Transmissible Gastroenteritis: Demonstration of the Virus from Field Specimens by Means of Cell Culture and Pig Inoculation

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ABSTRACT

Isolation of transmissible gastroenteritis virus was attempted from segments of jejunum collected from piglets submitted for diagnosis of transmissible gastroenteritis. The virus was isolated more frequently in susceptible piglets than in pig kidney or pig thyroid cells. Practically, both cell systems were equally capable of demonstrating the virus when the tissue suspensions were sonicated. The pig thyroid cells prepared with glands collected from minimal disease pigs were preferred to the pig kidney cells for initial virus isolation because of their ability to respond to transmissible gastroenteritis virus with a progressive cytopathic effect. However, the pig thyroid cells, prepared from pool of glands collected in abattoirs, were often contaminated with parvoviruses and could not be used for diagnostic work. Controlled ultrasound treatments of the inoculum increased the frequency of virus isolation in both cell systems.

RÉSUMÉ

Cette étude rapporte les résultats des tentatives d'isolement du virus de la gastro-entérite transmissible, à partir des segments de jéjunum des porcelets, soumis pour fin de diagnostic. Nous avons isolé le virus plus souvent à la suite de l'administration orale à des porcelets susceptibles que par l'intermédiaire de cultures de cellules rénales et thyroïdiennes. La susceptibilité des deux types cellulaires s'est avérée semblable, mais nous préférons les cellules thyroïdiennes surtout parce qu'elles montrent, d'emblée, des effets cytopathogènes avec tous les isolements du virus de la gastro-entérite transmissible. Les cellules thyroïdiennes, préparées avec des glandes récoltées aux abattoirs, sont souvent inutilisables par suite de leur contamination par des parvovirus. Le traitement contrôlé des suspensions tissulaires, au moyen d'ultrasons, a aussi augmenté le nombre d'isolements du virus dans les deux systèmes cellulaires utilisés.

INTRODUCTION

In 1963, Harada *et al* (10) described Japanese strains of transmissible gastroenteritis (TGE) virus which were cytopathic for primary pig kidney (PK) cells. Since that time, other investigators have also isolated the virus of TGE in PK cells (1, 4, 7, 14) but most isolates required several passages before detectable CPE occurred.

In their search for a better cell culture assay for TGE, Witte and Easterday (19) and Witte (20) observed that all field strains of TGE virus inoculated on pig thyroid (PTh) cells showed a rapid and progressive CPE upon initial inoculation.

Requests to confirm clinical cases of TGE are frequently made in our laboratory. Suitable specimens are stained with immunofluorescent conjugates and examined for TGE antigens in the tissues. However, a number of specimens are not preserved adequately for immunofluorescence staining and, on such tissues, the diagnosis has to be confirmed by other means. This paper reports our experience in the isolation of TGE virus from clinical cases of TGE by inoculation to piglets, PK and PTh cells.

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MATERIALS AND METHODS

PIGS

The pigs used in these experiments were obtained from the "minimal-disease" (MD) pig herd kept at the Animal Diseases Research Institute. This herd was established sections and subsequently maintained in originally with pigs procured by cesarean isolation (7).

CELL CULTURES

The procedures followed for the collection of the thyroid glands[1] and for the preparation of the thyroid cells were those described by Witte and Easterday (19). The kidney cells were prepared according to the procedure described by Greig *et al* (9). The growth medium used for the thyroid and the kidney cells was a modified Hanks lactalbumin hydrolysate (9) while the maintenance medium was that of Eagle's (MEM) in Earle's balanced salt solution supplemented with 2% fetal bovine serum (FBS).

VIRUS

Two isolates of TGE virus were used for the preparation of reagents: an American cell culture-adapted virus[2] and a Canadian field isolate (ADRI-1) from a clinical case of TGE (7).

PREPARATION OF STOCK TGE VIRUS

Cell culture stocks — The American and the ADRI-1 TGE viruses were prepared in actively growing primary PK cells according to a method described by McClurkin (14). Endpoint titers were determined on primary PTh cells in 15 x 125 mm disposable glass tubes using four tubes per dilution and $\log_{10}$ dilution steps. The infectivity titer was estimated by the Reed and Muench method (17) and found to be $10^{5.2}$ and $10^{5.0}$ $TCID_{50}$/0.1 ml for the American and ADRI-1 viruses respectively. The American isolate was used as the virus challenge in the serum neutralization (SN) tests while the cell culture-adapted ADRI-1 virus was used to hyperimmunize pigs.

