Use of enzyme-linked immunosorbent assays (ELISA) for serotyping rotavirus and "Breda" virus

by

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ABBREVIATIONS

ELISA	Enzyme-linked immunosorbent assay
HAHI	Hemagglutination-hemagglutination inhibition
IEM	Immune electron microscopy
MW	Molecular weight
IF	Immunofluorescence
CF	Complement fixation
GD	Gel diffusion
RIA	Radioimmunoassay
OD	Optical density
PHA	Passive hemagglutination
IEOP	Immunoelectrosmophoresis
BSA	Bovine serum albumin
PTA	Phosphotungstic acid
NT	Neutralization test
PMA	Polyclonal monospecific antiserum
HI	Hemagglutination inhibition
GC	Gnotobiotic calf
DPI	Days post-infection
LI	Large intestine
K9	Canine

INTRODUCTION

Rotaviruses derived from different host species have been distinguished antigenically by the ELISA blocking test as described by Yolken et al. (1978a). By this method, homologous antibody blocked the homologous antigen with a 50% blocking (BL50) at least 10 times higher than the heterologous system.

The new "Breda" virus isolated in Iowa (Woode et al., 1982) and an isolate from Ohio (Saif et al., 1981) have been compared by immunofluorescence, HAHI, IEM and ELISA. By HAHI and by ELISA two distinct serotypes, "Breda" virus 1 and "Breda" virus 2 with Ohio as serotype 2, have been proposed (Woode et al., 1983a).

The purpose of this study was to investigate the use of ELISAs for serotyping animal rotaviruses and "Breda" virus isolates 1 and 2 which had been shown by serum neutralization (Gaul et al., 1982) and by hemagglutination inhibition test (Woode, G. N., personal communication, VMPM, Iowa State University) respectively, to be different serotypes of their particular group of viruses.

LITERATURE REVIEW

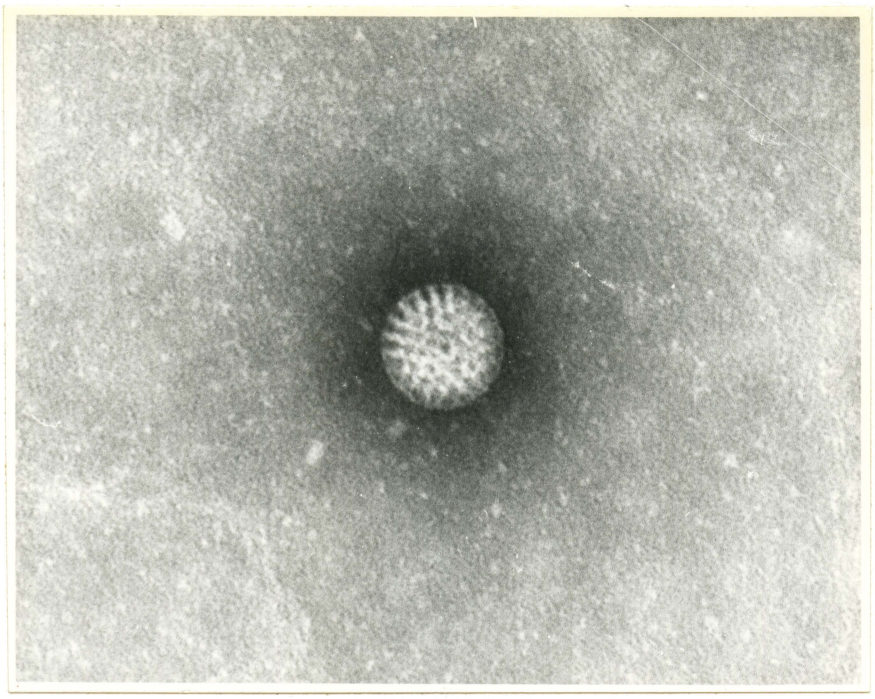
Definition of Rotavirus

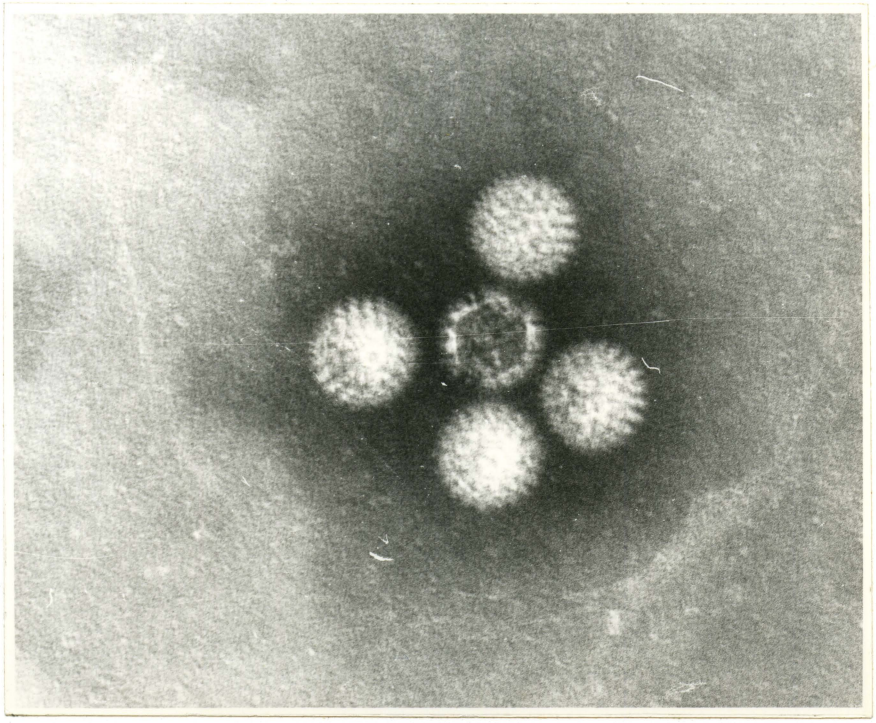
Rotaviruses are classified among the animal virus genera within the family Reoviridae (Flewett and Woode, 1978; McNulty, 1978; Matthews, 1982; Holmes, 1983). Nucleic acid contains 11 pieces of linear double-stranded (ds) ribonucleic acid (RNA) with molecular weights (MWs) equivalent to 0.2-2.2 x 10^6 daltons and with a total MW equivalent to 12-20 x 10^6 which comprise 14-22% of the weight of virus. There are 8-10 polypeptides in the virion, including transcriptase and other enzymes, with MWs equivalent to 15-130 x 10^3. Some polypeptides may contain a small amount of carbohydrate. There are no lipids present. Effective buoyant density in CsCl = 1.36-1.39g/cm^3. Infectivity is stable at pH 3 and they are relatively heat stable and resistant to ether. Rotavirus is an icosahedral particle (P = 3; T = 3) with diameter 65-75 nm, there is no lipoprotein envelope but there are two protein coats (Figure 1). The particle with the outer coat removed (Figure 2) is termed the core. The core has 12 spikes with 5-fold symmetry arranged icosahedrally. Replication of the virus occurs in the cytoplasm. Viroplasms in cytoplasm of infected cells sometimes contain virus particles in crystalline arrays.

Transmission occurs horizontally and biological vectors are not considered important. Rotaviruses have been isolated from man, cattle, mouse (EDIM), guinea pig, sheep, goat, pig, monkey (SA11), horse, antelope, bison, deer, rabbit, cat, dog, chicken, turkey, etc.

Figure 1. Electron micrograph of a porcine rotavirus particle (400,000X) with the outer protein coat present (Courtesy Dr. D. E. Reed, VMRI, ISU, Ames, IA 50011)

Figure 2. Porcine rotavirus. Micrograph shows virus particles without the outer protein coat (incomplete virus particles) (400,000X) (Courtesy Dr. D. E. Reed, VMRI, ISU, Ames, IA 50011)





Rotaviruses are not host specific as cross infection was shown experimentally with human and bovine in pigs, and human rotavirus in calves (Hall et al., 1976; Davidson et al., 1977; Mebus et al., 1977) and similarly the calf rotavirus infected pigs (Woode et al., 1976). Experimental disease has been caused by homologous virus in humans, mice, calves, piglets, foals, lambs and puppies (Holmes, 1983), some cross-infections have induced disease for example bovine, equine, simian and some human rotavirus isolates in piglets (Woode and Bridger, 1975; Woode et al., 1976). All rotavirus isolates are similar in appearance on the electron microscope and appear to be pathogens exclusively of enterocytes of the small intestine (Flewett and Woode, 1978) with the exception of lamb rotavirus which also infects the large intestine (Holmes, 1983). All the known rotaviruses share a common antigen associated with the inner capsid layer (Flewett et al., 1974; Woode et al., 1976; Bridger, 1978; Flewett and Woode, 1978; McNulty, 1978) as demonstrated by immunofluorescence (IF), complement fixation (CF), gel diffusion (GD) and immune electron microscopy (IEM). Serotypes are probably numerous and show some cross-reaction.

Definition of "Breda" Virus

"Breda" virus as described by Woode et al. (1982) was either spherical (89 $\pm$ 7 nm x 75 $\pm$ 9 nm with peplomers 7.6-9.5 nm long) (Figures 3 and 4) or kidney shaped (120 $\pm$ 15 nm x 32 $\pm$ 8 nm) (Figure

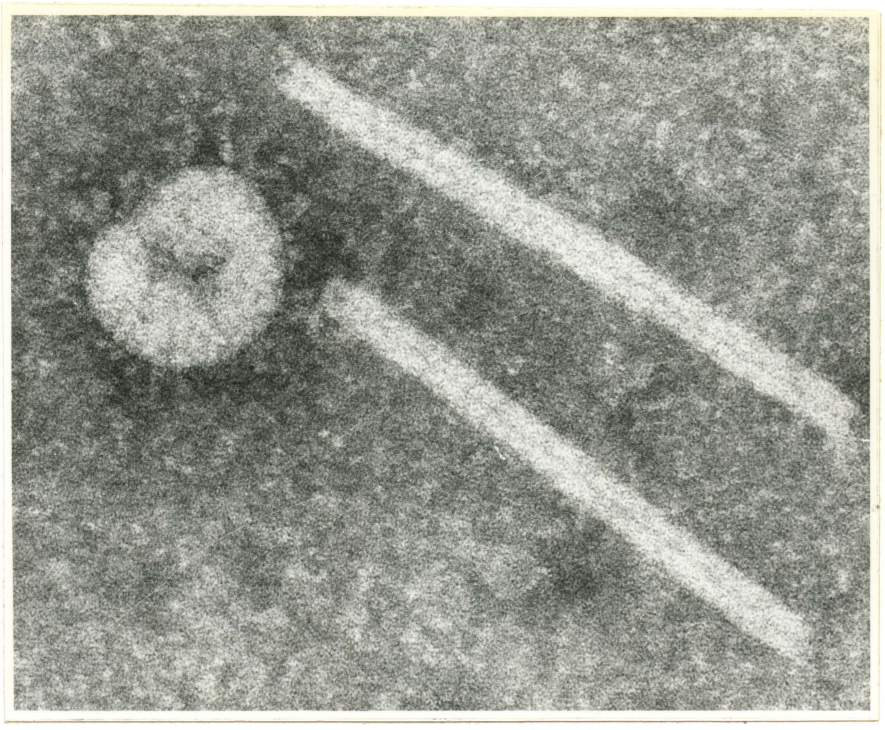
5) with similar peplomers as the spherical shaped particle. This morphology is superficially similar to coronavirus but it was considered different to coronavirus in that their peplomers were of 7-9 nm in contrast to the 17-20 nm of coronavirus. The hemagglutinin was antigenically distinct from coronavirus and "Breda" virus was antigenically distinct from other common bovine viruses. Attempts to isolate the virus in cell cultures, intestinal and tracheal organ cultures and embryonated eggs were unsuccessful. For cultivation of the virus, gnotobiotic calves were inoculated orally and virus obtained from intestinal and fecal samples.

Serotypes of Rotavirus

Rotaviruses have been shown to differ antigenically from each other despite the presence of one or more common antigens (Flewett et al., 1974; Woode et al., 1976; Thouless et al., 1977). Serotype specific antigens are associated with the outer capsid layer of rotaviruses (Bridger and Woode, 1976; Bridger, 1978). Type specific antigens have been distinguished from each other by complement fixation (Zissis and Lambert, 1980), by a hemagglutination inhibition test (Spence et al., 1978) and by serum neutralization test (Flewett et al., 1976; Woode et al., 1976; Bridger, 1978; Thouless et al., 1978; Gaul et al., 1982). Enzyme-linked immunosorbent assay (ELISA) was employed by Yolken (Yolken et al., 1978a) to serotype various

Figure 3. Bovine "Breda" virus and Tobacco mosaic virus. Photomicrograph shows a spherical shaped "Breda" virus particle (400,000X)
(Courtesy Dr. D. E. Reed, VMRI, ISU, Ames, IA 50011)

Figure 4. Bovine "Breda" virus spherical shaped particle (400,000X)
(Courtesy Dr. D. E. Reed, VMRI, ISU, Ames, IA 50011)



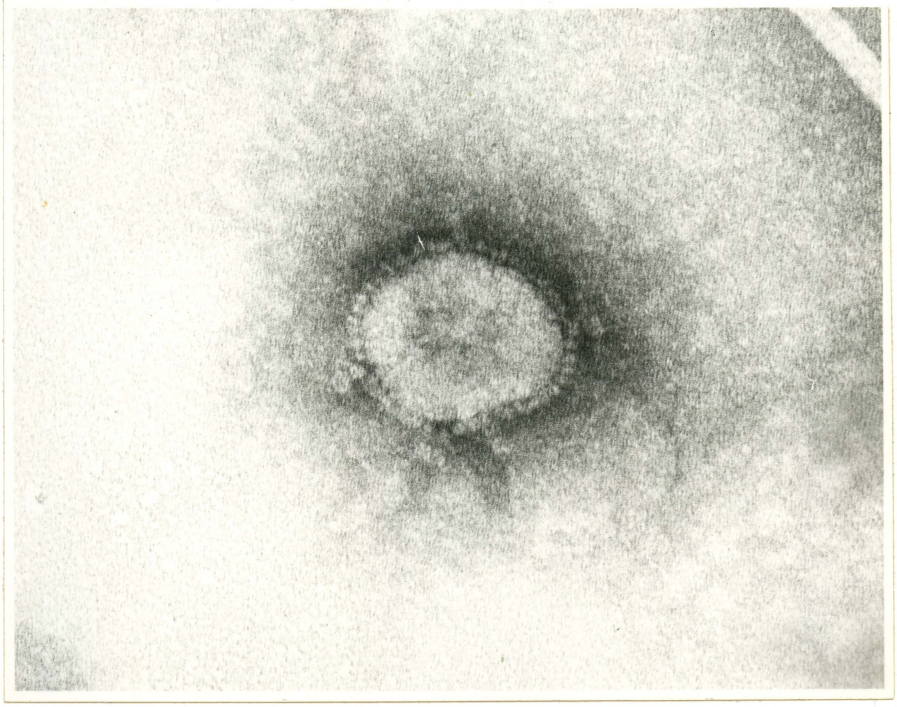
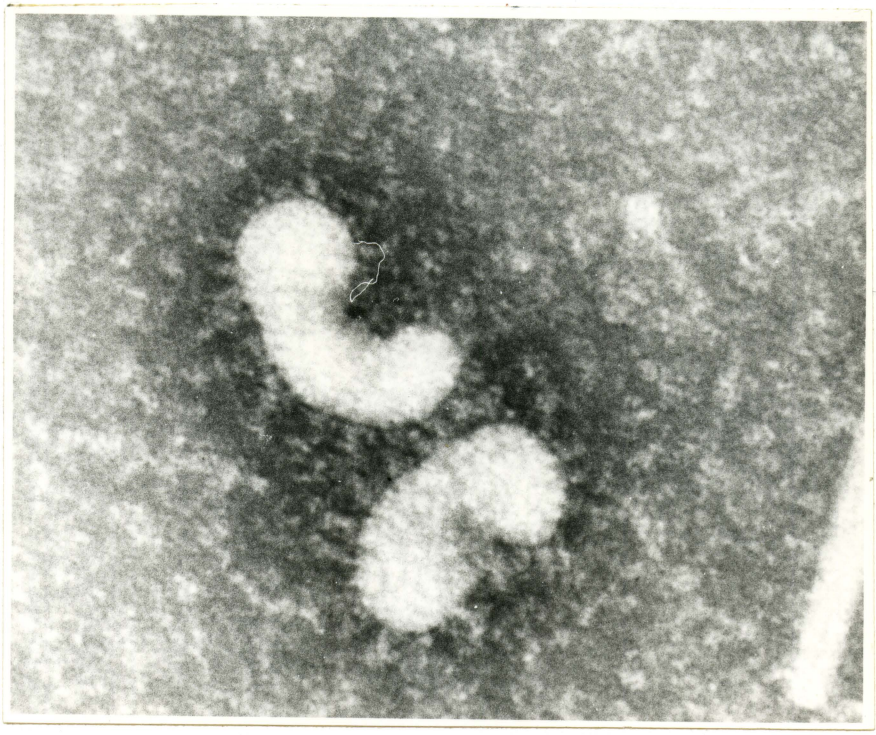


Figure 5. Electron micrograph of bovine "Breda" virus kidney shaped particles (400,000X)
(Courtesy Dr. D. E. Reed, VMRI, ISU, Ames, IA 50011)



human and animal rotaviruses. In the same year, Yolken et al. (1978c) reported an ELISA for differentiating human serotype 1 from human serotype 2. Zissis and Lambert, in 1980, serotyped human rotavirus by the double antibody sandwich ELISA. This latter method was followed with success by Thouless et al., in 1982, to serotype human rotavirus.

Review of ELISA

Introduction

Advances in molecular biology and the understanding of the molecular basis of disease, generated a need for new methods which are quantitative, specific and even more sensitive. Traditionally, infectious diseases have been diagnosed by the cultivation of the infecting agent in an in vitro system or laboratory animal. There are limitations to this approach for those viral agents that can not be cultivated outside the natural animal host. In order to obtain a rapid diagnosis of an infectious disease, there has been considerable interest in the development of assays capable of detecting infectious agents directly in clinical specimens. Most of these assays are based on the fact that infectious agents can be identified by a specific antigen-antibody reaction. An immunoassay that has attained widespread usage for this purpose is the solid-phase radioimmunoassay (RIA), which is highly sensitive and objectively interpreted. Disadvantages of RIA are associated with the use of radioactive reagents and with the costly equipment necessary for the tests

(Yolken, 1982). Other assay systems based on immunodiffusion, immunoelectrophoresis, agglutination and immunofluorescence have attained widespread use but these assay systems are often less sensitive than RIA, are not readily quantified and may require subjective interpretations of the antigen-antibody indicator system (Yolken, 1982).

