Journal of Virological Methods 223 (2015) 88–95

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Isolation and propagation of the animal rotaviruses in MA-104 cells—30 years of practical experience

Peter H. Otto[a], Jochen Reetz[b], Werner Eichhorn[c], Werner Herbst[d], Mandy C. Elschner[a,*]

[a] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Naumburger Str. 96A, 07743 Jena, Germany
[b] Federal Institute for Risk Assessment, Diedersdorfer Weg 1, 12277 Berlin, Germany
[c] Ludwig-Maximilians-Universität München, Institut für Infektionsmedizin und Zoonosen, Infektions- und Seuchenmedizin, Veterinärstraße 13, 80539 München, Germany
[d] Justus-Liebig-Universität Gießen, Institut für Hygiene und Infektionskrankheiten der Tiere, Frankfurter Str. 87-89, 35392 Gießen, Germany

Article history:
Received 1 December 2014
Received in revised form 27 July 2015
Accepted 28 July 2015
Available online 31 July 2015

Keywords:
Cell culture
Diarrhoea
Isolation
Rotavirus
Trypsin
MA-104 cells

A B S T R A C T

A total of 136 rotavirus positive samples from diarrhoeic animals of different species were submitted for isolation and cultural propagation of rotavirus on MA-104 cells. The samples were collected from animals with diarrhoea, between 1980 and 2010, originating from herds or farms located in several parts of Germany. Rotaviruses of species A were isolated from 102 faecal samples in cultures of MA-104 cells under the following conditions: pre-treatment of virus with trypsin, incorporation of trypsin into culture medium, use of roller cultures, and centrifugation of the samples on the cells. The cell culture adapted viruses produced a cytopathic effect, accompanied by the release of cells from the glass surface of the cultivation vessels. After 10 passages the virus isolates yielded titres between $10^{5.5}$ and $10^{7.5}$ ml^{-1} $TCID_{50}$.

Isolation and serial propagation of the virus in MA-104 cells was confirmed by immunofluorescence assay, transmission electron microscopy, and polyacrylamide-gel electrophoresis of viral dsRNA. Eight (5.9%) of the electrophoretic profiles were characteristic of species B or D rotaviruses, which were not replicated in MA-104 cells.

1. Introduction

Rotavirus (RV) infections have been associated with enteritis and diarrhoea in a variety of mammalian and avian species (Estes and Greenberg, 2013). RVs are non-enveloped icosahedral particles containing a genome of 11 segments of double –stranded (ds) RNA (Estes and Greenberg, 2013). The virion is comprised of three layers of viral structural proteins (VP), with VP2 forming the core, VP6 the inner layer with species-specific epitopes, and VP7 and VP4 forming the outer shell which contains the serotype/genotype-specific epitopes. RVs are classified serologically by a scheme that distinguishes multiple species (A to H) and multiple serotypes/genotypes within each species. RVs of species A (RVA) represent the most common cause of gastroenteritis in humans and animals. A comparison of RVAs from avian hosts with their counterparts from mammals showed that both groups are distantly related. The most obvious differences between avian and mammals RVA strains are found in genome segment 5, which has a slower migration in mammalian RVA strains (Schumann et al., 2009). RVs of species B, C and H are found in humans and animals, whereas RVs of species D, E, F and G have been found only in animals; RVs of species D, F and G have been detected only in various avian species (Ghosh and Kobayashi, 2011; Matthijnssens et al., 2012; Wakuda et al., 2011).

The first RV strains that could be propagated in cell cultures were the murine EDIM strain and simian SA-11 strain (Adams and Kraft, 1963; Malherbe and Strickland-Cholmley, 1967). Bovine and human RVs were first isolated in cell cultures in 1971 and 1980, respectively (Mebus et al., 1971; Wyatt et al., 1980). Although a wide variety of techniques for the detection of RVs in faecal samples have been developed, the use of cell cultures for replicating these viruses is limited. By addition of gastrointestinal enzymes to the cell culture systems, it was possible to enhance RV infectivity, resulting in increased isolation rates of RV strains. The pre-treatment of RV particles with enzymes prior to

Abbreviations: RV, rotavirus; VP, viral structural proteins; RVA, rotavirus of species A; MA-104 cells, rhesus monkey kidney cell line; CaCo-2, human colon carcinoma cell line; HepG2 cells, human hepatocellular liver carcinoma cell line; PBS, phosphate buffered saline solution; DMEM, Dulbecco's modified eagle medium; CPE, cytopathic effect; DEAE, diethylaminoethyl; IFA, immunofluorescence assay; TEM, transmission electron microscopy; PAGE, polyacrylamide gel electrophoresis; U, units.