Pig virus stock — A fourth pig passage of ADRI-1 virus was made in two three day old MD piglets. A 10% suspension of mucosal scrapings was prepared in Dulbecco's phosphate buffered saline (PBS) (8) as described by Witte and Easterday (19). The titer of this stock was greater than 10^6 pig infective doses/ml. The virus suspension was further concentrated according to the method described by McClurkin (14) and purified on a discontinuous sucrose gradient.

ANTISERUM

Antiserum against the ADRI-1 virus was prepared as follows: three five day old pigs were given orally 2 ml of bacteria free suspension of the pig virus stock. Three weeks later, the animals were inoculated intravenously with 15 ml of the ADRI-1 cell culture-adapted virus and intramuscularly with 10 ml of partially purified ADRI-1 pig virus. This dosage was repeated four times at weekly intervals and the animals were exanguinated 21 days after the last inoculation.

SEROLOGICAL TESTS

Serum neutralization (SN) — The SN tests were done in PTh cells, grown in tubes or in micro plates (21) according to the classic variable serum-constant virus (100 $TCID_{50}$) method. The variable virus-constant serum method was used to identify new isolates.

Modified direct complement fixation test (MDCF) — The MDCF test was done as described previously (2). The procedure for preparing TGE antigen has been published elsewhere (7). This test was used primarily as an indicator of quality of TGE antiserum prior to conjugation with fluorescein isothiocyanate (FITC). The test was also used to detect CF antibodies to TGE virus from the swine serums submitted along with the specimens.

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[1]Some glands were supplied by the abattoir of C. H. Thomas Ltd., 70 Corkstown Road, Ottawa, Ontario, while the remaining were from the MD swine herd.

[2]Dr. G. Lambert, United States Department of Agriculture, Agricultural Research Service, National Animal Diseases Laboratory, Post Office Box 70, Ames, Iowa 50010.

Fluorescent antibody technique (FAT) — Antiserums against TGE virus and porcine parvovirus were conjugated with FITC, adsorbed and preserved as described previously (3). The conjugated parvovirus antiserum was used to screen the PTh cells for parvovirus contaminants.

Specimens

Origin — Frozen or refrigerated specimens were submitted by provincial veterinarians of Nova Scotia, Prince Edward Island, Quebec and Ontario. Segments of jejunum were collected at necropsy from pigs showing clinical signs compatible with those described for TGE (6).

Isolation of TGE virus — Segments of jejunum were minced with scissors and suspended in an equal volume of ice cold Dulbecco's PBS containing 200 I.U. of penicillin and 200 μg of streptomycin per ml. Each tissue suspension in 20 ml volume, was homogenized in a 50 ml stainless steel chamber of a Sorval Omni-mixer[3] operated at full speed for five min. One half of the homogenized tissues was further sonicated as described by Witte (20). The fluids from the homogenized and sonicated tissues were further diluted 1:10 and occasionally 1:100 with cold PBS and added to an equal volume of inactivated FBS. Thus the final dilution of the tissues was 1:40 (w/v) and occasionally 1:400.

Each dilution of the homogenized and homogenized-sonicated preparations was inoculated into five to ten disposable 15 x 125 mm glass tubes and six Leighton tubes of primary PK and PTh cells. Occasionally, subcultures were also used in addition to the primary cells. After an adsorption period of one and one-half hours at 37°C the inoculum was removed and replaced with maintenance medium and the tubes incubated at 37°C in stationary racks. At the beginning of the experiments, the tubes were also maintained in roller drums but this procedure was discontinued when found unsatisfactory. The cells were examined daily for five days for the presence of CPE. For the fluorescent antibody test the cells, grown on glass coverslips in Leighton tubes, were fixed with cold (–10°C) acetone when approximately 25% of the thyroid cells showed CPE or alternatively five days after inoculation. After five days the fluids and the cells in the tubes were frozen, thawed and a second passage made onto fresh cells. A 10% tissue suspension prepared from each specimen was also administered orally to one two day old MD piglet. The piglets were kept in isolation either until clinical signs compatible with those of TGE (6) were observed or alternatively for four days. The animals were then killed and their jejunum used to demonstrate the presence of TGE virus by FAT, applied directly on tissues, and virus isolation in cell cultures as already described.

[3]Ivan Sorval Inc., Newton, Connecticut 06470.