Enzyme-linked immunosorbent assay (ELISA) has been found to be as sensitive as RIA and offers many theoretical advantages and few disadvantages. It does not require expensive equipment, it can be performed in any diagnostic laboratory, the results are highly reproducible and it is highly adaptable to mass screening and automation (Saxinger, 1981). Since a single molecule of enzyme can catalyze the conversion of a large number of molecules of substrate, its sensitivity is partly explained (Clark and Engvall, 1980; Yolken, 1982). ELISA was first described by Engvall and Perlmann, 1971 as a method for antigen determination, with immunoglobulin G (IgG) from rabbit as antigen, and conjugates made with alkaline phosphatase and glutaraldehyde. The mass of published work since then in which the ELISA is employed is evidence of its broad applicability and potential.

The ELISA is based on measuring the binding of antigen to antibody by reacting the mixture with an enzyme labeled antibody to one or the other of the reactants. The enzyme then catalizes the substrate added from colorless to a colored product. The various ELISAs have been classified as either competitive or non-competitive

(Engvall, 1980; Yolken, 1982). A competitive assay involves a reaction step in which unlabeled and labeled antigen (or antibody) compete for a limited number of antibody (or antigen) sites. In a non-competitive assay, the antigen (or the antibody) to be measured is first allowed to react with antibody (or antigen) on a solid phase followed by measurement of the binding of enzyme-labeled immune reactant. For both assays, the enzymatic activity in the bound or free fraction is quantified by enzyme-catalyzed conversion of a relatively non-chromatic substrate to a highly chromatic product. In order to choose the assay design to be employed, several factors should be considered (Clark and Engvall, 1980). For the competitive ELISA, purified antigen is required for the preparation of the enzyme-antigen conjugate. The incubation of enzyme-labeled antigens or antibodies with test solutions containing protein modifying enzymes such as proteases and non-competitive enzyme inhibitors constitute a serious disadvantage since all of which may alter the activity of the enzyme label in the subsequent incubation with the enzyme substrate (Maggio, 1980). This problem is avoided in the non-competitive ELISA where the incubation with the test solution is separated from the incubation with enzyme-labeled antigens or antibodies.

The sensitivity of an enzyme immunoassay is directly related to the amplification effect imparted by the enzyme moiety (the formation of many product molecules per test antigen molecule) (Clark and Engvall, 1980; Yolken, 1982). The enzyme used in the preparation of the conjugate should be relatively stable at 25-37^{o}C with a shelf life

of at least 6 months at 4°C, should be commercially available and relatively inexpensive, its activity should be easily measurable by colorimetric methods, it should have a high substrate turnover number and in the case of competitive ELISAs the enzyme should not be affected by biological components of the test sample (Maggio, 1980). The enzymes that best satisfy these criteria according to Clark and Engvall (1980); and to Yolken (1982) are alkaline phosphatase from calf intestine, horseradish peroxidase and β-galactosidase. Alkaline phosphatase activity may be quantitated using nitrophenyl phosphate as substrate, β-galactosidase activity can be measured using nitrophenyl galactose as a substrate. Horseradish peroxidase activity involves several sensitive redox reactions with H_2O_2 and 2,2-azino-di-(3-ethylbenzothiazolinsulfone-6) diammonium salt (ABTS) as substrate.

Antibody and antigen have been covalently attached to cellulose, agarose and polyacrylamide, however solid phase carriers such as beads, discs and tubes facilitate washing and separation steps. Antigens and antibodies have been physically adsorbed to plastic carriers (polystyrene, polyvinyl, polypropylene, polycarbonate) and to silicone rubber or treated glass (Clark and Engvall, 1980, Burrels and Dawson, 1982). Most proteins adsorb to plastic surfaces, probably as a result of hydrophobic interactions between non-polar protein substructures and the non-polar plastic matrix (Clark and Engvall, 1980). The rate and extent of coating will depend on the diffusion coefficient of the adsorbing molecule, the ratio of the surface area to be coated to the volume of coating solution, the concentration of

the adsorbing substance, the temperature and the duration of the adsorption reaction (Clark and Engvall, 1980). The most commonly used buffer for adsorption to solid phase is a 0.05 M carbonate buffer pH 9.6. The reported volumes of reagents added to microplate wells vary from 50 µl through 100 µl, 150 µl, 200 µl, 250 µl and 300 µl. With tubes and cuvettes the volume generally used is 1 ml (Burrels and Dawson, 1982). Adsorption may vary from incubation at 4°C, room temperature and 37°C overnight, 37°C for 3 hours and 37°C for 30 minutes. Fluids used for washing vary from tap water, distilled water, deionized water, saline, phosphate buffered saline and 0.2 M tris buffer. Most of them incorporate Tween 20 or Tween 80 in order to decrease the non-specific binding (Burrels and Dawson, 1982). Buffers used during the specific incubations can vary. They can be the same ionic salt solutions or buffers used as washers or they can incorporate a small amount of protein to avoid any non-specific binding.

Factors affecting the specificity as well as the sensitivity of the ELISA have been reviewed by Clark and Engvall (1980); and by Yolken (1982). As a general conclusion, two major factors limit the sensitivity of ELISA: first the binding affinity between antigen and antibody and second, the level of detection of the enzyme employed as label. The most sensitive ELISA is, in theory, the two site or sandwich ELISA which probably results from the use of excess reagents in each step of the procedure. The principal determinant of specificity in ELISA is the antibody. A second critical factor is the purity of the antigen used as immunogen and as assay standard.

The ELISA results have been expressed (Burrels and Dawson, 1982) as: end point titers with samples to be assayed serially diluted, positive or negative (qualitative purposes), titers vs. absorbance value where the OD of the test sample is usually compared with the OD of a known positive sample included in each test, ratio where OD of a test sample is divided by the mean OD of a group of known negative samples, in comparison with a standard curve.

Variables of the ELISA include: types of solid phase employed, conditions for adsorption to the solid phase, wash solutions, wash procedures, conditions during specific reaction steps, methods by which assays are read and results expressed. All these variations do not suggest that any one modification is superior to others. Once the test has been standardized, the main factors governing test procedures are quality and volume of reagents, availability of ancillary equipment, importance of rapid results and the ability of the test system to fit into a normal working time span.

Applications of ELISA

General Recent articles (Voller et al., 1978) or books Wardley (1982); summarize the fields of application of the ELISA. Briefly, in the microbiology field ELISA has been developed for the detection of antibodies against microorganisms such as brucella (Thoen et al., 1979); serology of parasitic diseases (Duffus, 1982), helminthology (Sinclair, 1982) and mycotic infections (Voller et al.,

1978). As reviewed by Voller et al. (1978) ELISA has also been developed for the detection of antigens from microorganisms including brucella, yersinia and salmonellae as well as for measuring hormones (Saver et al., 1982) serum proteins, cancer antigens, drugs and allergens (Schuurs and vanWeemen, 1980).

Viruses The development of efficient cultivation techniques has allowed reliable detection of a large number of agents. However, for some infectious diseases, standard cultivation techniques have not yielded an etiologic agent (Yolken, 1980). In addition, in the case of many viral agents, diagnosis based on the cultivation of an agent often can not be made with sufficient rapidity to be used in the management of an acute illness. Because of these shortcomings, solid-phase immunoassays have been developed for the direct detection of viral antigens in clinical specimens and also for the detection of viral antibodies. Bidwell et al. (1977) reported the successful use of ELISA for the detection of antibodies to rubella, measles, adenovirus, coxsackievirus, herpesvirus, respiratory syncytial virus and Newcastle virus. Indirect ELISA has been employed for the detection of IgG, IgA and IgM antibodies to rinderpest virus (Anderson and Rowe, 1982), for African swine fever antibodies (Sanchez-Vizcaino et al., 1982), for antibodies against swine vesicular disease (Hamblin and Crowther, 1982). The ELISA has been employed for detection of antibodies to infectious bovine rhinotracheitis virus (IBR) in milk samples (Bommeli and Kihm, 1982) and also to study local immunity such

as detection of IgA and IgG in tracheal fluids of infected chickens with infectious bronchitis virus (IBV) (Darbyshire, 1982). The ELISA blocking assay (sandwich ELISA) has been employed for the detection of antibodies to coronavirus-like agents in pigs (Debouck et al., 1982) and the double antibody sandwich blocking ELISA for the detection of antibodies against equine infectious anemia (Gielkens and Houwers, 1982). For the serodiagnosis of bovine enteric coronavirus as reported by Crouch and Raybould (1983) ELISA as well as passive hemagglutination assay (PHA) systems were suitable, however PHA was more rapid and economic.

In the case of the detection of an infectious agent, direct single antibody ELISAs have been used for the detection of Hepatitis A antigen (Yolken, 1980). Sandwich ELISAs have been employed for detection of canine parvovirus in fecal samples (Have, 1982), to detect bovine coronavirus in feces and intestinal contents in calves (Meyling, 1982) and for detecting bovine rhinotracheitis virus (Nettleton et al., 1982). Blocking sandwich ELISA was employed by Debouck et al. (1982) to detect coronavirus-like agents in feces of pigs with porcine epidemic diarrhea. Yolken and Stopa (1980) reported the relative sensitivity of seven different immunoassay systems for the measurement of cytomegalovirus (CMV). Double antibody methods were more sensitive than single antibody methods preferably when antisera were prepared in two different animal species. Successful application of the ELISA for the detection of FeLV antigens and antibodies in feline leukemia cases in which virus was not isolated

was reported by Saxinger (1981).

Serotyping Viruses by ELISA

The indirect ELISA may be successfully used for subtyping foot and mouth disease (FMD) virus (Rai and Lahiri, 1981). According to Ouldridge et al. (1982a and b) the complement fixation test commonly used to quantify the FMD immunogen detects both the immunogenic antigens as well as the capsid protein subunits which do not elicit an antibody response. In contrast, the indirect sandwich ELISA preferentially measured the major immunogenic site (neutralizing protein antigen). Indirect sandwich ELISA had greater specificity than the indirect ELISA since it readily distinguished intact virion from trypsin-cleaved virions, and heterotypic virion.

Serotyping rotavirus

All rotaviruses from whichever species look alike in the electron microscope and all appear, to date, to be pathogens of the enterocytes of the small intestine. Nevertheless, considerable antigenic diversity exists among them which is detectable by immune electron microscopy and ELISA but it is best reflected in the serum neutralization tests in which a 10-fold or greater titer is demonstrated in the homologous reaction as compared with the heterologous reaction (Flewett et al., 1974; Woode et al., 1976; Thouless et al., 1977; Thouless et al., 1982).

Woode et al. (1976) were able to partially distinguish animal and human rotavirus by immune electron microscopy. Thouless et al. (1977) were able to distinguish between different groups of animal rotaviruses by neutralization tests. Yolken et al. (1978a) distinguished rotavirus derived from different host species by post-infection serum blocking virus activity in an ELISA. Homologous antigen-antibody systems blocked with a BL50 at least 10 times higher than the heterologous by this ELISA blocking test. Specificity was noted only with sera containing antibody induced initially or solely by infection. Sera obtained from animals immunized parenterally with antigen reacted equally well with all the rotaviruses. This suggests that such sera contained large amounts of antibody directed against common viral determinants, while convalescent sera contained antibody directed primarily against specific determinants. The fact that the ELISA blocking test was able to distinguish viruses from different species but not viruses from the same species suggests that the RNA segments which differ among viruses from the same species code for proteins that are not involved in species specificity as measured by this method (Yolken et al., 1978a). Human rotavirus antibodies were measured by Yolken et al. (1978b) by the ELISA blocking assay. Goat anti-human rotavirus antiserum was employed as capturing antibody. Human rotavirus from gnotobiotic calf stool filtrate was reacted with the human test serum samples. The unreacted virus was measured by the ELISA antigen method. Results of the ELISA blocking assay correlated with those obtained with IF and ELISA. When NCDV (Nebraska calf

diarrhea virus), a bovine rotavirus, was employed as antigen, the ELISA was not as efficient indicating that rotaviruses derived from different animal species could be differentiated by the ELISA blocking test. The same authors (Yolken et al., 1978c) developed a double antibody sandwich ELISA to differentiate serotype-specific rotavirus antigen and antibody using type specific antibody (anti-human rotavirus in guinea pigs), homotypic human antigen from calves where the common rotaviral determinants were blocked with calf serum containing antibodies to NCDV. As final step, serum or milk test samples were reacted. Birch et al. (1979) reported that RIA and the double antibody sandwich ELISAs were the most sensitive methods for detecting human rotavirus as compared with EM and IF. Zissis and Lambert (1980) used the ELISA for serotyping human rotavirus (antigen) types 1 and 2 strains and compared it with complement fixation test in terms of specificity and sensitivity. Serotyping differentiation was achieved by determining a neutralization endpoint titer, either with a constant serum-varying antigen dilution method or vice versa. When the antibody sandwich consisted of two type-specific hyperimmune sera the typing procedure was much improved over other ELISAs employed. Grabaulle et al. (1981) differentiated human and bovine rotavirus in stools using a double-antibody sandwich ELISA. ELISA was the most sensitive method when compared with EM, IEOP and IF. Thouless et al. (1982) described and ELISA for serotyping and subgrouping rotaviruses. According to the authors, 128-fold difference in titer between rotavirus isolates in human feces was obtained. This difference was

observed after absorption of the typing antisera with incomplete particles of calf rotavirus and complete particles of heterologous human rotavirus isolates, reducing cross-reactivity to a great extent.

PART I: SEROTYPING ROTAVIRUS

INTRODUCTION

As reviewed earlier, rotaviruses obtained from different animal species and some isolates from the same species are antigenically different as determined by serum neutralization tests, and these are defined as serotypes. Some of these have been distinguished antigenically by the ELISA blocking test.

The purpose of this work was to compare different ELISAs for serotyping rotaviruses which had been isolated from canine, simian, porcine and bovine species and showed at least twenty-fold difference between the homologous and the heterologous neutralization test reactions.

MATERIALS AND METHODS

Rotavirus Isolates

The Rotavirus isolates used for this work were supplied by S. K. Gaul, T. F. Simpson, N. Kelso and G. N. Woode and the full details of their origin and subsequent passage history was reported by Gaul et al. (1982). Briefly, the porcine rotavirus OSU (OSU strain P:USA:77:1) was originally supplied by E. Bohl at passage 32 in MA104 cells. The simian Rotavirus S-USA (S:USA:79:2) was originally supplied by N. Schmidt at passage 15 in cell culture and plaque purified. The bovine rotavirus B14 (B:USA:78:1A,3cl.IVBp), the canine rotavirus K9 (LSU 79C-36,C:81:2), and the bovine rotavirus strain B223 were isolated in this laboratory by G. N. Woode. Fecal samples of rotavirus were obtained by sampling experimentally infected gnotobiotic calves.

Cell Culture

MA104 (Monkey kidney) cells[1] at the 26th passage were removed from 4 (75 cm^2) flasks (Linbro, Hamden, Conn.) with EDTA-Trypsin solution (20 µg/ml) washed and resuspended in 100 ml of growth medium for large-scale antigen production in 850 cm^2 roller bottles (Corning

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[1]Kindly supplied by Dr. Margaret Cholmey, Salt Lake City, Utah 84107.

Glass Works, Corning, NY). Cells were incubated at 37°C. The growth medium, as described by Gaul et al. (1982) consisted of Eagle Minimum Essential Medium (Modified) (MEM) (Flow Laboratories, Inc., Flow General, Inc.) complemented with 0.25% lactalbumin hydrolysate (DIFCO Laboratories, Detroit, Mich.), penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B [5 µg/ml, Fungizone (E. R. Squibb & Sons, Inc., Princeton, NJ)] and 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NY). Fetal bovine serum was omitted for maintenance of cells, and for the cultivation or assay of viruses pancreatin [4X N. F. 2.5% (10X); GIBCO Laboratories] was added at a final concentration of 0.1%.

Rotavirus Antigen Preparation

Viruses were grown in confluent 24 to 48 hours old monolayers of MA104 cells. The medium consisted of serum-free (SF) MEM with 0.1% pancreatin. Adaptation of fecal rotavirus to cell culture was described by Gaul et al. (1982). Virus-infected cells were incubated 24 to 48 hours at 37°C until cytopathic effect (CPE) was observed. Virus was freed from intact cells by two cycles freeze-thawing. The virus suspension was clarified by centrifugation at 122,047.74 x g (L5-65 Ultracentrifuge, SW 27 rotor, Beckman Instruments, Inc., Palo Alto, California) for 90 minutes at 4°C. Pellets were resuspended in 1.0 ml sterile phosphate-buffered saline (PBS) pH 7.2 overnight at 4°C. Further extraction was done with two cycles of an equal volume

of trichlorotrifluoroethane (Freon^{r} T. F., E. I. DuPont De Nemours & Company, Inc., Wilmington, Del.) each followed by centrifugation at 1000 x g for 10 minutes at 4°C. The virus was further purified by centrifugation at 122,047.74 x g for 4.0 hours at 4°C through 40% sucrose. The pellet obtained was resuspended in TNC-buffer (0.05 M Tris-0.1 M NaCl-0.001 M $CaCl \cdot 2 H_2O$) at pH 7.5 (Gaul et al., 1982). Virus was frozen until used.

Serology

Rotavirus antisera are listed in Table 1.

The guinea pig hyperimmune antisera was kindly supplied by S. K. Gaul (Gaul et al., 1982). Briefly, animals were inoculated twice in the footpad at 3 week intervals with 0.1 ml of cell-cultured rotavirus combined with an equal volume of Freund's incomplete adjuvant (GIBCO Laboratories, Grand Island, NY).

Hyperimmune antiserum to various rotavirus strains (porcine: OSU P:USA:77:1; simian: S:USA:79:2; bovine strain B14: B:USA:78:1A; canine: LSU 79C-36 and bovine: B223) was raised in a goat. The animal was inoculated three times intramuscularly at 3 week intervals with 0.5 ml (1:50 in PBS, pH 7.2) of each of the cell-cultured rotavirus combined with an equal volume of Freund incomplete adjuvant. Each of the viruses employed was prepared as described for rotavirus antigen preparation with a final concentration of approximately 1-10 x 10^{11} virus particles per ml.

Rabbit polyclonal monospecific antiserum to canine rotavirus was kindly supplied by M. Thouless. This was prepared by inoculating rabbits with single polypeptide preparation isolated from a polyacrilamide electrophoresis gel. This serum would distinguish by neutralization titer of canine from simian rotavirus which are closely related serotypes, in addition to distinguishing them from the other serotypes used (Gaul et al., 1982).