* Corresponding author. Tel.: +49 3641 804 2428; fax: +49 3641 804 2228.
E-mail address: mandy.elschner@fli.bund.de (M.C. Elschner).

http://dx.doi.org/10.1016/j.jviromet.2015.07.016
0166-0934/

their inoculation in cell cultures and its incorporation in the maintenance media has permitted their replication and successful passage in cell culture (Almeida and Hall, 1978; Clark et al., 1979; Graham and Estes, 1980; Theil and Bohl, 1980). Several studies demonstrated that the enzyme trypsin is responsible for cleaving the attachment protein VP4 into VP5* and VP8* (Estes and Greenberg, 2013). The use of enzymes alone has not always facilitated the direct adaptation of many RV strains. A procedure using enzymes, pre-treatment of highly permissive cells with DEAE-dextran and roller cultures has been more successful (Bingnan et al., 1991; Greenberg et al., 1986; Fukusho et al., 1981; McNulty et al., 1979; Superti et al., 1993; Theil et al., 1986; Urasawa et al., 1981; Yason and Schat, 1985). The propagation of RVs is generally performed in several cell types of kidney or colon adenocarcinoma (Arnold et al., 2009). MA-104 cells (Rhesus monkey kidney cell line) in addition to CaCo-2 (Human colon carcinoma cell line) and HepG2 cells (Human hepatocellular liver carcinoma cell line) are the cells of choice for RV growth (Arnold et al., 2009; Birch et al., 1983; Estes and Greenberg, 2013; Kitamoto et al., 1991; Lee et al., 2013; Legrottaglie et al., 1997; Minamoto et al., 1988; Urasawa et al., 1981).

Of the animal RV isolated in cell culture in recent times almost all belong to species A (Estes and Greenberg, 2013). Several attempts to serially propagate non-RVAs have been unsuccessful (Bohl et al., 1982; Terret and Saif, 1987). Very few non-RVA strains have been successfully propagated in cell culture: a single species C human, bovine and porcine RV strain (Saif et al., 1988; Shinozaki et al., 1996; Tsunemitsu et al., 1991; Welter et al., 1991), and a single species B porcine RV strain have been cultured (Sanekata et al., 1996).

The objective of this study is the detailed description of a recommendable routine method for the successful isolation and cultivation of RVs from several animal species in cell cultures. Tissue-culture adapted animal RV strains can be used to investigate the disease processes and immune mechanisms of RV infections and to develop new RV vaccines.

2. Materials and methods

2.1. Samples

136 specimens of faeces or intestinal contents were collected from (a) animals of seven mammalian species (66 calves, 19 piglets, 16 foals, 5 cats, 2 red river hogs, 2 dogs, 2 camels, 1 fox, and 1 goat) suffering from acute diarrhoea and (b) three avian species (18 chickens, 3 turkeys, and 1 pheasant). Samples in which rotavirus particles have been observed by electron microscopy, were used for this study. Based on the results of two studies the samples were stored at −80 °C until virus isolation in cell cultures (Holmes, 1988; Tietzova and Petrovicova, 1994).

An approximately 20% suspension of faecal samples was prepared in phosphate buffered saline solution (PBS, pH 7.4) and sonicated for 60 s at level 4 (USD 20, K.W. Meinhardt Ultraschalltechnik, Leipzig, Germany). This suspension was clarified by centrifugation at 3345 × *g* for 20–30 min (Megafuge 1.0 RS, Heraeus Instruments GmbH, Hanau, Germany). The supernatants were examined immediately as described below, or stored at 4 to 8 °C for not more than 24 h or at −80 °C for longer periods before use. Prior to the inoculation on the cells the supernatants were thawed and mixed with an equal quantity of Hanks solution pH 7.4 (Serva Electrophoresis GmbH, Heidelberg, Germany) supplemented with gentamicin (Sigma, Taufkirchen, Germany) at a final concentration of 500 μg/ml. After an incubation period of 60 min at room temperature, trypsin (Serva Electrophoresis GmbH, cat. # 37290) was added to a final concentration of 0.4 U/ml corresponding 100 μg/ml. Samples were used for inoculation after incubation for 60 min at 37 °C.