RESULTS

Virus Isolation in Piglets

Tissue suspensions prepared from 12 out of 16 specimens produced TGE following oral administration to piglets (Table I). Specimens No. 13 to 16 failed to yield the virus despite repeated isolation attempts. Signs of diarrhea and vomition occurred within 24 hours following oral administration of positive specimen material. At necropsy, these piglets also exhibited gross lesions of TGE. Villous atrophy was noted in segments of inverted jejunum when examined by stereomicroscopy. The FAT detected the presence of TGE antigens in the columnar epithelium of the jejunum of piglets inoculated with positive specimens. The TGE virus was also reisolated in cell culture from each of the 12 piglets and identified by SN test in PTh cells. Occasionally the presence of virus in cell culture was also verified by FAT.

Virus Isolation in Cell Cultures

TGE virus was demonstrated in nine of 15 specimens inoculated onto PTh cells grown in tubes. Specimens No. 10 and 12, which were positive in pigs, were negative in cell culture and specimens 13-16 remained negative in all systems (Table I). In PTh cells, CPE associated with the TGE virus was occasionally seen within 24 hours and was always obvious 48 hours postinoculation. In

TABLE I. Virus Isolation from Field Specimens in Pig Thyroid, Pig Kidney Cells and in Piglets

Case No.	Inoculation onto PTh Cells				Inoculation of Piglets	Inoculation onto PK Cells				Second Passage onto PK Cells
	Tissues Homogenized		Tissues Homogenized and Sonicated			Tissues Homogenized		Tissues Homogenized and Sonicated		
	1/40	1/400	1/40	1/400[a]		1/40	1/400	1/40	1/400[a]	
1	2/9[b]	ND	9/10	ND	+[c]	0/10	ND	0/11	ND	0/11
2	ND[e]	ND	ND	ND	+	0/10	ND	ND	ND	0/7
3	0/10	0/10	0/10	3/10	+	0/10	0/10	0/10	0/10	5/9
4	1/5	ND	5/5	ND	+	0/10	ND	0/10	ND	2/4
5	4/10	ND	9/9	ND	+	0/10	ND	0/10	ND	1/5
6	6/10	ND	10/10	ND	+	0/10	ND	0/10	ND	4/4
7	0/10	ND	7/10	ND	+	0/10	ND	0/10	ND	0/2[d]
8	0/10	ND	3/10	ND	+	0/10	ND	0/10	ND	4/7
9	9/9	10/10	10/10	10/10	+	0/10	0/10	0/10	0/10	4/5
10	0/6	ND	0/6	ND	+	0/10	ND	0/10	ND	0/9
11	ND	ND	5/5	2/10	+	ND	ND	0/10	0/10	3/5
12	0/5	0/10	0/10	0/10	+	0/10	0/10	0/10	0/10	0/6
13	0/11	ND	0/10	ND	−	0/9	ND	0/8	ND	0/4
14	0/9	ND	0/9	ND	−	0/9	ND	0/11	ND	0/9
15	0/9	ND	0/10	ND	−	0/10	ND	0/10	ND	0/5
16	0/10	ND	0/10	ND	−	0/9	ND	0/8	ND	0/5

[a] Dilution of the original tissues on a weight/volume basis

[b] Number of tubes with CPE / Number of tubes inoculated

[c] + = clinical signs in piglets
− = no clinical signs in piglets

[d] CPE was noted at the third passage

[e] ND = not done

unstained PTh cell cultures, the first sign of infection was the appearance of a few swollen, refractile cells that rapidly became necrotic and pleomorphic. These affected cells floated upon the partially disrupted monolayers seemingly held together, in clumps or irregular chains. Generally, the monolayers were 50% destroyed within 24 to 36 hours from the first appearance of CPE. Complete destruction of the monolayers was rare. That this CPE was associated with TGE virus was confirmed by FAT on the coverslips inoculated in parallel with the tubes (Table II). For the FAT, it was important to stain the affected cells soon after the CPE became detectable. When the cells were stained in an advanced stage of destruction, the fluorescence was generally less characteristic and more difficult to interpret than that seen early in the infection. The TGE fluorescence seen in PK and PTh cell cultures appeared as an irregular green coloration, slightly granular, located in the cytoplasm of the infected cells (Fig. 1). There was no intranuclear staining.

In PK cells, the virus was demonstrated from eight of 16 specimens. Contrary to what was seen in the PTh cells none of the new isolates were cytopathic for the PK cells at the first passage (Table I). However, virus replication occurred in these cells as demonstrated by positive FAT (Table II).