Table 1. Rotavirus antisera

Virus strain	Host species virus	Host species antiserum	Animal identification	Antiserum identification
LSU 79C-36				
C:USA:81:2	Canine	Guinea pig	19	K9
S:USA:79:2	Simian	Guinea pig	34	S-USA
B:USA:79:1A	Bovine	Guinea pig	38	B14
OSU-P:USA:77:1	Porcine	Guinea pig	44	OSU
None	None	Guinea pig	61	GP61
Pooled LSU 79C-36 C:USA:81:2 S:USA:79:2 B:USA:78:1A OSU-P:USA:77:1 B223	Canine Simian Bovine Porcine Bovine	Goat	75	Hyp. Goat
None	None	Goat	75	Pre. Goat
Data not available	Canine	Rabbit	2289	Hyp. Rabbit 2289[a]
None	None	Rabbit	2289	Pre. Rabbit 2289[a]

[a]Kindly supplied by Dr. Margaret Thouless, Seattle, WA 98195, U.S.A.

ELISAs for Antibody Assay

There are a number of ways in which ELISAs can be formulated to provide antibody measurement.

For this work non-competitive ELISAs, in which antigen is reacted with antibody and the extent of the antigen-antibody reaction is measured in a second step, were performed. In all assays one of the reactants was immobilized onto a solid phase (microtiter plate), the indicator conjugate was an enzyme-labeled anti-species class-specific immunoglobulin (anti-IgG), and as a final stage, the addition of the enzyme substrate yielding a colored product on reaction with the enzyme in the conjugate. The results were read photometrically and expressed as absorbance values at one dilution of test sample.

The indirect ELISA had the advantage of additional amplification because a single molecule of the second (or third) antibody can react with several molecules of the antiglobulin-enzyme conjugate (Kapikian et al., 1979).

For this work, rotavirus-serotype-specific guinea pig antisera were employed as the "typos" second antibody (Figure 6 step 2) in the indirect ELISAs which allowed these tests to be utilized for rotavirus serotyping. Performance of the indirect ELISAs are described below.

Indirect ELISA method

Immulon I, flat-bottomed plates (Dynatech Laboratories, Inc., Alexandria, Virginia) were coated with 50 µl of rotavirus antigen

diluted in 0.1 M carbonate buffer pH 9.6 (see page 32) and with 50 µl of Cyanamide (SIGMA Chemical Co., St. Louis, MO) (Hall and Thoen, verbal communication, VMPM, Iowa State University) diluted to 1 mg/ml in 0.1 M carbonated buffer pH 9.6. Plates were incubated at 4°C overnight in a humidified chamber and washed three times with ELISA buffer (see page 32) immediately before use. One hundred microliters of one percent bovine serum albumin (BSA) (BSA-GIBCO Laboratories, Grand Island, NY) or 1% of ovalbumin (Ovalbumin-SIGMA Chemical Co., St. Louis, MO) in ELISA buffer was added to each microtiter-plate well. Plates were incubated with agitation (Thomas shaking apparatus, Arthur H. Thomas Co., Philadelphia, PA) for 30 minutes at room temperature and then washed three times with ELISA buffer. Fifty microliters of serial two-fold dilutions of antiserum in ELISA buffer were added to the relevant microtiter-plate wells. The plates were incubated with agitation for 30 minutes at room temperature and then washed eight times with ELISA buffer. Peroxidase labeled conjugate affinity purified for species IgG (H + L) (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) was diluted to 1:200 in ELISA buffer and 50 µl were added to the wells. The plates were incubated with agitation for 30 minutes at room temperature and then washed eight times with ELISA buffer. One hundred microliters of the ELISA substrate solution (see page 32) were added to each microtiter-plate well. The plates were incubated for 60 minutes at room temperature. For this method and for all different ELISAs, optical densities were measured with a Dynatech MicroELISA Reader (Dynatech Laboratories,

Inc., Alexandria, VA) at 405 nm bandpass filter (Figure 6).

Preparation of ELISA reagents

Reagent	Component	Quantity	pH
ELISA buffer	NaCl	29.0 g	
	Na_2HPO_4	2.3 g	
	NaH_2PO_4	0.2 g	
	Tween 80	5.0 ml	
	H_2O	to 1,000.0 ml	7.5
ELISA substrate	Citric acid at 0.05 M	25.0 ml	4.0
	3% H_2O_2	100.0 µl	
	ABTS[1] at 40 mM	125.0 µl	

Double antibody sandwich ELISA method

The indirect ELISA method was modified by coating the microtiter-plate wells with 50 µl of pre- or post- goat hyperimmune serum (capturing antiserum) diluted in 0.1 M carbonate buffer pH 9.6 and with 50 µl Cyanamide. The plates were incubated at 4°C overnight

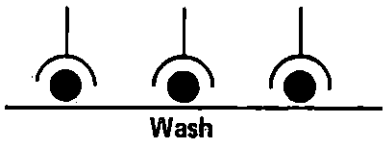
[1] 2.2'-Azino-Di-(3-Ethylbenzthiazoline sulfonic acid) (SIGMA Chemical Co., St. Louis, MO).

Figure 6. Indirect ELISA method

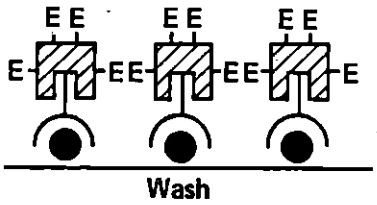
INDIRECT ELISA METHOD

1. Attachment of antigen to solid phase

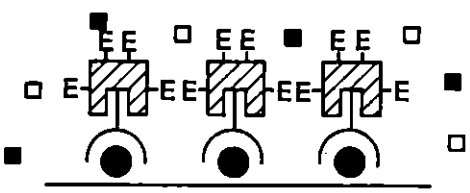
2. Test serum sample added



3. Enzyme anti-globulin conjugate added



4. Substrate added (□), incubate and measure product (■)



in a humidified chamber and washed three times with ELISA buffer immediately before use. One hundred microliters of 1% ovalbumin in ELISA buffer were added to each microtiter-plate well. The plates were incubated with agitation for 30 minutes at room temperature and then washed three times with ELISA buffer. Fifty microliters of the antigen diluted in ELISA buffer were added to the microtiter-plate wells. The plates were incubated with agitation for 30 minutes at room temperature and then washed eight times with ELISA buffer. The indirect ELISA method was followed when adding the serial two-fold dilutions of second antiserum (guinea pig) in ELISA buffer, the peroxidase-labeled conjugate and the ELISA substrate solution (Figure 7).

ELISA blocking methods

Triple antibody sandwich ELISA method The double antibody sandwich ELISA method was altered by the addition of a third antiserum (from a different animal species). The plates were then agitated for 1 minute at room temperature, incubated for 2 hours at 37°C and then washed eight times with ELISA buffer. The indirect ELISA method was followed from step 4 in Figure 7 except that an enzyme labeled antiglobulin to antiserum C (bovine serum) was used in place of the anti-guinea pig antiserum (Figure 8).

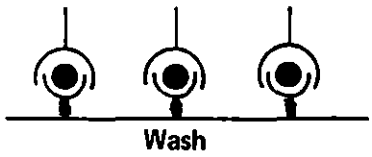
Figure 7. Double antibody sandwich ELISA method

DOUBLE ANTIBODY SANDWICH ELISA METHOD

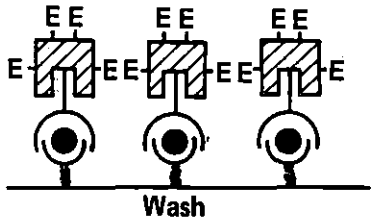
1. Attachment of specific "A" antibody (Goat) to solid phase

2. Antigen added

3. Specific antibody "B" of different species (Guinea pig) added.



4. Enzyme labeled anti-B globulin added (anti-guinea pig IgG)



5. Substrate added (□), incubate and measure product (■)

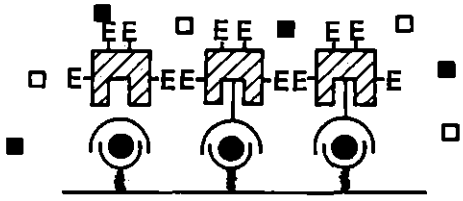


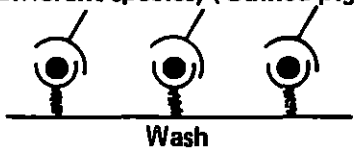
Figure 8. Triple antibody sandwich ELISA method

TRIPLE ANTIBODY SANDWICH ELISA METHOD

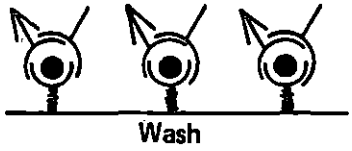
1. Attachment of specific antibody "A" (Goat) to solid phase

2. Antigen added

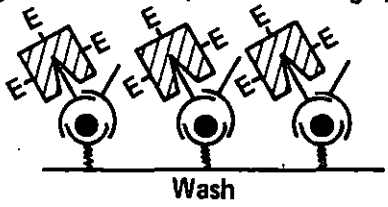
3. Specific antibody "B" of different species, (Guinea pig) added



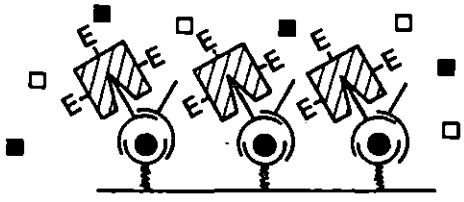
4. Specific antibody "C" of different species (Bovine) added



5. Enzyme labeled anti-C globulin added (anti-bovine IgG)



6. Substrate added (□), incubate and measure product (■)



Modified triple antibody sandwich ELISA method The various antisera were absorbed first with the relevant antigens and the unbound antigen assayed by the modified triple antibody sandwich ELISA method. In order to prevent adsorption of the antigen-antiserum to the plastic, polystyrene, round "U"-bottomed plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 200 µl of 1% egg albumin (SIGMA Chemical Co., St. Louis, MO) and incubated with agitation for 30 minutes at room temperature. After washing the plates three times with ELISA buffer, serial two-fold dilutions of guinea pig rotavirus antisera with an equal volume (50 µl) of the antigen diluted in ELISA buffer were added to each well. The plates were incubated with agitation for 1 minute at room temperature and without agitation for 2 hours at 37°C. The unbound antigen in the antigen-antibody complex was then assayed by the modified triple antibody sandwich ELISA method.

Microtiter-plate wells were coated with the capturing antiserum (goat) and ovalbumin treated as indicated in the double antibody sandwich ELISA method. After the plates were incubated with agitation for 30 minutes at room temperature and then washed three times with ELISA buffer, 50 µl of the antigen guinea pig antiserum complex was added to the microtiter-plate wells. Plates were incubated with agitation for 30 minutes at room temperature and then washed eight times with ELISA buffer. The indirect ELISA method was followed when adding the third antiserum (bovine) diluted in ELISA buffer, the peroxidase-labeled conjugate and the ELISA substrate solution (Figure 9).

Figure 9. Modified triple antibody sandwich ELISA method

MODIFIED TRIPLE ANTIBODY SANDWICH ELISA METHOD

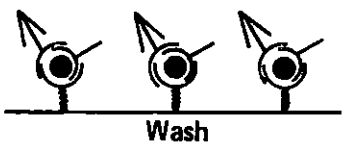
1. Attachment of specific antibody "A" (Goat) to solid phase

2. Antigen previously incubated with specific antibody "B" of different species (Guinea pig) is transfered to the well coated with antibody "A"

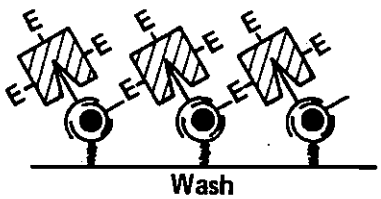
Transfer to precoated wells

Rotavirus antigen was incubated with dilutions of guinea pig serum. If the dilution contains Rotavirus antibody it will bind to the virus.

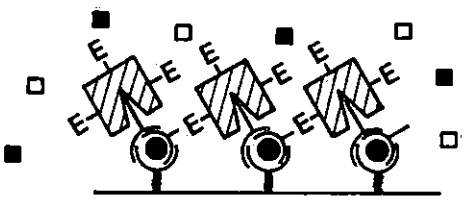
3. Specific antibody "C" of different species (Bovine) added



4. Enzyme labeled anti-C globulin added (anti-bovine IgG)



5. Substrate added (□), incubate and measure product (■)



The requisite controls were tested on at least 3 occasions for each antigen-antibody reaction in order to monitor the negative and the non-specific reactions. Antigens and antisera were employed at the lowest test dilution. The OD readings vs. absorbance obtained were equal or less than indicated below. Different controls were prepared according to the ELISA employed.

The various controls incorporated in the ELISAs are as follows:

Control composition	Control Identification Number																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Goat antiserum (capturing)							+	+	+	+	+	+	+	+	+	+	+	+
Ovalbumin or BSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Antigen					+		+			+		+	+	+	+			
Guinea pig antiserum	+			+														
Guinea pig antiserum (second)								+										
Guinea pig antiserum (blocking)												+	+	+				
Bovine antiserum (third)												+			+	+		
Anti-guinea pig IgG conjugate	+	+					+	+	+	+								
Anti-bovine IgG conjugate														+	+	+	+	
Substrate	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	
Substrate-conjugate						+												+

+ = reagent added

To determine non-specific adherence = control #1. To determine the specificity of the anti-guinea pig IgG (H + L) conjugate = controls #2, #7, #9 and #10. To determine the specificity of the anti-bovine IgG (H + L) conjugate = controls #14 and #17. To determine non-specific reactions of the substrate = controls #3, #4, #5, #11, #12 and #13. To determine the maximum positive reaction (spot check) = controls #6 and #18. To determine non-specific reactions of the guinea pig and bovine sera against goat serum = controls #8 and #16 respectively. To determine a positive reaction = control #15.

Control Identification Number	OD Reading Range
1	0.000-0.055
2	0.000-0.026
3	0.003-0.090
4	0.000-0.030
5	0.001-0.035
6	1.297-1.514
7	0.007-0.045
8	0.000-0.090
9	0.020-0.080
10	0.000-0.020

Control Identification Number	OD Reading Range
11	0.000-0.011
12	0.000-0.030
13	0.000-0.020
14	0.007-0.050
15	0.420-0.960
16	0.010-0.025
17	0.008-0.040
18	1.370-1.420

Electron Microscopy Method for the Quantitation of Rotavirus Particles Used for Cross-absorption

The electron microscopy method was performed as described by Woode et al. (1982) with some modifications. Briefly, one drop of the viral antigen was resuspended in 15 drops of distilled water and mixed with 2 drops of 4% phosphotungstic acid (PTA) pH 6.4 (Ted Pella, Inc., Tustin, CA) with 1 drop of 1% BSA and 1 drop of the latex beads (1.37 x 10^{11} latex beads/ml, Balzers Union, Hudson, NH). This mixture was incubated for 10 minutes at room temperature and sprayed onto carbon-collodion coated 200 mesh grids (LKB, Stockholm, Sweden) with a glass nebulizer (Ted Pella, Inc., Tustin, CA). Grids were examined with an electron microscope (Hitachi 12A) at 75 Kv and at 200,000X

magnification. The ratio of virus particles to latex beads was determined by counting 1,000 to 1,400 particles of virus. The concentration of virus particles was calculated from the known number of latex beads. A ratio of 1:1 was observed for incomplete to complete virus particles.

Cross-absorption of Guinea Pig Hyperimmune Rotavirus Antisera

Guinea pig hyperimmune rotavirus antisera diluted in ELISA buffer were absorbed with an equal volume of rotavirus antigens. The number of rotavirus particles used for absorption of antibody were recorded below and the method for virus particle determination has been described above. Antigen-antiserum mixtures were incubated for 30 minutes at 37°C and rocked overnight at 4.5 oscillations per minute (Rocker platform, Bellco Glass, Inc., Vineland, NJ) at 4°C. The mixtures were clarified by centrifugation at 10,000 x g (B20 High speed centrifuge, A147 rotor, International Equipment Co., Needham Heights, Mass.) for 60 minutes at 4°C.

Cross-absorption of guinea pig hyperimmune antisera

Antiserum Identification	Antigen Identification Used for Absorption
K9	OSU-P:USA:77:1[4.25(1.37×10^{11})particles/ml±.2]
S-USA	OSU-P:USA:77:1[4.25(1.37×10^{11})particles/ml±.2]
B14	OSU-P:USA:77:1[4.25(1.37×10^{11})particles/ml±.2]
OSU	OSU-P:USA:77:1[4.25(1.37×10^{11})particles/ml±.2]

OSU	LSU 79C-36,C:USA:81:2 [3.30(1.37x10^{11})particles/ml±.2]
B14	LSU 79C-36,C:USA:81:2 [3.30(1.37x10^{11})particles/ml±.2]
	OSU-P:USA:77:1[2.90(1.37x10^{11})particles/ml±.2]
K9	LSU 79C-36,C:USA:81:2 [3.30(1.37x10^{11})particles/ml±.2]

RESULTS AND DISCUSSION

Standardization of ELISA for Determination of Rotavirus Antibodies (Homologous Antigen-antibody Reactions)

Determination of optimal rotavirus antigen concentration

The optimal rotavirus antigen (B14, OSU, S-USA and K9) concentration was determined by the indirect ELISA method. Rotavirus antigens were serially diluted and adsorbed to the microtiter immulon I plate wells. A constant dilution for each of the guinea pig hyperimmune anti-rotavirus antiserum was selected (B14-1:2,000; OSU-1:20,000; S-USA-1:20,000; K9-1:4,000) based on preliminary tests by the same ELISA and also from their homologous neutralization titer (NT) (Gaul et al., 1982). According to Figure 10, a dilution equal to 1:500 was found to be the optimal working antigen dilution for all the antigens here tested after which the antigen titration curve showed a more pronounced decline. The following controls were included.

