéines M et S.

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