Effect of Sonication

As indicated in Tables I and II, sonication of the tissue suspension prior to inoculation onto cell culture improved the virus yield. In the PTh cells, this improvement was shown by an increase in the number of tubes affected and by a more extensive CPE observed in each tube. The amount of fluorescent antigen detected was generally greater in cells inoculated with homogenized-sonicated inocula than in those inoculated with homogenized material. Following sonication, the TGE virus was demonstrated in three cases (No. 3, 7 and 8) where homogenization alone failed to do so. While sonication also reduced the incidence of bacterial contamination in the inoculated cultures, it occasionally made the initial inoculum cytotoxic. Cytotoxicity was not encountered when the inoculum was diluted to 1:400. Occasionally CPE appeared earlier

TABLE II. Fluorescent Antibody Results on Segments of Jejunum of Experimental Pigs and on Cell Cultures Inoculated with Field Specimens

Case No.	Inoculation to PTh Cells: Tissues Homogenized 1/40	Inoculation to PTh Cells: Tissues Homogenized 1/400	Inoculation to PTh Cells: Tissues Sonicated 1/40	Inoculation to PTh Cells: Tissues Sonicated 1/400[a]	Inoculation of Piglets	Inoculation to PK Cells: Tissues Homogenized 1/40	Inoculation to PK Cells: Tissues Homogenized 1/400	Inoculation to PK Cells: Tissues Sonicated 1/40	Inoculation to PK Cells: Tissues Sonicated 1/400[a]	Second Passage in PK Cells
1	++[b]	ND	++	ND	+	−	ND	−	ND	−
2	ND	ND	ND	ND	+	−	ND	ND	ND	−
3	−	+	−	+	+		+	−	++	++++
4	++++	ND	++++	ND	+	ND	ND	ND	ND	++++
5	++++	ND	++++	ND	+	+++	ND	+++	ND	++++
6	++	ND	+	ND	+	+	ND	++	ND	++
7	−	ND	++	ND	+	−	ND	++	ND	++
8	−	ND	+++	ND	+	−	ND	++	ND	+++
9	+++	++++	++	++	+	−	+	++	++	++++
10	−	−	−	−	−	−	−	−	−	−
11	ND	−	+++	−	+	ND	ND	ND	−	++++
12	−	−	−	−	ND	−	−	−	−	−
13	−	ND	−	ND	−	−	ND	−	ND	−
14	−	ND	−	ND	ND	−	ND	−	ND	−
15	−	ND	−	ND	ND	−	ND	−	ND	−
16	−	ND	−	ND	−	−	ND	−	ND	−

[a]Dilution of the original tissues on a weight/volume basis

[b] − = no fluorescence
+ = one to five typical fluorescent cells in a few fields
++ = approximately five typical fluorescent cells in many fields
+++ = approximately ten typical fluorescent cells in many fields
++++ = numerous typical fluorescent cells in most fields

in tubes inoculated with the more diluted inoculum and in case No. 3, virus was isolated repeatedly from the inoculum diluted at 1:400 but could not be demonstrated at at dilution of 1:40 (Table I).

Serological Tests

Neutralizing antibodies were detected in 54 serum samples submitted with the specimens. The results obtained with the tube and the micro SN test were always in agreement with one another. Antibodies were not detected in these same serums by the MD-CF test.

DISCUSSION

The susceptible piglet was the most sensitive indicator of the presence of TGE virus in tissues. The cell culture systems were also capable of demonstrating the virus in the majority of cases. In two instances, (cases No. 10 and 12) when the specimens had been collected from two week old pigs that were convalescent from TGE, the cell culture systems did not demonstrate the virus. At such a late stage of the disease it is known that the amount of virus in the intestine is low (16). Furthermore, the antibodies derived from the milk or those acquired

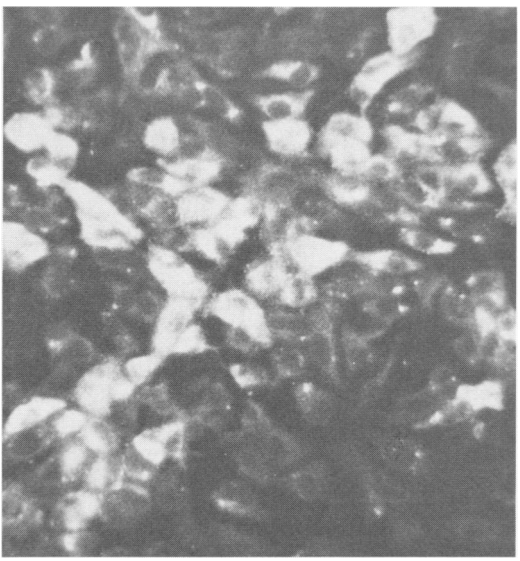

Fig. 1. Transmissible gastroenteritis virus in primary porcine cell cultures stained by immunofluorescence. X480.

actively by the piglets following infection may interfere with the virus replication in cell culture (18). The difficulties encountered in the isolation of the virus with specimen No. 3 is believed to be the result of interference by antibodies or other virus inhibitors present in the inoculum diluted at 1:40. At 1:400 these interfering substances have apparently been diluted beyond their level of activity. In pigs infected orally, it is possible that the low pH prevailing in the stomach could dissociate the virus-antibody complex thus explaining the greater susceptibility of piglets compared to cell culture.