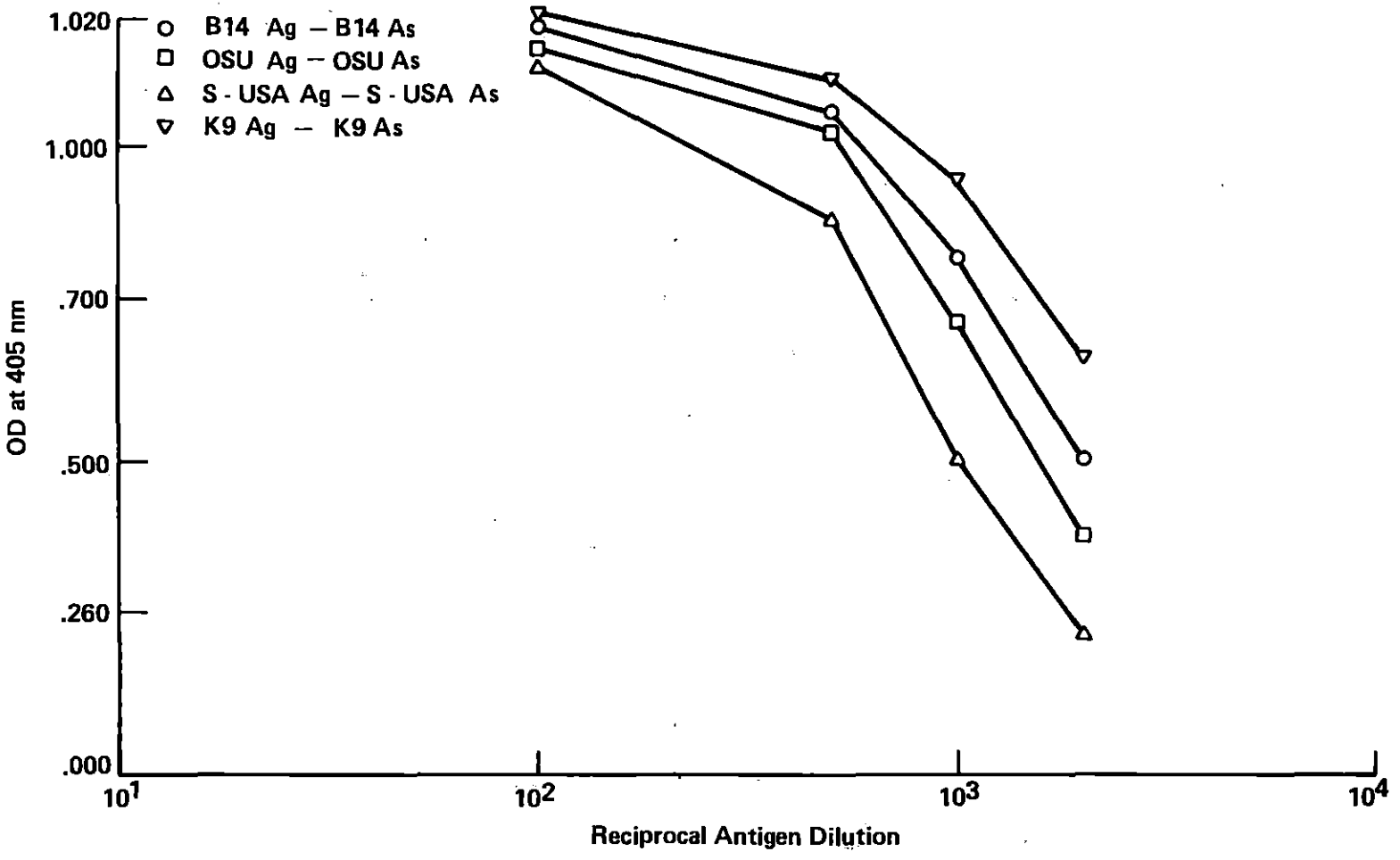
Titration of a weak positive guinea pig antiserum against rotavirus antigens by the indirect ELISA method

In order to standardize the ELISA a negative serum was required. None of the guinea pig sera available, including prebleeds of the hyperimmune animals, were negative for rotavirus antibody.

Figure 10. Determination of optimal rotavirus antigens concentration by the indirect ELISA method

Controls included for Figure 10		OD Reading (405 nm)
#1	for B14 antiserum (1:2,000)	.051
#1	for OSU antiserum (1:20,000)	.025
#1	for S-USA antiserum (1:20,000)	.018
#1	for K9 antiserum (1:4,000)	.011
#2		.000
#3		.003
#6		1.387

One percent BSA and peroxidase labeled goat to guinea pig IgG (H + L) conjugate were employed.


1.020
1.000
.700
.500
.260
.000
OD at 405 nm
B14 Ag – B14 As
OSU Ag – OSU As
S - USA Ag – S - USA As
K9 Ag – K9 As
10^1
10^2
10^3
10^4
Reciprocal Antigen Dilution

One guinea pig (GP61) had the lowest titer for rotavirus antibody and this serum was used as a negative serum when the serum was diluted.

Serial two-fold dilutions of the guinea pig antiserum (GP61), from 1:10 up to 1:5,200, were tested by the indirect ELISA method against OSU, K9, S-USA and B14 antigens diluted 1:500. One percent BSA and 1:200 dilution of peroxidase labeled goat to guinea pig IgG (H + L) conjugate were employed. Titer of each antiserum was determined by reading its titration curve at an OD .500 selected as the point of the slope with the lowest curve variability. A titer equal to 24 was obtained with OSU antigen while titers lower than 10 were observed with K9, S-USA and B14 antigens. As no rotavirus antibody negative guinea pig antisera were obtained, GP61 was used as the lowest titered serum available. Controls included were #1, #3, #5, #6 and #7.

Titration of guinea pig hyperimmune antisera against their respective rotavirus antigens

Serial two-fold dilutions of hyperimmune rotavirus antisera to OSU, S-USA, K9 and B14 were tested with their respective rotavirus antigens, diluted to 1:500, by the indirect ELISA method. In addition to the relevant controls, rotavirus antibody negative GP61 antiserum was titrated against each of the rotavirus antigens. Titers of the antisera were obtained from their titration curves at an OD reading of .500 (Figure 11) giving values equal to 500,000, 72,000, 380,000 and 84,000 for OSU, S-USA, K9 and B14 antisera respectively. An average OD reading for each GP61 antiserum dilution against all rotavirus

antigens was calculated and plotted in Figure 11. Controls used included.

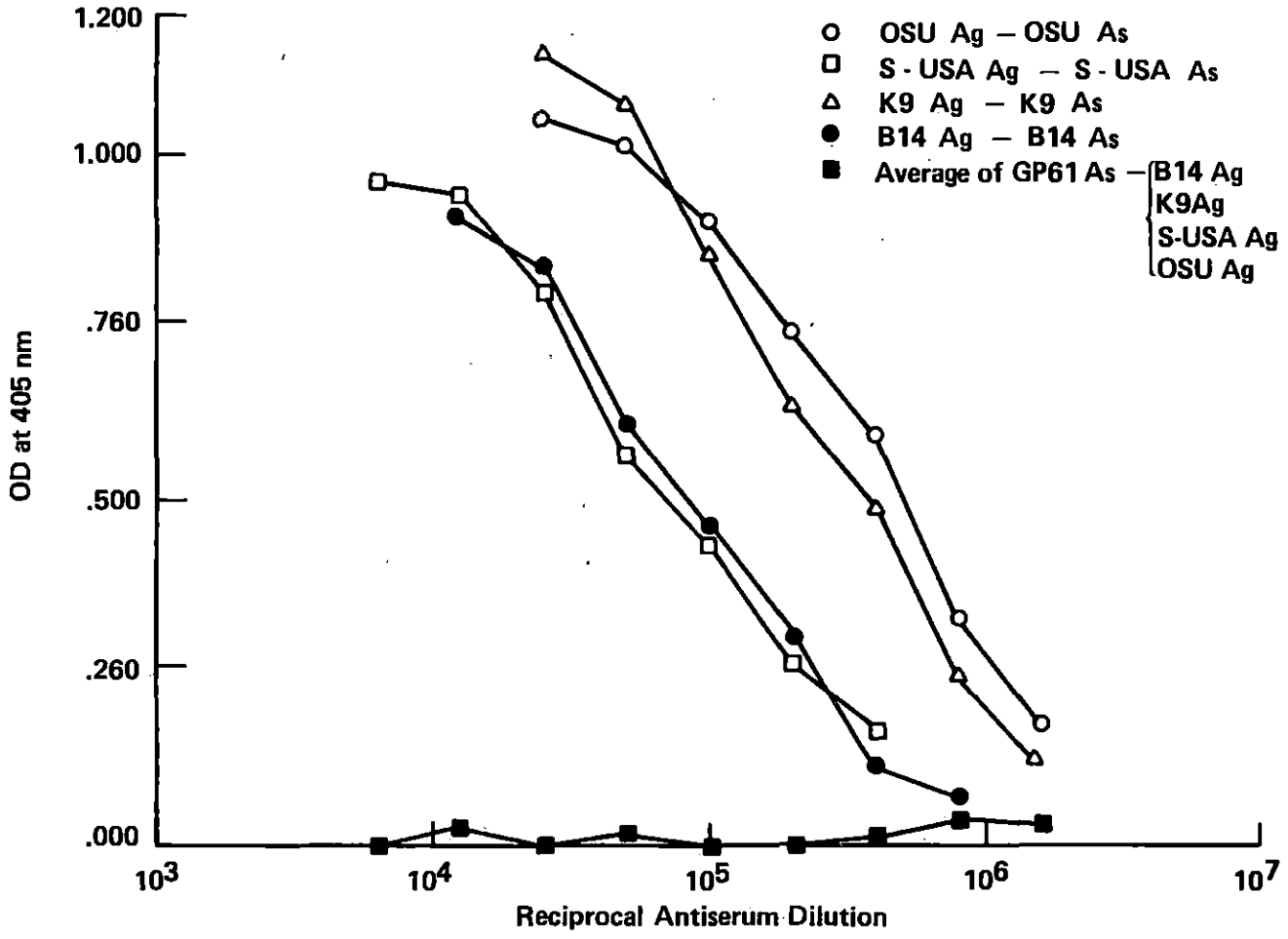
Determination of the optimal conjugate dilution

Two-fold dilutions, starting from 1:200 up to 1:32,000, of horseradish-peroxidase labeled affinity purified goat antibody to guinea pig IgG (H + L) conjugate were tested against K9 antigen (1:500)-K9 antiserum (1:102,400), B14 antigen (1:500)-B14 antiserum (1:25,600), OSU antigen (1:500)-OSU antiserum (1:102,400) and S-USA antigen (1:500)-S-USA antiserum (1:25,600) by the indirect ELISA method, where dilutions of the guinea pig hyperimmune antisera were one third to one fourth the titers obtained from Figure 11. To control the OD readings of the negative reactions, the negative GP61 serum was diluted to 1:25,600 and tested against each rotavirus antigen. These all gave negative OD readings. The 1:200 conjugate dilution gave the highest OD readings (OD = .780) with a rapid decline shown with higher dilutions. For economic reasons, the conjugates were employed at this dilution for all subsequent tests.

Figure 11. Titration of guinea pig hyperimmune rotavirus antisera against their respective rotavirus antigens by the indirect ELISA method

Controls included for Figure 11		OD Reading (405 nm)
#1	for OSU antiserum	.000
#1	for S-USA antiserum	.000
#1	for K9 antiserum	.011
#1	for B14 antiserum	.000
#1	for GP61 antiserum	.015±.019 (from 4 readings)
#3		.031±.033 (from 4 readings)
#5	for OSU antigen	.009
#5	for S-USA antigen	.031
#5	for K9 antigen	.020
#5	for B14 antigen	.022
#6		1.409±.024 (from 4 readings)
#7	for OSU antigen	.020
#7	for S-USA antigen	.019
#7	for K9 antigen	.040
#7	for B14 antigen	.038

One percent BSA and peroxidase labeled goat to guinea pig IgG (H + L) conjugate were employed.


1.200
1.000
.760
.500
.260
.000
OD at 405 nm
10^3
10^4
10^5
10^6
10^7
Reciprocal Antiserum Dilution
OSU Ag – OSU As
S - USA Ag – S - USA As
K9 Ag – K9 As
B14 Ag – B14 As
Average of GP61 As – B14 Ag
K9Ag
S-USA Ag
OSU Ag

Rotavirus Serotyping by ELISAs

In order to determine whether the ELISAs could be used to serotype rotavirus strains, it was decided to use the simplest test available namely the indirect ELISA method and compare homologous versus heterologous titers. To be a usable test in practice, it was considered that the homologous titer should be at least 10-fold higher than the heterologous titer.

Direct comparison between homologous and heterologous reactions

In order to compare the homologous and heterologous titers of each hyperimmune guinea pig antiserum, as well as the reproducibility of the test, each plate was adsorbed with one of the rotavirus antigens and tested against dilutions of the four guinea pig hyperimmune antisera. As a second approach, each plate was adsorbed with the four rotavirus antigens and each antigen tested against its homologous antiserum. On each plate, one heterologous antigen-antiserum assay was performed. In addition to the proper controls, the low titered GP61 antiserum was included in each test to monitor the negative reaction.

Single antigen and four antisera per plate The rotavirus antigens K9, OSU, S-USA and B14 diluted to 1:500 were adsorbed to the plates, one per plate, and tested against serial two-fold dilutions of K9, OSU, S-USA, B14 and GP61 antisera by the indirect ELISA method.

Controls denominated #1, #2, #3, #6 and #7 were included in each assay. One percent BSA and peroxidase labeled goat to guinea pig IgG (H + L) conjugate were employed in these tests. Titers of the guinea pig hyperimmune rotavirus antisera were determined by reading their titration curves at .500 OD as this point on the graph showed less variability among titration-curve slopes (Fig. 12) and are summarized in Table 2. The low titered GP61 serum diluted to 1:1,000 had OD readings lower than .100.

Table 2. Titers of guinea pig hyperimmune rotavirus antisera by the indirect ELISA method (single antigen, four antisera per plate)

Antigen	Antiserum			
	K9	OSU	S-USA	B14
K9	230,000	640,000	100,000	16,000
OSU	140,000	580,000	90,000	42,000
S-USA	300,000	130,000	490,000	100,000
B14	150,000	105,000	150,000	56,000

Four homologous and a single heterologous antigen-antiserum complexes per plate Rotavirus antigens K9, OSU, S-USA and B14 diluted to 1:500 were tested with their homologous and heterologous guinea pig hyperimmune antisera respectively. The titers obtained were read at .500 OD (Figure 13) and summarized in Tables 3 and 4. Controls #1, #3, #5, #6 and #7 were included in each assay.

Figure 12. Rotavirus serotyping by the indirect ELISA method.
Single antigen four antisera per plate

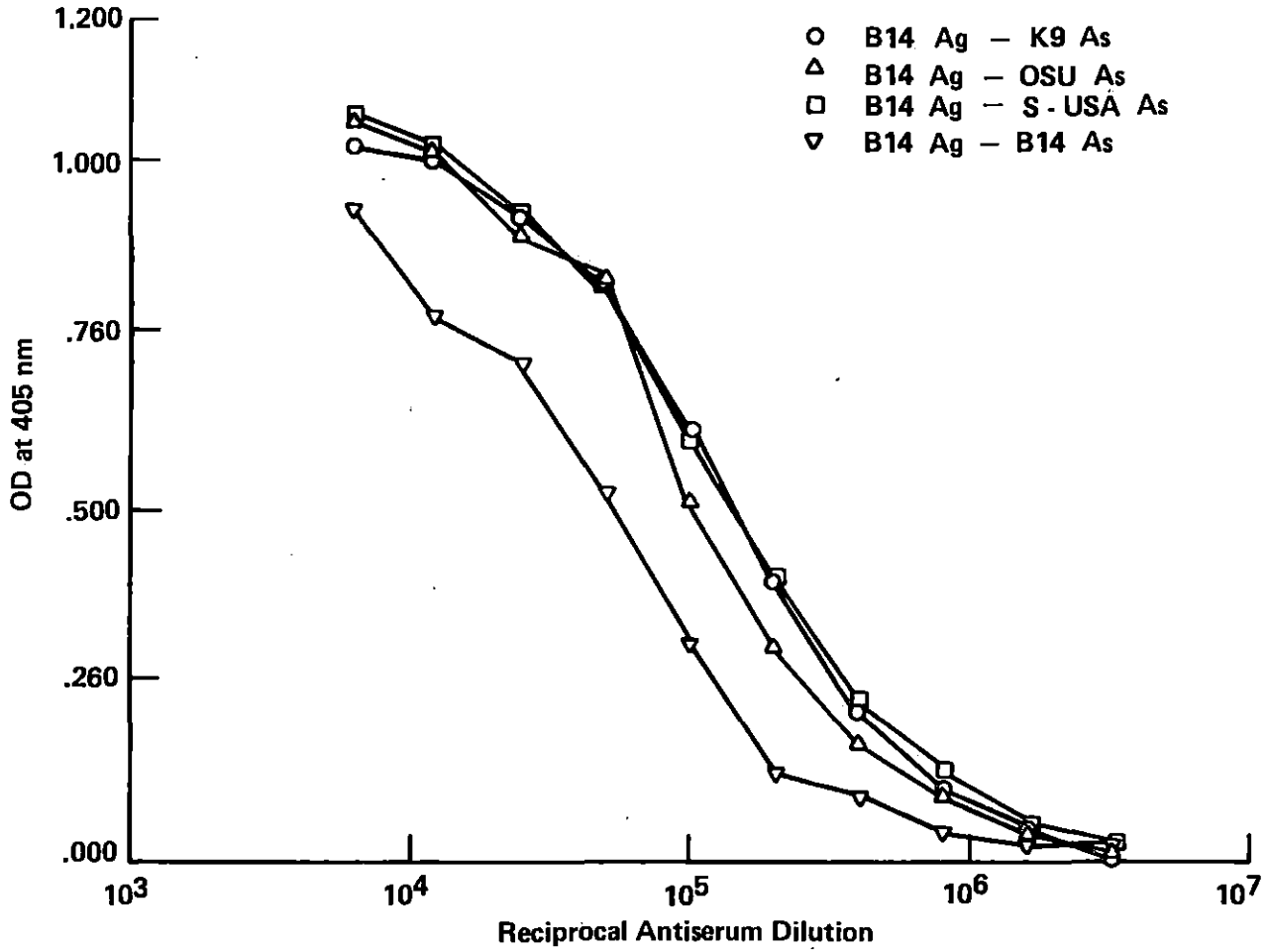

1.200
1.000
.760
.500
.260
.000
OD at 405 nm
B14 Ag – K9 As
B14 Ag – OSU As
B14 Ag – S-USA As
B14 Ag – B14 As
10^3
10^4
10^5
10^6
10^7
Reciprocal Antiserum Dilution

Figure 13. Rotavirus serotyping by the indirect ELISA method. Four homologous and a single heterologous antigen-antiserum complexes per plate