2.2. Cells and virus inoculation

A seed culture of the permanent MA-104 cell line, free of *Mycoplasma hyorhinis*, was provided from the Friedrich-Loeffler-Institut, Collection of Cell Lines in Veterinary Medicine, Isle of Riems, Germany (cat. # Rie 142). For the isolation experiments cell passage numbers 3–50 were used. Cells (10^5/ml) were seeded in Dulbecco's modified eagle medium (DMEM) with L-glutamine and 4.5 g/l glucose without bicarbonate (ICN Biomedicals, Inc., Aurora, USA, cat. # 1033120) supplemented with 10% of foetal bovine serum in 24-multiwell plates (Becton Dickinson Labware, Le Pont de Clair, France, cat. # 30479), 96-well culture plates (Nunc, Roskilde, Denmark, cat. # 167008), 25 ml-bottles (Schott AG, Mainz, Germany) or flasks (BD Biosciences Labware, Heidelberg, Germany) were incubated for 3 days at 37 °C in a 5% CO_2-air atmosphere (WTP, Binder Labortechnik GmbH, Tuttlingen, Germany). The growth medium was decanted, and the cell cultures were washed once with Hanks solution (Biochrom KG, Berlin, Germany) to remove any residual serum before inoculation with virus and were pre-treated with diethylaminoethyl (DEAE)-dextran solution (Serva Electrophoresis GmbH, Heidelberg, Germany, cat. # 18686; 40 mg/l) in DMEM at 37 °C for 30 min. 500 μl of faecal suspension or 1 ml of virus from a cell culture suspension were used as inocula. The bottles or flasks were incubated at 37 °C in a humidified air atmosphere with 5% CO_2 for 60 min to allow virus for adsorption, while the plates were centrifuged at 1000 × *g* for 1 h at room temperature. The inoculum was replaced by DMEM containing 0.04 U/ml trypsin and 50 μg/ml gentamicin and incubated at 37 °C for 1–5 days. The cell cultures were examined daily for up to 5 days for cytopathic and morphological changes using an inverted light microscope (IX 70, Olympus Optical Co. GmbH, Hamburg, Germany). The cultures were harvested five days after infection or when the cytopathic effect (CPE) could be observed.

From the 2nd to the 10th passage, stationary growing cells were used in roller bottles, and the bottles were rolled only after virus inoculation. In a few cases, in which the cell cultures were contaminated with bacteria during the first two virus passages the isolation procedure was repeated after filtration of the supernatant of faecal samples using a membrane filter with a pore size of 0.2 μm (Sartolab plus, Sartorius GmbH, Göttingen, Germany). If the virus showed a distinctive CPE, the further viral passages were performed under stationary conditions as follows. The virus-infected cultures either 24 well plates, bottles or flasks were harvested using two freeze-thaw cycles, and stored at −20 °C until needed. Subsequent virus passages were performed as described. To avoid virus cross contamination, the strains were handled separately in a safety cabinet.

2.3. Virus quantification and clone purification

Viral infectivity was assayed at passage level for all isolates by inoculating four cell culture wells per $\log_{10}$-dilution using standard procedure. Final reading of the CPE was carried out after an incubation period of 4 or 5 days. The infectious titre was expressed as 50% tissue culture infective dose ($TICD_{50}$)/ml according to the method of Spearman and Kaerber (Mayr et al., 1974).

All isolates were clonally-purified as described for avian rotaviruses (Yason and Schat, 1985) with minor modifications. The virus suspension was $\log_2$-diluted and the virus harvested by scraping the cells from the wells of those virus dilutions at which only one rotavirus-positive plaque was observed. The harvested virus suspensions were titrated in a second and third cycles.

2.4. Transmission electron microscopy (TEM)

The faecal samples and intestinal contents were investigated by TEM using negative staining. Briefly, the sample supernatants were