Under identical test conditions, the frequency of virus isolation in the PTh and the PK cells was very similar. However, every field isolate of TGE virus was cytopathic for PTh cells without additional blind passages while one and occasionally two passages were needed to demonstrate the virus by its CPE in PK cells. Other investigators from different parts of the world have also reported similar findings with the PTh cells infected with TGE virus (19, 20). For the easily recognizable CPE to occur, it was essential to incubate the tubes in stationary positions. When the tubes were agitated or rolled in drums, only vague cellular alterations were noted in both cell systems.

Although the majority of the tests were conducted with primary PTh cells occasional tests were also done with secondary PTh cells without noticeable differences in susceptibility. When further subcultures of PTh cells were made however, their ability to produce CPE in response to TGE virus declined such that by the fifth and subsequent subcultures only vague CPE was noted. With the PK cells it was essential to use fresh, actively growing, primary cells in order to have reproducible CPE. Primary kidney cells, kept on the maintenance medium for more than three to four days, as well as secondary cells, did not regularly show detectable CPE following inoculation with the newly isolated TGE viruses.

Several lots of PTh cells, prepared from pools of glands collected in abattoirs, could not be used for diagnostic work, because of parvovirus contamination. The contamination was always subtle and, at times, was detectable only by immunofluorescence after two or three subcultures. It is unlikely that the trypsin was the source of parvovirus contamination because other primary porcine cells and porcine cell lines were dispersed with the same lot of trypsin and remained free of parvovirus. This high incidence of parvovirus contamination in PTh cells, has not been reported by other investigators (12, 19-21) though the virus is known to occur in populations of conventionally-raised pigs (5, 11, 13, 15).

The MDCF test developed previously (7) for the diagnosis of TGE could not detect antibodies in pigs convalescent from an infection in the field. However, the MDCF test has been useful in selecting appropriate swine serums for conjugation with fluorescein isothiocyanate. It was found that sera with SN titers were not necessarily good for conjugation while serums from hyperimmunized pigs and having MDCF titers of 1:40 or above and SN titers of above 1:512 gave good immunofluorescent conjugates. Further work is needed to elucidate the nature of these differences.

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BOOK REVIEW

LABORATORY DIAGNOSIS OF MYCOPLASMOSIS IN FOOD ANIMALS. *Edited by Ole H. V. Stalheim. A special report of the Mycoplasmosis Committee American Association of Veterinary Laboratory Diagnosticians. Published by The American Association of Veterinary Laboratory Diagnosticians. 1976. 156 pages. Price $7.50 Postpaid.*

Information on the significance of mycoplasma in animal diseases has been increasing for the past 28 years. During this time a new specialist has emerged, the mycoplasmologist. Because of the special requirements, care and procedure required for isolation and identification of mycoplasma they have been a somewhat obscure group for many. This book provides an up-to-date review of this important group of microorganisms and will do much to remove the aura of mystery that has kept them unfamiliar.

In the book, mycoplasma associated with the bovine receive the greatest attention probably because a definite role for mycoplasma in specific conditions has been established. Nonetheless the association of mycoplasma with disease in sheep, goats, pigs and poultry have been adequately covered.

Approximately one-third of the book is devoted to laboratory procedures and sufficient details are provided to enable those laboratories not now working with mycoplasma to enter this field.

The reports, while written in the style of summaries are complete and indicate clinical signs and macroscopic changes, diagnosis, isolation and identification and indicate serological tests of value. Each section has been documented with references. Four hundred and fifty-four references have been provided in addition to those for most of the various media listed.

Some may question the use of the term L-forms as opposed to L-phase variants while others may suggest media which they consider more appropriate for a particular purpose rather than those described. Still others may suggest that certain areas should have been expanded. These comments are bound to occur in a field which is developing as rapidly as mycoplasmology.

This book contains a wealth of information written in a clear and comprehensive manner. It is highly recommended for anyone wishing a ready reference on mycoplasma of food animals, the diseases produced and the isolation and identification of the organism involved. — *R. B. Truscott.*