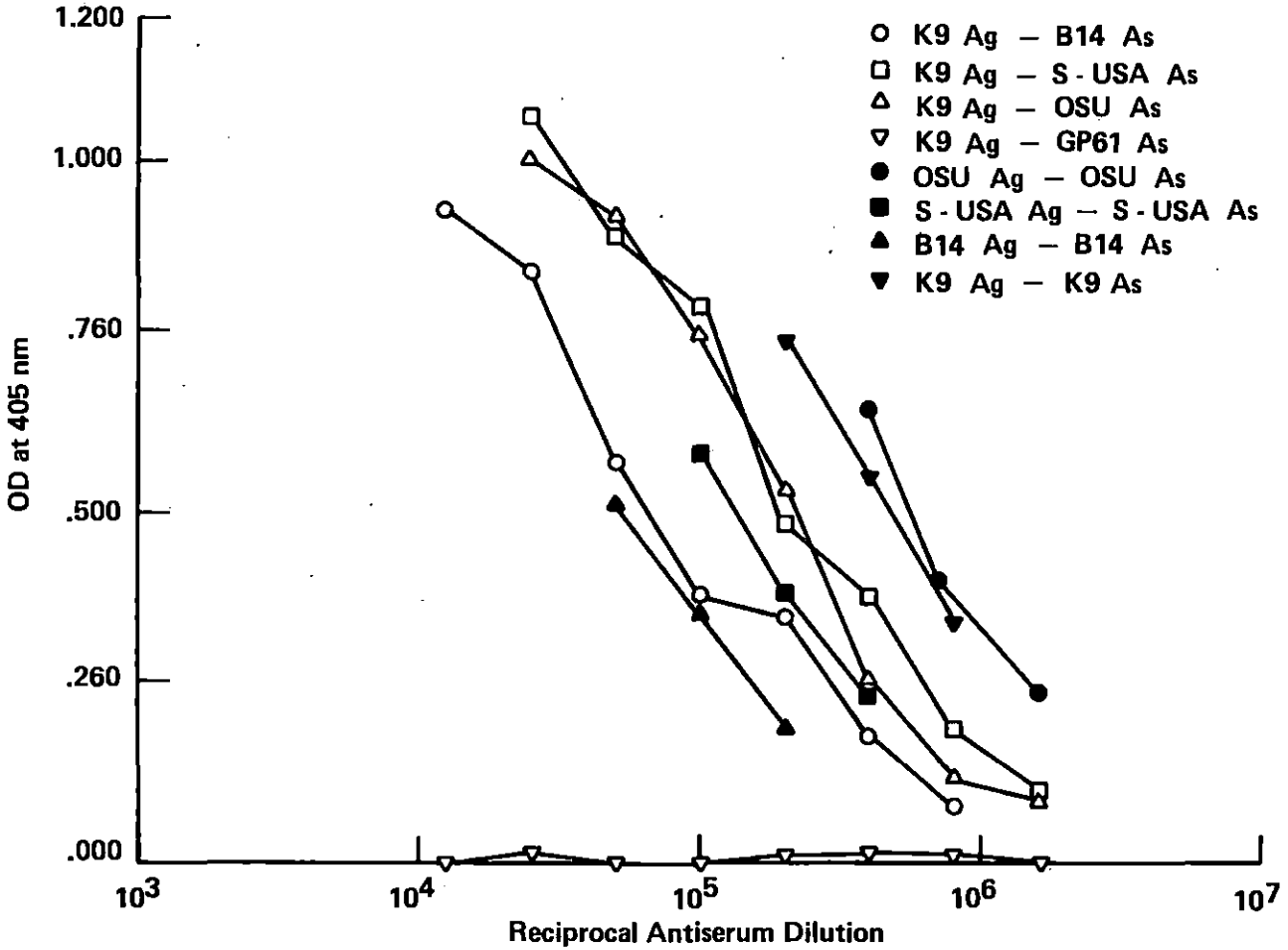

1.200
1.000
.760
.500
.260
.000
OD at 405 nm
10^3
10^4
10^5
10^6
10^7
Reciprocal Antiserum Dilution
K9 Ag – B14 As
K9 Ag – S-USA As
K9 Ag – OSU As
K9 Ag – GP61 As
OSU Ag – OSU As
S-USA Ag – S-USA As
B14 Ag – B14 As
K9 Ag – K9 As

Table 3. Homologous titers of guinea pig hyperimmune rotavirus antisera by the indirect ELISA method (four homologous and a single heterologous antigen-antiserum complexes per plate)

Antigen	Antiserum			
	K9	OSU	S-USA	B14
K9	480,000 160,000 200,000 800,000			
OSU		600,000 580,000 540,000 540,000		
S-USA			135,000 110,000 350,000 350,000	
B14				52,000 54,000 37,000 155,000

Table 4. Homologous and heterologous titers of guinea pig hyperimmune rotavirus antisera by the indirect ELISA method (four homologous and a single heterologous antigen-antiserum complexes per plate)

Antiserum	Antigen			
	K9	OSU	S-USA	B14
K9	410,000 ±296,423.12	160,000	155,000	66,000
OSU	200,000	565,000 ±30,000	155,000	120,000
S-USA	400,000	82,000	236,250 ±131,743.12	120,000
B14	300,000	165,000	320,000	74,500 ±54,200.25

According to the data reported by Gaul et al. (1982), the guinea pig sera had a serotype specific neutralization titer much higher than the common antigen antibody titer detected by IF. This fact led one to expect that the serotype specific antigen-antibody reaction by ELISA would also show a much higher antibody titer. This was not so (see Tables 2, 3 and 4). The homologous titer was not always higher than the heterologous one and a possible explanation is that the quantity of serotype specific antigen was low when compared with the common rotavirus antigen. Thus, the ELISA appears to have measured the common antigen.

The virus preparation used as antigen contained complete (presumably carrying serotype specific antigen) and incomplete

particles in a ratio of 1:1. If we had used only complete particles, the serotype specific antigen concentration may still have been too low to show the required differences. Based on these initial conclusions, the blocking ELISA was performed (as indicated below) in order to increase the sensitivity of the method.

Standardization of the ELISA Blocking Test

Titration of pre-immune and hyper-immune rotavirus goat antisera as capturing antibody by the double antibody sandwich ELISA method

In order to determine the optimal concentration of the goat antisera as capturing antibody, the procedure for the double antibody sandwich ELISA method was followed. Serial two-fold dilutions from 1:200 of the two goat sera samples were prepared in 0.1 M carbonate buffer pH 9.6 and adsorbed to the microtiter plates. These dilutions were tested for reactivity with B14 antigen (1:500)-B14 antiserum (1:12,800), OSU antigen (1:500)-OSU antiserum (1:102,400), K9 antigen (1:500)-K9 antiserum (1:51,200) and S-USA antigen (1:500)-S-USA antiserum (1:102,400). The dilutions of the guinea pig hyperimmune rotavirus antisera were selected as approximately one fourth the titers obtained from the "single antigen four antisera per plate" approach as previously described. Titers of the goat antisera were determined from the titration curves at .500 OD at 405 nm (Figure 14). Pre-immune goat antiserum and the relevant controls were included in order to monitor the negative and the non-specific reactions

respectively. Pre-immune goat antiserum had capturing antibody titers of less than 200 when tested against all the four homologous rotavirus antigen-antibody complexes and the non-specific reactions had OD readings up to .089. Rotavirus hyper-immune goat antiserum had capturing antibody titers equal to 25,600; 53,000; 75,000 and 27,000 against the homologous antigen-antibody OSU, K9, B14 and S-USA reactions respectively. A dilution equal to 1:25,600 was selected as the working dilution for the hyper-immune goat antiserum.

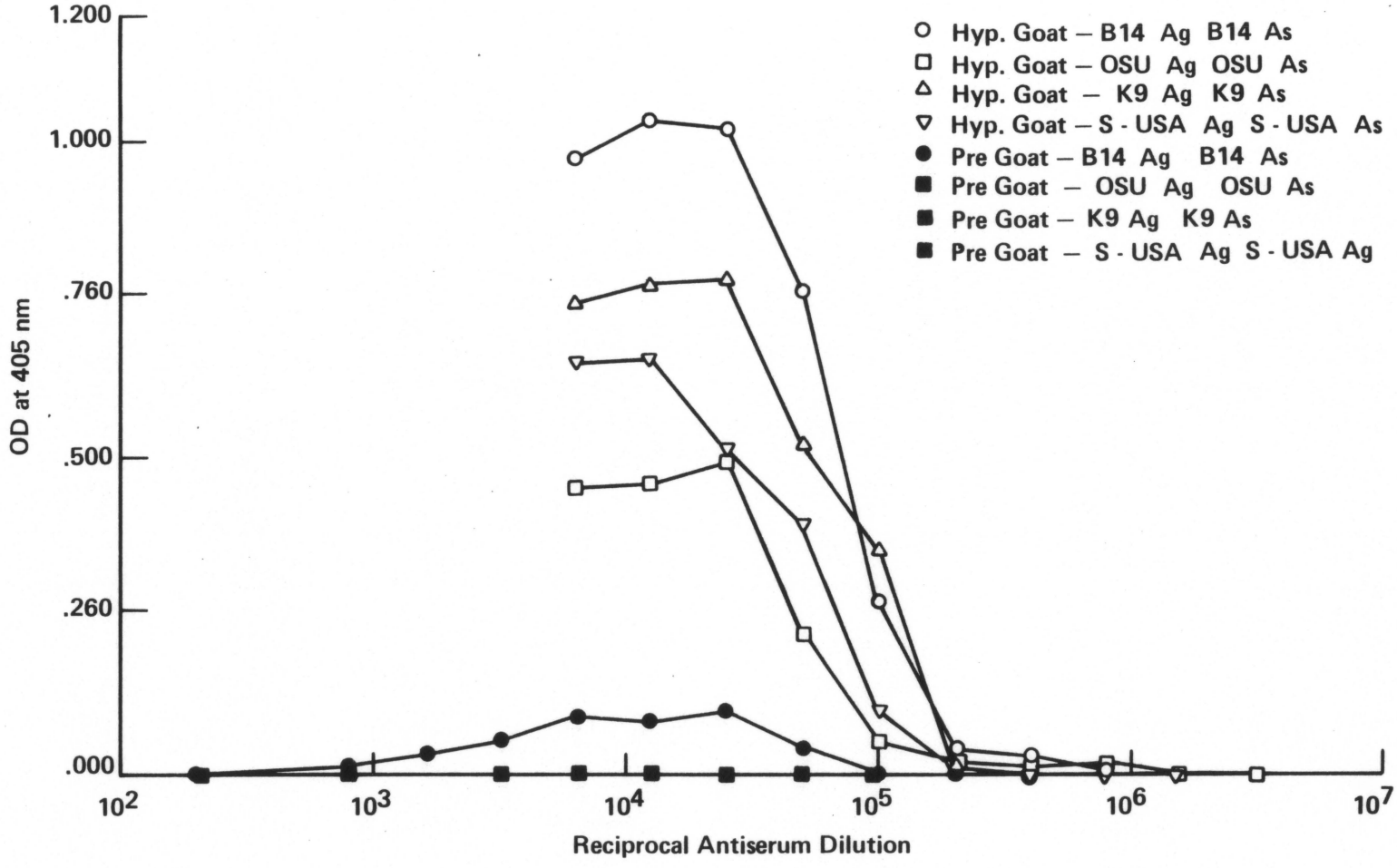
Determination of the optimal OSU, B14, K9 and S-USA rotavirus antigen dilutions by the double antibody sandwich ELISA method

Microtiter-plate wells were coated with either pre-immune goat serum or rotavirus hyperimmune goat serum diluted to 1:25,600 in the 0.1 M carbonate buffer. The rotavirus antigens OSU, K9, B14 and S-USA were diluted serially two-fold and reacted with their homologous guinea pig antisera at dilutions of 1:102,400, 1:51,200, 1:12,800 and 1:102,400 respectively following the double antibody sandwich ELISA method. A negative binding by the pre-immune goat antiserum was obtained. Controls denominated #3, #6, #8, #9, #10 and #11 were included in each test. One per cent ovalbumin and peroxidase labeled goat antiserum to guinea pig IgG (H + L) conjugate were employed. From the titration curves obtained and based on the same criteria used for the initial antigen titration of this work, 1:500 was selected for all the antigens as the working dilution to be use in the triple and the modified triple antibody sandwich ELISA methods.

Figure 14. Titration of pre-immune and hyperimmune rotavirus goat antisera as capturing antibody by the double antibody sandwich ELISA method

Controls included for Figure 14	OD Reading (405 nm)
#6	1.305±.053
#8 for Pre-goat (1:200),OSU antiserum	.055
#8 for Pre-goat (1:200),B14 antiserum	.000
#8 for Pre-goat (1:200),K9 antiserum	.000
#8 for Pre-goat (1:200),S-USA antiserum	.040
#8 for Hyp-goat (1:6.400),OSU antiserum	.089
#8 for Hyp-goat (1:6.400),B14 antiserum	.000
#8 for Hyp-goat (1:6.400),K9 antiserum	.000
#8 for Hyp-goat (1:6.400),S-USA antiserum	.023
#9 for Pre-goat (1:200)	.022±.027
#9 for Hyp-goat (1:6.400)	.035±.040

One percent ovalbumin and peroxidase labeled goat to guinea pig IgG (H + L) conjugate were employed.


1.200
1.000
.760
.500
.260
.000
OD at 405 nm
10^2
10^3
10^4
10^5
10^6
10^7
Reciprocal Antiserum Dilution
Hyp. Goat – B14 Ag B14 As
Hyp. Goat – OSU Ag OSU As
Hyp. Goat – K9 Ag K9 As
Hyp. Goat – S - USA Ag S - USA As
Pre Goat – B14 Ag B14 As
Pre Goat – OSU Ag OSU As
Pre Goat – K9 Ag K9 As
Pre Goat – S - USA Ag S - USA Ag

Titration of a known bovine rotavirus positive antiserum and a known bovine rotavirus negative serum against OSU, K9, B14 and S-USA antigens by the indirect ELISA method

For the third antiserum in the ELISA sandwich test, a gnotobiotic calf antiserum to bovine rotavirus (GC5) lacking antibodies to other bovine viruses was selected. To control this antiserum, a serum sample lacking antibody to rotavirus was used (SB219). These sera were obtained from gnotobiotic calves infected orally 21 days previously with bovine rotavirus and bovine "Breda" virus respectively. These sera were titrated by the indirect ELISA method. Serial two-fold dilutions, from 1:10 up to 1:327,680 of the bovine antisera were tested against the four rotavirus antigens diluted to 1:500. One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate were employed. Titers of both sera were determined by reading from their titration curves at .500 OD on the graph. Antiserum GC5 had titers to OSU, K9, B14 and S-USA antigens equal to 2,200; 1,900; 2,900 and 2,100 respectively. Titers lower than 10 against the four rotavirus antigens were obtained with SB219 antiserum. Approximately one fourth of the titers here obtained were selected as the working dilutions, being equal to 1:640 for GC5 antiserum against OSU, B14 and S-USA antigens, and 1:320 for GC5 against K9 antigen. As the pre-inoculation gnotobiotic calf serum samples lack immunoglobulins they were not considered to be adequate controls for this test, despite being negative for rotavirus antibodies by ELISA. In contrast, the calves, one convalescent to

rotavirus and the other convalescent to "Breda" virus, both possessed immunoglobulins of different ELISA specificities.

Comparison of ELISA Triple and the Modified ELISA Triple Antibody Sandwich Methods

Two blocking ELISAs for antibody assay, the triple and the modified triple antibody sandwich methods were compared in order to determine the assay with the best blocking of the antigen by the serum test sample. Briefly, serial two-fold dilutions of guinea pig hyperimmune rotavirus antiserum (B14) and guinea pig rotavirus negative antiserum (GP61) were tested against rotavirus B14 antigen (1:500) by these two ELISA blocking methods. Goat hyperimmune rotavirus antiserum diluted to 1:25,600 in 0.1 M carbonate buffer was employed as the capturing antibody and bovine rotavirus positive antiserum GC5 diluted 1:640 in ELISA buffer as the third antiserum in the sandwich for both methods. The percentage blocking at each dilution was determined by the equation $(1-A_2/A_1) \times 100$, where A_1 and A_2 were the absorbancies at 405 nm of the ELISA reaction after incubation with GP61 antiserum (A_1) and GC5 antiserum (A_2) (Yolken et al., 1978a). Titers of B14 antiserum were determined by reading the titration curves at 50% blocking (Figure 15). The modified triple antibody sandwich ELISA method was selected for further rotavirus serotyping since it gave with B14 a titer approximately four-fold dilutions higher than the one obtained with the triple antibody

sandwich ELISA method.

Titration of guinea pig hyperimmune antisera to OSU, B14, K9 and S-USA rotavirus antigens against their homologous antigens by the modified triple antibody sandwich ELISA method

Following the ELISA blocking method described above and with the same reagents, the titers of the hyperimmune guinea pig antisera to OSU, B14, K9 and S-USA antigens were determined. Titers of the guinea pig hyperimmune rotavirus antisera were determined by reading their titration curves at 50% blocking (Figure 16). Initial titers equal to 6,000, 2,300, 3,700 and 5,600 were obtained for OSU, B14, K9 and S-USA antisera respectively against their homologous antigens.

Comparison of Homologous and Heterologous Titers by the Modified Triple Antibody Sandwich ELISA Method

Serial two-fold dilutions of guinea pig rotavirus hyperimmune antisera B14, OSU, K9 and S-USA as well as serial two-fold dilutions of guinea pig negative GP61 serum were tested against B14, OSU, S-USA and K9 antigens, diluted 1:500, by the modified triple antibody sandwich ELISA method. Goat hyperimmune rotavirus antiserum diluted to 1:25,600 in 0.1 M carbonate buffer was employed as capturing antibody and bovine rotavirus positive antiserum (GC5) as the third antiserum in the sandwich. The blocking titration curves had a regular comportment similar to that included in Figure 17. Titers of

Figure 15. Comparison of ELISA triple and modified ELISA triple antibody sandwich methods using B14 rotavirus homologous antigen-antibody complexes

Controls included for Figure 15	OD Reading (405 nm)
#11 for Hyp-goat	.007±.009
#12 for Hyp-goat,B14 antiserum GC5 antiserum	.002±.002
#12 for Hyp-goat, GP61 serum GC5 antiserum	.004±.006
#13 for Hyp-goat, B14 antiserum	.007±.010
#13 for Hyp-goat, GP61 serum	.006±.008
#14 for Hyp-goat, B14 antiserum	.019±.001
#14 for Hyp-goat, GP61 serum	.017±.006
#16 for Hyp-goat, GC5 antiserum	.012±.016
#17 for Hyp-goat	.009±.012
#18 for Hyp-goat	1.375±.006

One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate were employed.

Controls to monitor the negative reaction in Figure 15

Controls composition												
Hyp-goat(1:25.600)	+		+									
Pre-goat(1:25.600)					+		+		+		+	
B14 antigen (1:500)	+		+		+		+		+		+	
B14 antiserum (1:100)	+				+				+			
GP61 serum (1:100)			+				+				+	
SB219 antiserum(1:640)		+		+						+		+
GC5 antiserum (1:640)					+		+					
Conjugate (1:200)	+		+		+		+		+		+	
Substrate	+		+		+		+		+		+	
OD Readings (405 nm) obtained	.010± .002	.000	.005± .004		.007± .009		.004± .000		.003± .004			

+ = reagent present

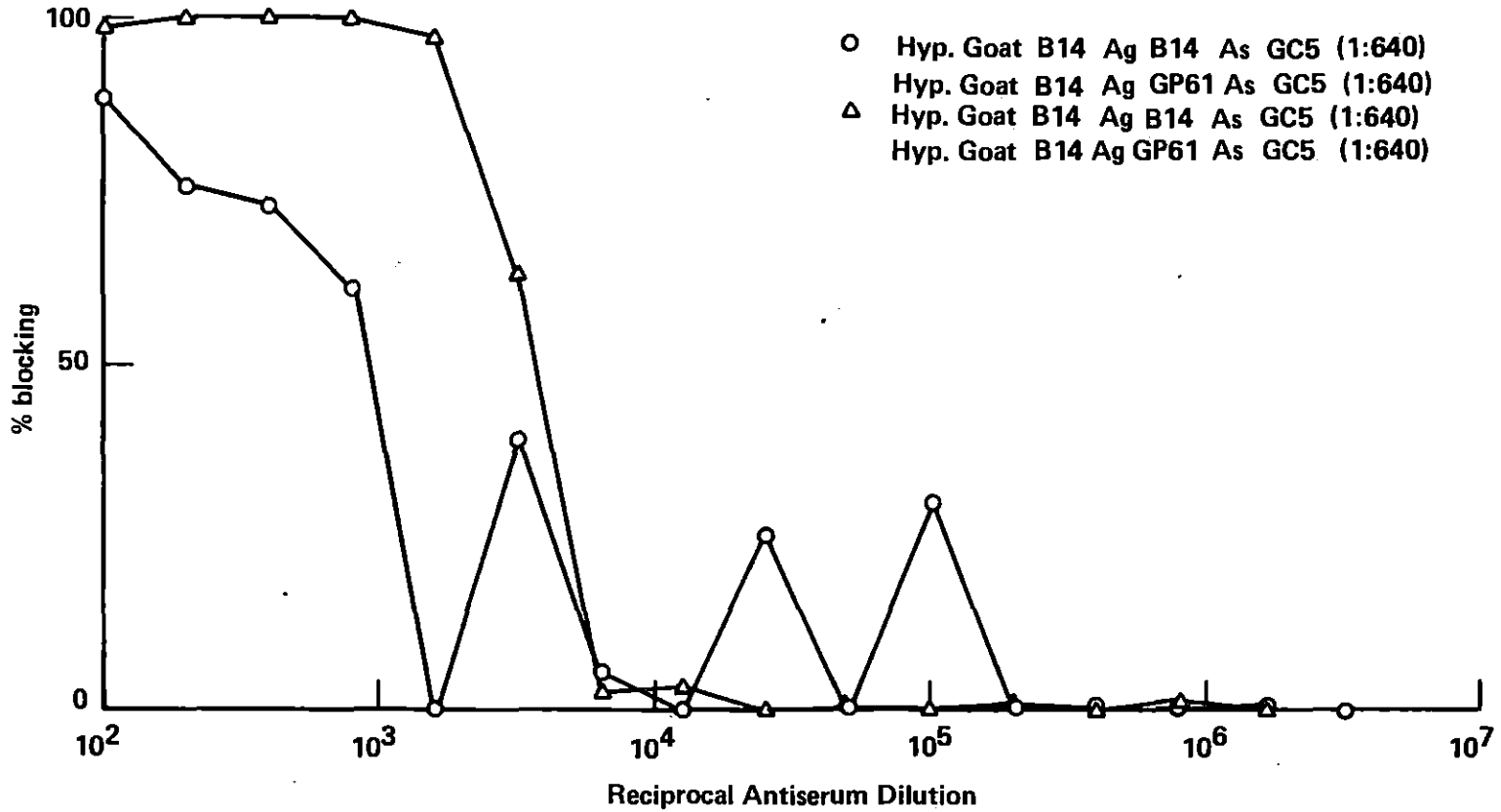
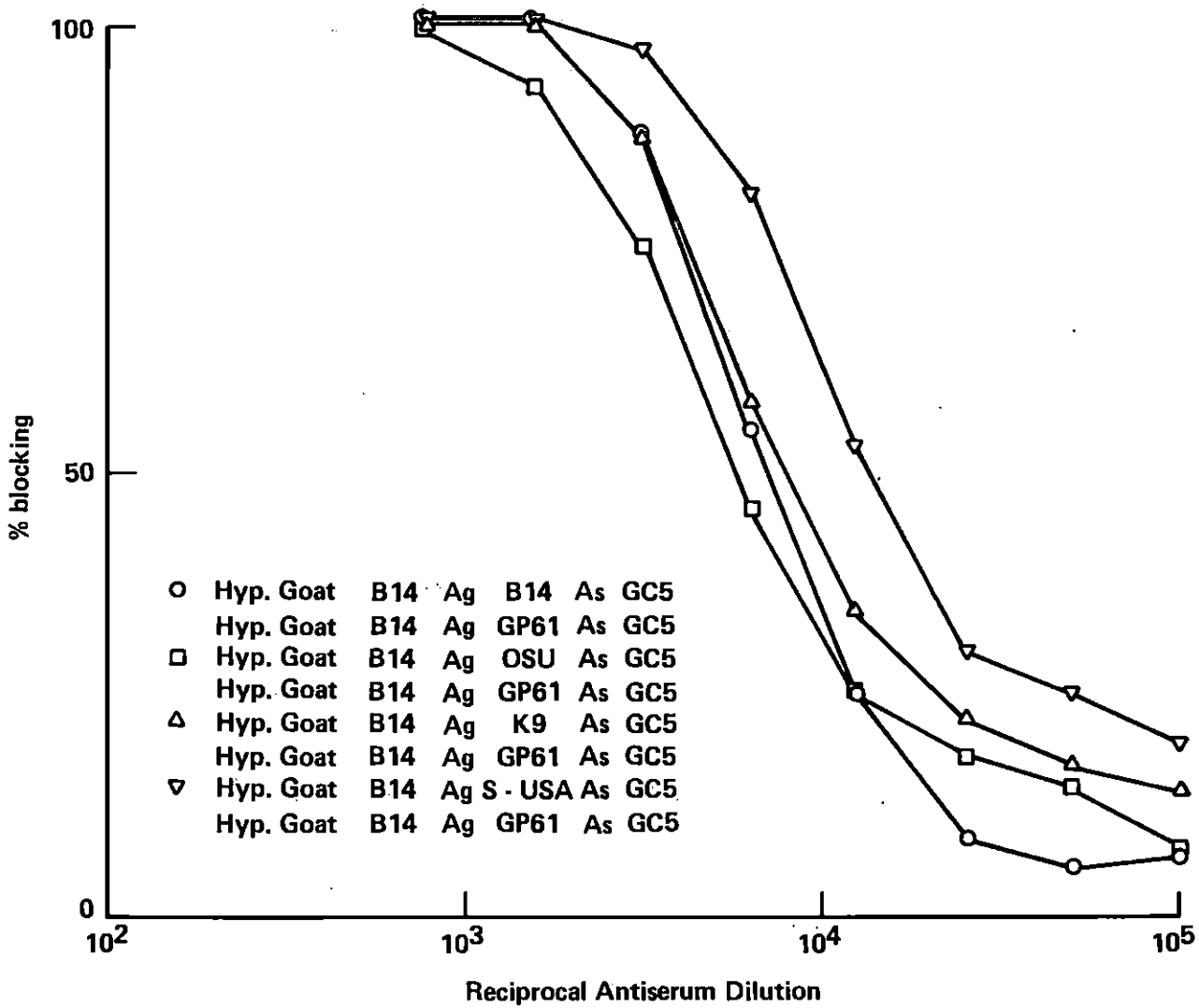

100
50
0
% blocking
10^2
10^3
10^4
10^5
10^6
10^7
Reciprocal Antiserum Dilution
Hyp. Goat B14 Ag B14 As GC5 (1:640)
Hyp. Goat B14 Ag GP61 As GC5 (1:640)
Hyp. Goat B14 Ag B14 As GC5 (1:640)
Hyp. Goat B14 Ag GP61 As GC5 (1:640)

Figure 16. Titration of guinea pig hyperimmune rotavirus antisera against their homologous antigens by the modified triple antibody sandwich ELISA method


100
50
0
% blocking
Hyp. Goat B14 Ag B14 As GC5
Hyp. Goat B14 Ag GP61 As GC5
Hyp. Goat B14 Ag OSU As GC5
Hyp. Goat B14 Ag GP61 As GC5
Hyp. Goat B14 Ag K9 As GC5
Hyp. Goat B14 Ag GP61 As GC5
Hyp. Goat B14 Ag S - USA As GC5
Hyp. Goat B14 Ag GP61 As GC5
10^2
10^3
10^4
10^5
Reciprocal Antiserum Dilution

Controls included for Figure 16	OD Reading (405 nm)
#11 for Hyp-goat	.004±.006
#12 for Hyp-goat, OSU antigen OSU antiserum, GC5 antiserum	.000
#12 for Hyp-goat, OSU antigen GP61 serum, GC5 antiserum	.001
#12 for Hyp-goat, B14 antigen B14 antiserum, GC5 antiserum	.000
#12 for Hyp-goat, B14 antigen GP61 serum, GC5 antiserum	.000
#12 for Hyp-goat, K9 antigen K9 antiserum, GC5 antiserum	.029
#12 for Hyp-goat, K9 antigen GP61 serum, GC5 antiserum	.002
#12 for Hyp-goat, S-USA antigen S-USA antiserum, GC5 antiserum	.001
#12 for Hyp-goat, S-USA antigen PG61 serum, GC5 antiserum	.008
#13 for Hyp-goat, OSU antigen OSU antiserum	.018
#13 for Hyp-goat, OSU antigen GP61 serum	.010
#13 for Hyp-goat, B14 antigen B14 antiserum	.000
#13 for Hyp-goat, B14 antigen GP61 serum	.000
#13 for Hyp-goat, K9 antigen K9 antiserum	.004
#13 for Hyp-goat, K9 antigen GP61 serum	.000

Figure 16 (continued)

#13 for Hyp-goat, S-USA antigen S-USA antiserum	.000
#13 for Hyp-goat, S-USA antigen GP61 serum	.005
#14 for Hyp-goat, OSU antigen OSU antiserum	.025
#14 for Hyp-goat, OSU antigen GP61 serum	.011
#14 for Hyp-goat, B14 antigen B14 antiserum	.020
#14 for Hyp-goat, B14 antigen GP61 serum	.013
#14 for Hyp-goat, K9 antigen K9 antiserum	.007
#14 for Hyp-goat, K9 antigen GP61 serum	.029
#14 for Hyp-goat, S-USA antigen S-USA antiserum	.037
#14 for Hyp-goat, S-USA antigen GP61 serum	.049
#16 for Hyp-goat, GC5 antiserum	.024±.024
#16 for Hyp-goat, GC5 antiserum	.041
#17 for Hyp-goat	.020±.015
#18 for Hyp-goat	1.381±.014

One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate were employed.

Figure 16 (continued)

Controls to monitor the negative reaction in Figure 16

Control composition						
Hyp-goat	+	+				
Pre-goat			+	+	+	+
OSU antigen	+	+	+	+	+	+
OSU antiserum (1:100)	+		+		+	
GP61 serum (1:100)		+		+		+
SB219 antiserum(1:640)	+	+			+	+
GC5 antiserum (1:640)			+	+		
Conjugate (1:200)	+	+	+	+	+	+
Substrate	+	+	+	+	+	+
OD readings (405 nm) obtained	.025	.018	.009	.004	.001	.004

Controls to monitor the negative reaction in Figure 16

Control composition						
Hyp-goat	+	+				
Pre-goat			+	+	+	+
B14 antigen	+	+	+	+	+	+
B14 antiserum	+		+		+	
GP61 antiserum (1:100)		+		+		+
SB219 antiserum (1:640)	+	+			+	+
GC5 antiserum (1:640)			+	+		
Conjugate (1:200)	+	+	+	+	+	+
Substrate	+	+	+	+	+	+
OD readings (405 nm) obtained	.011	.000	.008	.000	.004	.000

Figure 16 (continued)

Controls to monitor the negative reactions in Figure 16

Control composition						
Hyp-goat	+	+				
Pre-goat			+	+	+	+
K9 antigen	+	+	+	+	+	+
K9 antiserum (1:100)	+		+		+	
GP61 serum (1:100)		+		+		+
SB219 antiserum (1:640)	+	+			+	+
GC5 antiserum (1:650)			+	+		
Conjugate (1:200)	+	+	+	+	+	+
Substrate	+	+	+	+	+	+
OD Readings (405 nm) obtained	.000	.000	.008	.020	.015	.007

Controls to monitor the negative reaction in Figure 16

Control composition						
Hyp-goat	+	+				
Pre-goat			+	+	+	+
S-USA antigen	+	+	+	+	+	+
S-USA antiserum (1:100)	+		+		+	
GP61 serum (1:100)		+		+		+
SB219 antiserum (1:640)	+	+			+	+
GC5 antiserum (1:640)			+	+		
Conjugate (1:200)	+	+	+	+	+	+
Substrate	+	+	+	+	+	+
OD Readings (405 nm) obtained	.070	.111	.052	.032	.031	.013

Figure 16 (continued)

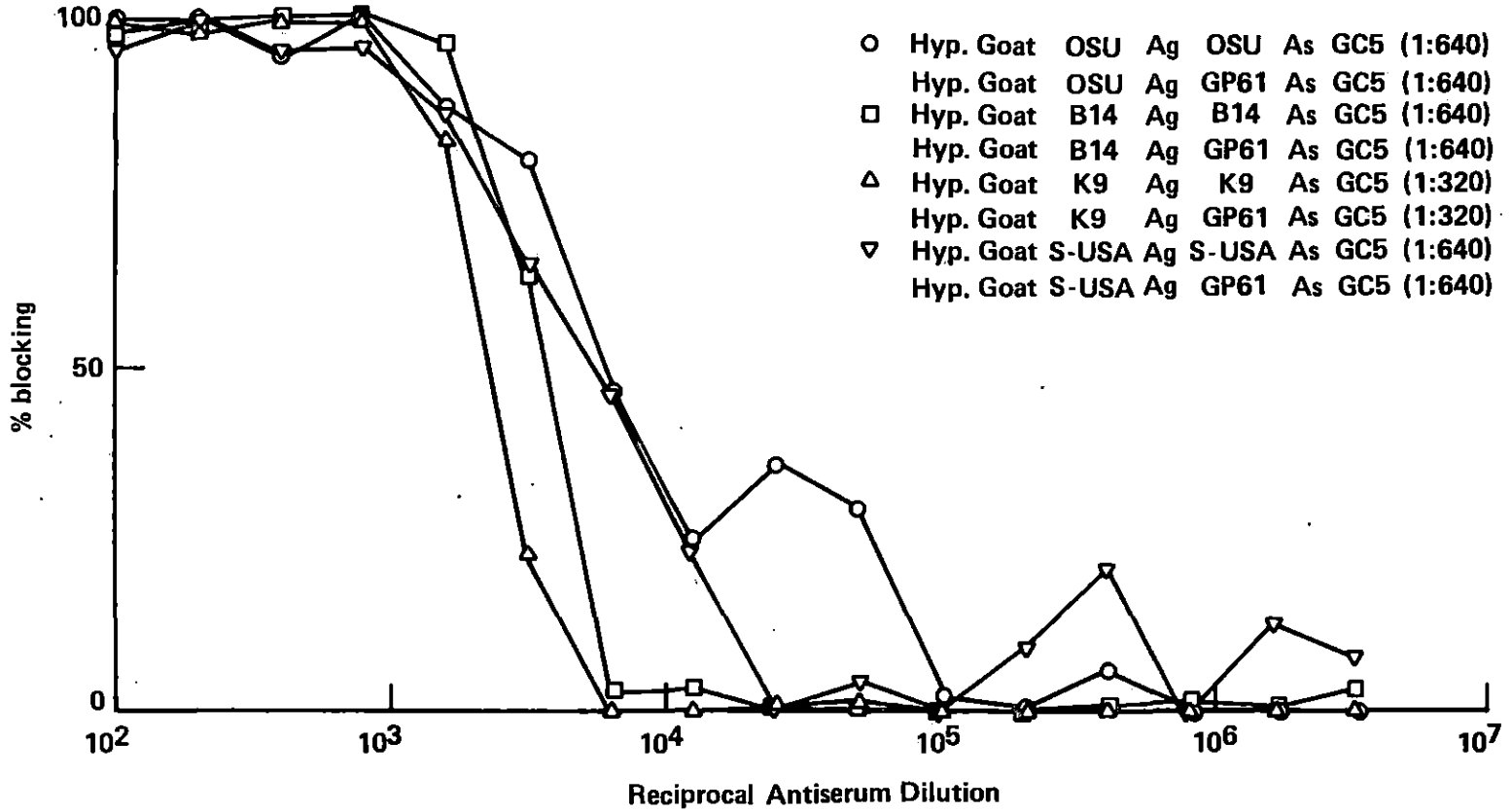
Figure 17. Rotavirus serotyping by the modified triple antibody sandwich ELISA method

Controls included for Figure 17	OD Reading
#11 for Hyp-goat	.001
#14 for Hyp-goat, OSU antiserum (1:400)	.022
#14 for Hyp-goat, K9 antiserum (1:400)	.035
#14 for Hyp-goat, S-USA antiserum (1:400)	.038
#15 for Hyp-goat, GC5 antiserum (1:640)	.960
#18 for Hyp-goat	1.419

One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate were employed.

Controls to monitor the negative reactions in Figure 17

Control composition		
Hyp-goat	+	+
B14 antigen	+	+
B14 antiserum (1:400)	+	
GP61 serum (1:400)		+
SB219 antiserum (1:640)	+	+
Conjugate (1:200)	+	+
Substrate	+	+
OD Readings (405 nm) obtained	.004	.008


100
50
0
% blocking
10^2
10^3
10^4
10^5
10^6
10^7
Reciprocal Antiserum Dilution
Hyp. Goat OSU Ag OSU As GC5 (1:640)
Hyp. Goat OSU Ag GP61 As GC5 (1:640)
Hyp. Goat B14 Ag B14 As GC5 (1:640)
Hyp. Goat B14 Ag GP61 As GC5 (1:640)
Hyp. Goat K9 Ag K9 As GC5 (1:320)
Hyp. Goat K9 Ag GP61 As GC5 (1:320)
Hyp. Goat S-USA Ag S-USA As GC5 (1:640)
Hyp. Goat S-USA Ag GP61 As GC5 (1:640)

the guinea pig antisera were determined by reading their titration curves at 50% blocking and summarized in Table 5.

Table 5. Homologous and heterologous titers of guinea pig hyperimmune rotavirus antisera by the modified triple antibody sandwich ELISA method

Antigen	Antiserum titer			
	B14	OSU	K9	S-USA
B14	6,400	5,800	8,400	13,000
	5,600	6,000	7,400	14,000
	7,800			
$\bar{X}$	6,600	5,900	7,900	13,500
±SD	±1,113.6	±141.4	±707.0	±707.1
OSU	7,800	27,000	14,000	11,500
	4,400	16,000	6,800	10,000
$\bar{X}$	6,100	21,500	10,400	10,750
±SD	±2,404.2	±7,778.2	±5,091.2	±1,060
K9	9,200	6,000	5,900	11,000
	5,800	3,100	3,700	9,600
$\bar{X}$	7,500	4,550	4,800	10,300
±SD	±2,404.2	±2,050.6	±1,555.6	±989.9
S-USA	16,000	9,400	16,000	21,000
	6,200	3,600	9,400	20,000
$\bar{X}$	11,100	6,500	12,700	20,500
±SD	±6,929.6	±4,101.2	±4,666.9	±707.1

Discussion

From the results summarized in Tables 2, 3 , 4 and 5 we must conclude that there were no significant differences detected between heterologous and homologous reactions. The variability of the tests can result in a range of 4- to 5-fold from the lowest to the highest titer of the same reaction (i.e. Table 3, K9-K9 homologous reaction). The greatest difference observed (between K9-K9 and K9-B14 reactions) was of the order of 6.2-fold. Although this may be significant, it is obviously too small a difference to be usable in a diagnostic serotype test when the variability of the test is considered. In addition to this observation, the homologous reaction was not always the highest (i.e. B14-B14, Table 4 and 5) and this may again reflect the variability of the test. Differences of at least 10-fold between homologous and heterologous reactions are necessary to be confident that these tests will demonstrate serotype differences.

As discussed above, the quantity of serotype specific antigen was insufficient to demonstrate a serotype specific antigen-antibody reaction by the indirect ELISA method. This is further complicated by the different antibody titers of the different guinea pig hyperimmune rotavirus antisera used. Finally, the goat catching antiserum may have had low titer for the various serotypes specific antigens, and there may have been insufficient binding of the specific antigen in the unblocked preparations.

There are other methods by which serotypes possibly may be demonstrated. These include:

(a) Purification of serotype specific antigen by:

(a.1) selection of complete particles by density gradient centrifugation but these would still have more common antigens or

(a.2) by removing the serotype specific antigen from the virus particle by trypsin and low Ca^{++} treatment and purify the soluble outer capsid layer antigen.

(b) The specificity of the antisera used in these tests could be improved by:

(b.1) removing the antibodies to the common antigens by cross-absorption and then demonstrating ELISA reactive antibodies to serotype specific antigens

(b.2) by the use of polyclonal monospecific antiserum raised against the purified protein antigen stimulating the serotype specific neutralizing response or

(b.3) by monoclonal antibodies with serotype specific neutralizing activity.

It was decided to investigate (b.1) and (b.2) as these were the only methods which were readily available for this study and may be available for the average diagnostic laboratories as the requisite reagents could be obtained on request from reference laboratories.

Polyclonal Monospecific Rotavirus Antiserum for Rotavirus Serotyping

Using a polyclonal monospecific antiserum (PMA) in rabbits to the K9 specific neutralization antigen, the indirect ELISA method was executed. It was predicted that there would be an absence of cross-reaction between rabbit K9 rotavirus polyclonal monospecific antiserum and B14, OSU and S-USA rotavirus antigens and a positive one when tested against K9 rotavirus antigen.

Serial two-fold dilutions, from 1:10 up to 1:5120, of rabbit 2289 pre-immune K9 rotavirus antiserum and from 1:100 up to 1:51,200 of the rabbit 2289 after K9 rotavirus immunization, were tested against K9, B14, OSU and S-USA antigens (1:500) by the indirect ELISA method. Titers of the rabbit antisera were determined by reading their titration curves at .500 OD and summarized in Table 6. To monitor the non-specific reactions, controls #1 for each rabbit antiserum, #3, #5, #6 and #7 were included in each test. One percent ovalbumin and peroxidase labeled goat to rabbit IgG (H + L) conjugate were employed.

The PMA to K9 rotavirus when tested with its homologous antigen had ELISA titers of 1.6, 11.5, and 3.8 folds higher than the ones obtained when tested against B14, OSU and S-USA antigens respectively. This test showed a greater rise in titer of the antiserum to K9 antigen than to B14, OSU and S-USA antigens probably showing specificity for K9. When considering that an error of the test of 2-3-fold is possible, again this difference was too small to be considered as significant for the K9-B14, K9-OSU and K9-S-USA

reactions. This test appears to be measuring K9 serotype specific antigen. The rise in titer obtained to B14 antigen was about same fold as for S-USA antigen.

Table 6. Rabbit K9 antisera titers by the indirect ELISA method

Antigen	Antiserum Titers	
	Pre-immune Rabbit 2289 serum	Polyclonal monospecific Rabbit 2289 antiserum
K9	1,900	15,000
B14	3,500	9,200
OSU	620	1,300
S-USA	1,300	4,000

Cross-absorption

To study whether cross absorption of the guinea pig hyperimmune rotavirus antisera could be used to remove antibodies reacting with the common or with the heterotypic antigens, but leaving the homotypic antigen reactive antibodies intact, the four antisera were absorbed with porcine (OSU) and or canine (K9) antigen. Absorbed and non-absorbed antisera were tested against the rotavirus antigens by the indirect ELISA method and the OD readings obtained after each absorption are summarized in Tables 7, 8, 9 and 10. Two different preparations of OSU antigen with the same passage number and one of K9

antigen were employed for the cross-absorption of the typing antisera.

In the first study (Table 7), the guinea pig hyperimmune rotavirus antisera were absorbed with OSU antigen (5.8×10^{11} particles/ml $\pm$ .2) and then reacted by the indirect ELISA method with each of the four rotavirus antigens. The OSU antigen removed both heterotypic and homotypic reactions from the OSU antiserum, it removed all or part of the antibodies in K9, S-USA and B14 antisera which reacted with OSU antigen. In contrast, the heterotypic reactions of K9, S-USA and B14 sera were either not diminished or only to a limited extend.

In an attempt to improve these results, the K9 antiserum was absorbed again with OSU antigen (Table 8). The reactions with K9 and S-USA antigens (which are of the same serotype subgroup) were virtually unaffected as well as the heterotypic reaction with B14 antigen was not diminished significantly.

For further studies, two absorptions of sera were carried out with freshly prepared antigens (K9 = 4.5×10^{11} particles/ml $\pm$ .2, and OSU = 4.0×10^{11} particles/ml $\pm$ .2) (Tables 9 and 10). The OSU serum was absorbed with K9 antigen to confirm that the removal of OSU antibodies by OSU antigen (Table 7) was a specific reaction. To test whether more of the heterotypic reaction antibodies could be removed by absorption, B14 antiserum was absorbed with a mixture of K9-OSU antigens and the K9 serum was absorbed with K9 antigen in the expectation that all reactions would be removed (Table 9). The K9 antigen removed most of the heterotypic reactions of OSU serum but

left the homotypic reaction intact. The K9-OSU antigen mixture reduced both the B14 serum homotypic and the heterotypic reactions, but the B14 serum reactions with K9 and OSU antigens were reduced to a greater extent. The K9 antigen removed most of the homotypic and heterotypic reactions of K9 serum. These sera, diluted two-fold for reasons of economy, were absorbed for a second time (Table 10) with K9 and or OSU antigens. This second absorption reduced further the various heterotypic reactions, it completely eliminated the K9 serum reactions but left intact the OSU homotypic reactions.

Discussion

The use of ELISA for serotyping animal rotaviruses (as based on the neutralization test) may not be possible or at least it will be very expensive, as cross absorption may be the method of choice and the serum may require several cross-absorptions.

It is interesting to observe that the strength of the homotypic reaction was not reduced by the heterotypic absorption (OSU serum with K9 antigen and K9 serum with OSU antigen). This implies that the ELISA with complete and incomplete particles as antigen measures antibodies in the homotypic reaction directed at the 2 or 3 surface antigens, and that the common antigens are minor. In contrast, the antibodies reacting in the heterotypic test are almost exclusively related to common antigens which were removed by cross-absorption.

Table 7. Absorbances at 405 nm of cross-absorbed guinea pig hyperimmune rotavirus antisera (one absorption)

Antiserum	Antigen			
	OSU	K9	S-USA	B14
K9 1:2,000	1.168	1.204	Not tested	Not tested
K9 1:2,000[OSU[a] absorbed]	.411	1.167	Not tested	Not tested
S-USA 1:20,000	.963	1.065	.977	1.063
S-USA 1:20,000[OSU[a] absorbed]	.000	.971	.869	.880
B14 1:2,000	1.051	1.114	.993	.944
B14 1:2,000[OSU[a] absorbed]	.353	.991	.881	1.017
OSU 1:20,000	1.066	1.021	.830	.995
OSU 1:20,000[OSU[a] absorbed]	.026	.088	.071	.075

[a]OSU antigen for absorption = 5.8×10^{11} particles/ml $\pm$ .2

Table 8. Absorbances at 405 nm of cross-absorbed guinea pig hyperimmune rotavirus antisera (two absorptions)

Antiserum	Antigen			
	OSU	K9	S-USA	B14
K9 1:4,000	1.081	1.150	1.121	1.138
K9 1:4,000 [OSU[a] absorbed two times]	.103	1.135	.985	.987

[a]OSU antigen for absorption = 5.8×10^{11} particles/ml $\pm$ .2

Table 9. Absorbances at 405 nm of cross-absorbed guinea pig hyperimmune rotavirus antisera (one absorption)

Antiserum	Antigen			
	OSU	K9	S-USA	B14
OSU 1:20,000	1.058	.976	1.009	1.042
OSU 1:20,000[K9[a] absorbed]	1.016	.127	.427	.455
B14 1:2,000	1.049	1.098	1.035	1.112
B14 1:2,000[K9[a]–OSU[b] absorbed]	.204	.373	.584	.825
K9 1:4,000	1.103	1.153	1.107	1.122
K9 1:4,000[K9[a] absorbed]	.152	.178	.133	.194

[a]K9 antigen for absorption = 4.5×10^{11} particles/ml $\pm$ .2

[b]OSU antigen for absorption = 4.0×10^{11} particles/ml $\pm$ .2

Table 10. Absorbances at 405 nm of cross-absorbed guinea pig hyperimmune rotavirus antisera (two absorptions)

Antiserum	Antigen			
	OSU	K9	S-USA	B14
OSU 1:40,000	1.061	.957	.716	.845
OSU 1:40,000U[K9[a] absorbed]	1.064	.019	.127	.210
B14 1:4,000	1.060	1.110	.974	1.073
B14 1:4,000[K9[a]–OSU[b] absorbed]	.027	.087	.128	.438
K9 1:8,000	1.087	1.166	1.053	1.058
K9 1:8,000 [K9[a] absorbed]	.005	.013	.020	.049

[a]K9 antigen for absorption = 4.5×10^{11} particles/ml $\pm$ .2

[b]OSU antigen for absorption = 4.0×10^{11} particles/ml $\pm$ .2

The observation that B14 shares common antigens with the different serotypes K9, S-USA but not OSU, shows that there are antigens not common to all rotaviruses which are not directly related to the neutralizing antigen.

These data suggest that the sera differed in their specificity. Antiserum to OSU rotavirus had specific antigens which are not removed by cross-absorption with the heterologous antigen K9 and this serum could be used to serotype OSU with respect to K9, S-USA and B14 antigens. In contrast, K9 antiserum appeared to possess specific antibodies which reacted with K9, S-USA and B14 antigens but not with OSU. The K9 and S-USA viruses are of the same serotype subclass but the strong cross reaction of a serotype nature with B14 antigen was surprising as these are of different serotypes. Cross-absorption, as conducted here, was unable to produce sera which would serotype B14, although the OSU antigen did remove most of the B14 and OSU reaction. It is possible that K9 may have been a better antigen for absorbing B14 antiserum (as for absorbing OSU antiserum), but this absorption was not performed. All these studies suggested a sharing of ELISA reactive antigens between K9, S-USA and B14.

PART II: DEVELOPMENT OF AN ELISA FOR SEROLOGIC STUDIES ON "BREDA" VIRUS

INTRODUCTION

A new transmissible agent which caused diarrhea in newborn calves in Iowa was described by Woode et al. (1982) and shown to be a virus ("Breda" virus). This virus was antigenically unrelated to other bovine enteric viruses (rotavirus, coronavirus and bovine pestivirus-BVD) and to bovine parainfluenza III virus. The morphology of the virus, although superficially similar to coronavirus, was considered different and the peplomers were short (8 nm) in contrast to the coronavirus group in which the peplomers are 12-24 nm (Matthews et al., 1981). Subsequently, this virus was shown to be antigenically related to an Ohio isolate (Saif et al., 1981) and to another Iowa isolate. Studies with immunofluorescence (IF) and the hemagglutination inhibition (HI) reaction, demonstrated that the three isolates shared common antigens (IF) but were subclassifiable into two serotypes. Serotype I was the first Iowa isolate ("Breda" virus Iowa I) and serotype II group were the "Breda" virus Iowa II isolate and the Ohio isolate (Woode et al., 1983b). There was no cross-reaction by HI between the two serotype groups (Woode, personal communication, VMPM, ISU). "Breda" virus Iowa I and II are antigenically related to an equine virus (Weiss et al., 1983).

In the above studies, the HI test had not proved ideal for the serological survey for the incidence of infection in the bovine due to the widespread distribution of HI inhibiting sera and the relatively low titer of the convalescent antibody response (rising from 1:4 to

1:24). It was decided to develop an ELISA antibody assay system to demonstrate the similarities and possible differences between the three "Breda" virus isolates and subsequently to use the test for a serological survey of human, bovine and other animal sera.

MATERIALS AND METHODS

Virus Isolates

Samples of bovine diarrheic feces were kindly supplied by Dr. G. N. Woode. These fecal samples had been collected from rotavirus, coronavirus and "Breda" virus orally infected gnotobiotic calves (GC) as indicated in Table 11. Gnotobiotic calves were produced and maintained by open Caesarean section as described by Matthews et al. (1981).

Table 11. "Breda" virus, bovine rotavirus and bovine coronavirus isolates

Viral inoculum for GC calf	Fecal Identification	Days post-infection (DPI)
Breda 1 (5 ml undiluted unfiltered LI contents plus 5 ml (1:3) unfiltered supernatant fecal in PBS)	GC2	6
Breda 2 (3 ml (1:5) .45 m filtered supernatant fecal in PBS)	GC32	4
Bovine Rotavirus (4 ml (1:10) .45 m filtered supernatant fecal in PBS)	GC26	2
Bovine Coronavirus (10 ml (1:3) .45 m filtered supernatant fecal in PBS	GC28	4

Serology

The pre-immune and convalescent antisera are listed in Table 12.

Table 12. Serology for "Breda" virus serotyping

Virus strain	Host species originally isolated	Host species antiserum raised	Animal identification	Antiserum identification	DPI[a]
None	Bovine	Bovine	GC0	SB216	0
Breda 1	Bovine	Bovine	GC0	SB217	7
Breda 1	Bovine	Bovine	GC0	SB218	15
Breda 1	Bovine	Bovine	GC0	SB219	21
Breda 2 and rotavirus	Bovine	Bovine	GC21	GC21(15)	15
Breda 2 and rotavirus	Bovine	Bovine	GC21	GC21(21)	21
None	Bovine	Bovine	GC15	GC15(0)	0
Breda 2	Bovine	Bovine	GC15	GC15(21)	21
B:USA:78:1A	Bovine	Bovine	GC5	GC5	45
Breda 2	Bovine	Bovine	GC37	GC37(2)	2
Breda 2	Bovine	Bovine	GC37	GC37(9)	9
Breda 2	Bovine	Bovine	GC37	GC37(14)	14
Breda 2	Bovine	Bovine	GC37	GC37(21)	21
Coronavirus[b]	Bovine	Bovine	?	Corona	?
None[c]	Bovine	Bovine	GC35	GC35(0)	0

[a]Days post-infection.

[b]Serum kindly supplied by Dr. Torres Medina.

[c]Serum kindly supplied by Dr. J. Pohlenz.

Method for the Virus Purification from Fecal Materials

Samples of diarrheic feces were diluted 1:4 (v/v) in phosphate buffered saline (PBS) pH 7.2, centrifuged at 6,000 x g (B20 High Speed centrifuge, A147 rotor, International Equipment Co., Needham Heights, Mass.) for 60 minutes at 4°C. The supernatants were pooled and pelleted at 122,047.74 x g (L5-65 Ultracentrifuge, SW27 rotor, Beckman Instruments, Inc., Palo Alto, CA) for 2 hours at 4°C. Pellets were resuspended in 1.0 ml Tris-Ca buffer [.1 M Tris (Hydroxymethyl) Aminomethane - 1.5 M $CaCl_2 \cdot 6\ H_2O$] pH 7.2 overnight at 4°C. The pellets were pooled and further purified by centrifugation at 122,047.74 x g for 4 hours at 4°C through 20% sucrose. The pellet obtained was resuspended in 1.0 ml Tris-Ca buffer and tested for the presence of the agent by electron microscopy (EM) (see section 2) and by HA. The virus was frozen at -20°C until used.

Indirect ELISA Method

The indirect ELISA method for antibody assay here employed has already been described in Part I, Materials and Methods (see page 29).

RESULTS AND DISCUSSION

Standardization of ELISA for Determination of "Breda" Virus Antibodies

The indirect ELISA method was standardized using "Breda" virus 1 and 2 as antigens, their respective convalescent gnotobiotic calf antisera and as negative controls sera, convalescent gnotobiotic calf antisera to bovine coronavirus and bovine rotavirus as well as their respective antigens purified from gnotobiotic fecal preparations.

Based on Part I of this paper and on previous results peroxidase labeled conjugates were diluted to 1:200.

Determination of optimal GC2 (Breda 1) and GC32 (Breda 2) antigen dilutions

Determination of the optimal GC2 and GC32 antigen dilutions was performed by the indirect ELISA method as previously described. Breda virus 1 and Breda virus 2 antigens were serially diluted two-fold in 0.1 M carbonate buffer, adsorbed to the microtiter-immulon-plate wells and tested against SB216, SB217, SB218, SB219, GC21(15), GC21(21), GC5 and corona antisera each diluted to 1:200 in ELISA buffer. To monitor the non-specific reactions, controls #1, #3, #6 and #7 were included in each test. One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate (1:200) were employed. Dilutions equal to 1:1,000 and to 1:1,500 were found as the optimal working dilutions for GC2 and for GC32 antigens respectively. Rotavirus and coronavirus

antisera had OD readings below .200.

Determination of optimal GC26 (rotavirus) and GC28 (coronavirus) antigen dilutions

Determination of the optimal GC26 and GC28 antigen dilutions was performed by the indirect ELISA method. Rotavirus and coronavirus antigens were serially two-fold diluted in 0.1 M carbonate buffer, adsorbed to the microtiter-plate wells and tested against rotavirus antiserum (GC5) and coronavirus antiserum, both diluted 1:200 in ELISA buffer. To monitor the non-specific reactions controls were included, as for GC2 and GC32 titration previously described, with OD readings up to .012. One percent ovoalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate (1:200) were employed. Dilutions equal to 1:800 and to 1:500 were found as optimal working dilutions for rotavirus and coronavirus antigens respectively.

Titration of experimental bovine antisera against GC26(rotavirus) and GC28(coronavirus) antigens

Serial two-fold dilutions of experimental bovine antisera [GC5, SB216, SB217, SB219, Corona, GC21(15), GC21(21), GC35(0), GC37(2), GC37(9), GC37(14) and GC37(21)] in ELISA buffer were tested against GC26 (rotavirus) and GC28 (coronavirus) diluted to 1:800 and to 1:500, in 0.1 M carbonate buffer, respectively. Titers of the antisera were determined by reading their titration curves at .500 OD at 405 nm (Figures 18 and 19) and summarized in Table 13. Absence of cross-

reaction between convalescent rotavirus (GC5), at 1:10 dilution, and coronavirus, at 1:100 dilution, antisera against Breda 1 and Breda 2 antigens was observed as well as between Breda 1 and Breda 2 antisera against either rotavirus or coronavirus antigens. Titers of the antisera were determined at OD values equal to .500, readings equal or below OD .200 were considered negative and or non-specific.

Table 13. Titers of experimental bovine antisera to GC26 and to GC28 antigens by the indirect ELISA method

Antiserum	Antigen	
	Rotavirus (GC26)	Coronavirus (GC28)
GC5	2,500	<10
SB216	<10	<10
SB217	<10	<10
SB219	<10	<10
Corona	<100	540
GC21(15)	<10	<10
GC21(21)	<10	<10
GC35(0)	<10	<10
GC37(2)	130	130
GC37(9)	<10	<10
GC37(14)	<10	<10
GC37(21)	<10	Not-tested

Figure 18. Titration of experimental bovine antisera against GC26 (rotavirus) antigen by the indirect ELISA method

Controls included for Figure 18	OD Readings (405 nm)
#1 for GC5 (1:100)	.015
#5	.007
#6	1.297
#7 for GC26 antigen	.015

One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate were employed.

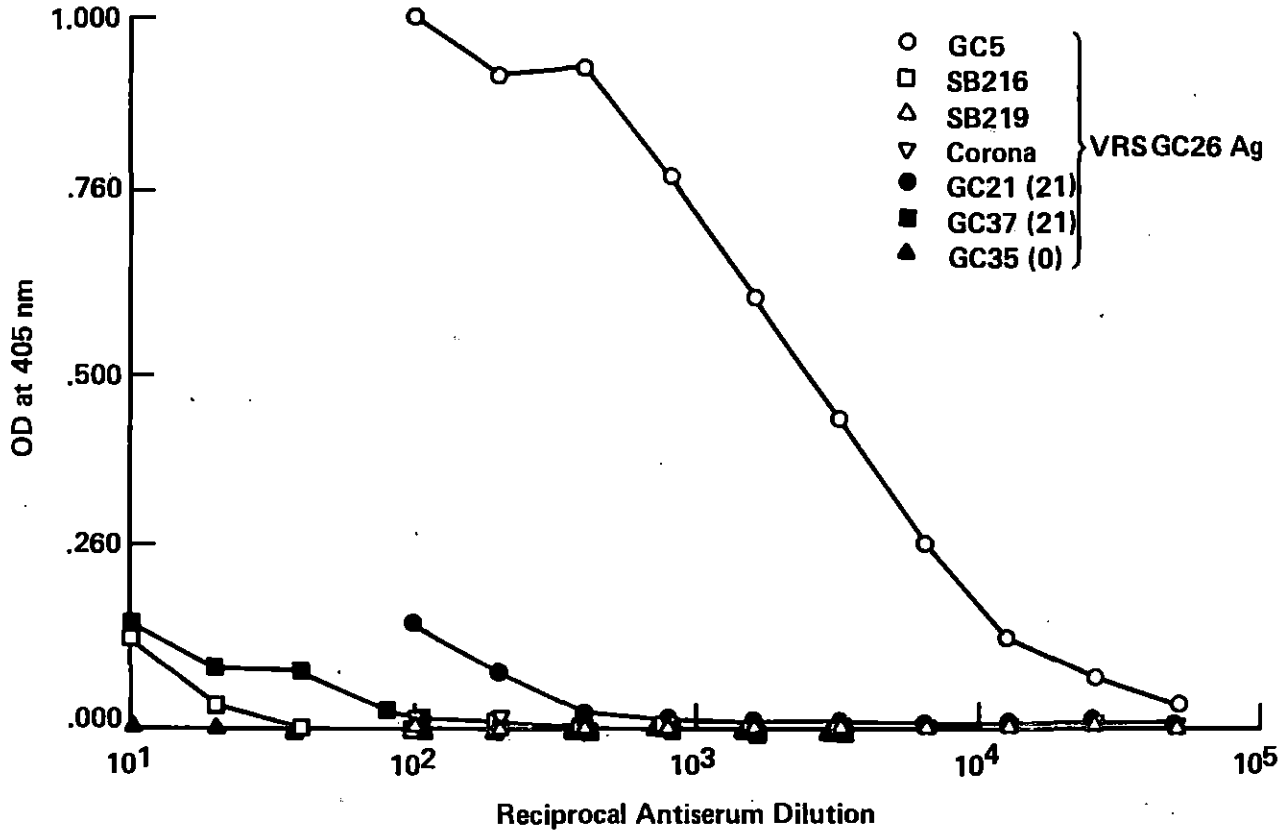
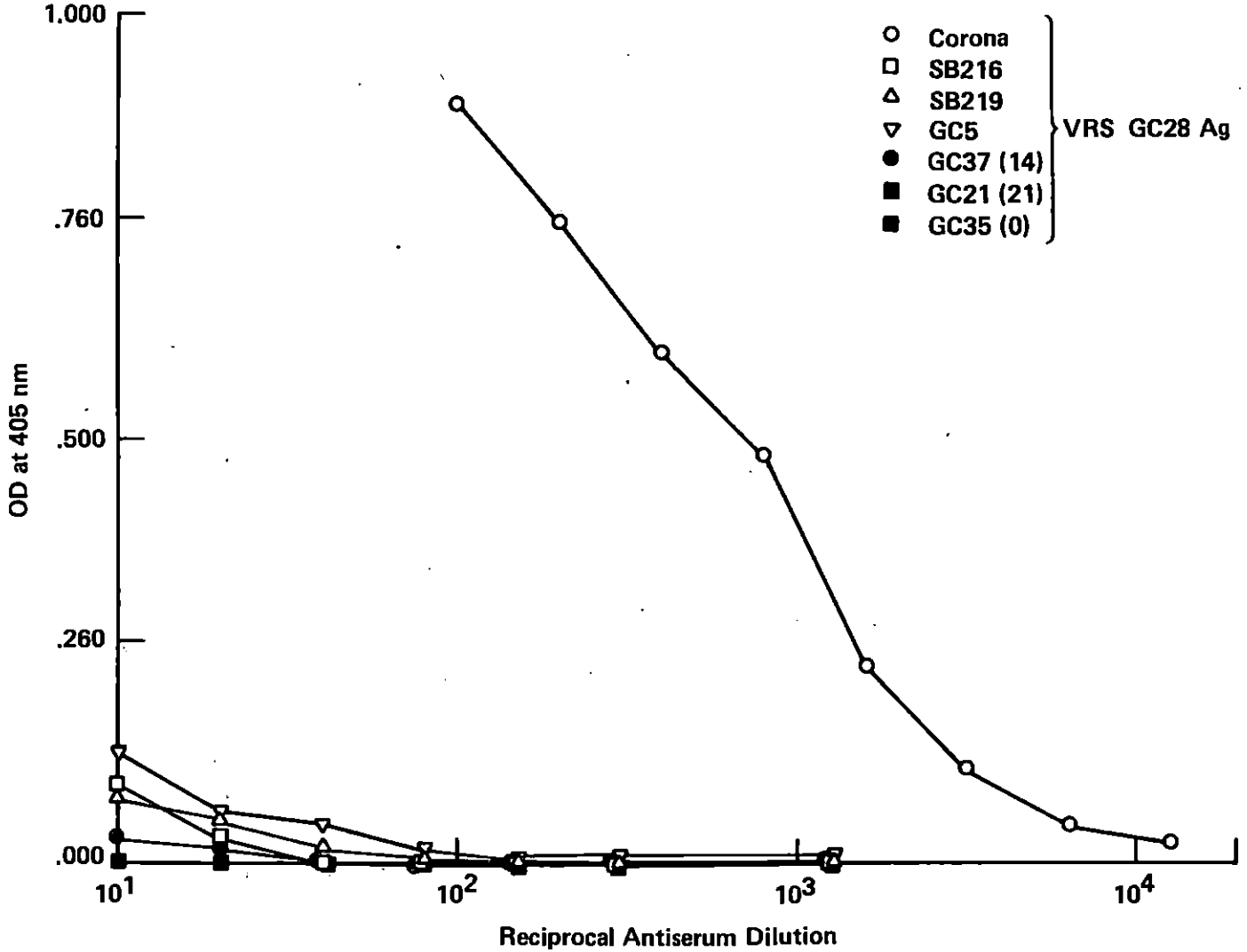

1.000
.760
.500
.260
.000
OD at 405 nm
10^1
10^2
10^3
10^4
10^5
Reciprocal Antiserum Dilution
GC5
SB216
SB219
Corona
GC21 (21)
GC37 (21)
GC35 (0)
VRS GC26 Ag

Figure 19. Titration of experimental bovine antisera against GC28 (coronavirus) antigen by the indirect ELISA method

Controls included for Figure 19	OD Readings (405 nm)
#1 for corona antiserum	.005
#5	.001
#6	1.392
#7 for GC28 antigen	.008

One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate were employed.


1.000
.760
.500
.260
.000
OD at 405 nm
10^1
10^2
10^3
10^4
Reciprocal Antiserum Dilution
Corona
SB216
SB219
GC5
GC37 (14)
GC21 (21)
GC35 (0)
VRS GC28 Ag

Breda Virus Serotyping by the ELISA

Direct comparison between homologous and heterologous reactions with experimental bovine antisera

Serotyping of experimental bovine antisera against GC2 (Breda 1) and GC32 (Breda 2) antigens was accomplished by the indirect ELISA method. Serial two-fold dilutions of experimental bovine antisera in ELISA buffer were tested against GC2 and GC32 antigens diluted to 1:1,000 and to 1:1,500 in 0.1 M carbonate buffer respectively. Titers of the antisera were determined from the titration curves at .500 OD at 405 nm. To test the reproducibility of the test, homologous antigen-antibody complexes were performed more than once and their OD readings summarized in Tables 14 and 15. Heterologous titers were determined as shown in Figures 20 and 21 respectively against GC2 and GC32 antigens and their OD values indicated in Table 16. Corona and GC5 antisera had titers lower than 10. Homologous titers for SB219, GC21(21) and GC37(21) were 5.7, 9.6 and 5.1 fold different respectively from the heterologous reaction. These gave an overall average of 6.8 fold difference indicating that the indirect ELISA method was able to distinguish these two "Breda" virus serotypes and confirming the data obtained by HI tests. As this approach had been successful (unlike rotavirus studies) no further attempts were made to develop ELISAs to distinguish "Breda" virus 1 and 2 but more data are necessary in order to be able to consider these results statistically significant.

Table 14. Homologous titers of experimental bovine antisera to GC2 ("Breda" virus 1) antigen by the indirect ELISA method

Antigen	Antiserum		
	SB216	SB217	SB219
Breda virus I (GC2)	<10	<10	1,280
	<10	<10	1,000
	<10	<10	800
	<10	<10	780
$\bar{X}$	<10	<10	965
$\pm$SD			232.3

Table 15. Homologous titers of experimental bovine antisera to GC32 ("Breda" virus 2) antigen by the indirect ELISA method

Antigen	Antiserum			
	GC35(0)	GC37(9)	GC37(21)	GC21(21)
Breda virus 2 GC32	<10	35	660	2,200
	<10	28	650	2,900
	<10	28	620	1,700
	<10	23	540	2,000
$\bar{X}$	<10	28.5	617.5	2,200
$\pm$SD		$\pm$4.9	$\pm$54.4	$\pm$509.9

Figure 20. "Breda" virus 1 (GC2) serotyping by the indirect ELISA method

Controls included for Figure 20	OD Readings (405 nm)
#1 for SB219 antiserum (1:100)	.005
#1 for GC21(21) antiserum (1:100)	.004
#5	.002
#6	1.453
#7 for GC2 antigen	.011

One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate were employed.

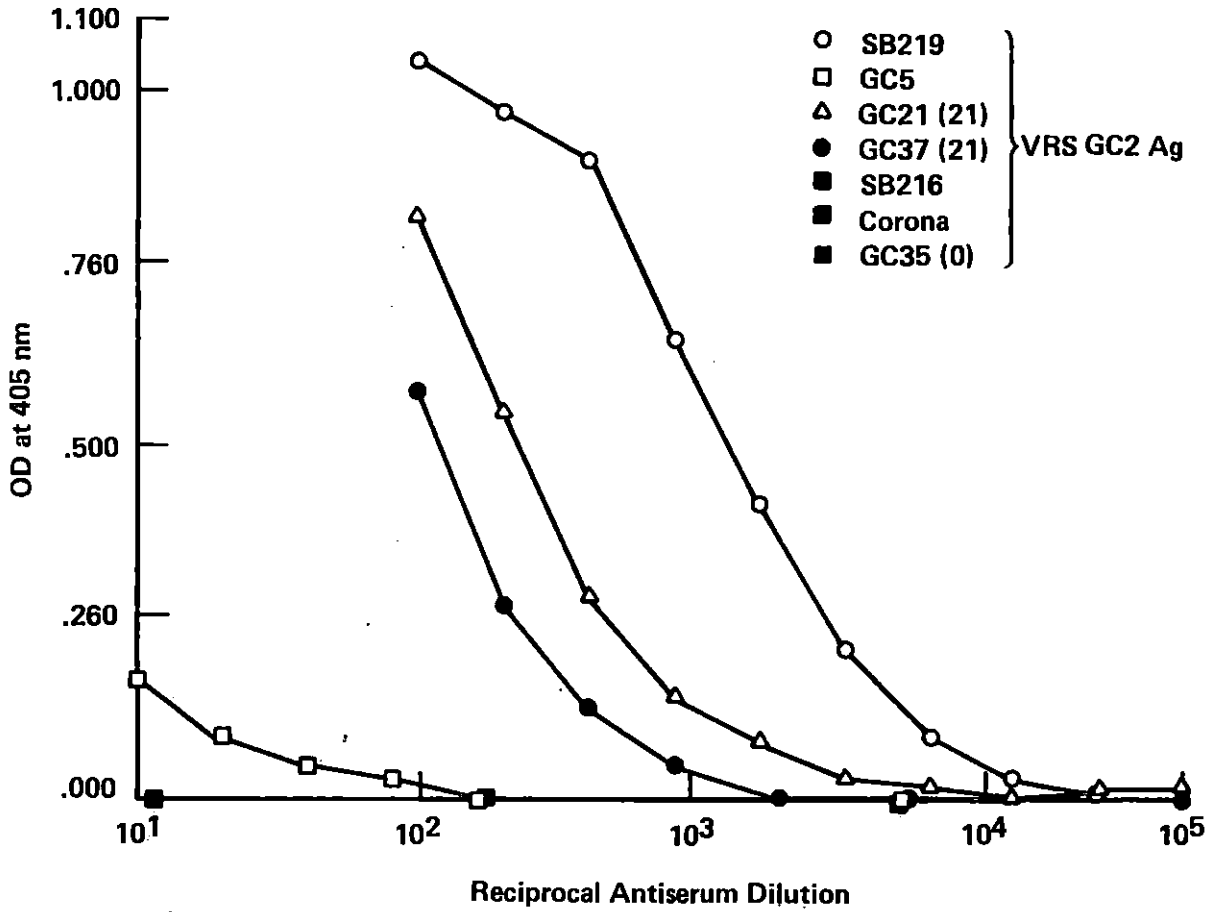

1.100
1.000
.760
.500
.260
.000
OD at 405 nm
10^1
10^2
10^3
10^4
10^5
Reciprocal Antiserum Dilution
SB219
GC5
GC21 (21)
GC37 (21)
SB216
Corona
GC35 (0)
VRS GC2 Ag

Figure 21. "Breda" virus 2 (GC32) serotyping by the indirect ELISA method

Controls included for Figure 21	OD Readings (405 nm)
#1 for SB219 antiserum (1:100)	.019
#1 for GC21(21) antiserum (1:100)	.019
#5	.016
#6	1.484
#7 for GC32 antigen	.031

One percent ovalbumin and peroxidase labeled goat to bovine IgG (H + L) conjugate were employed.

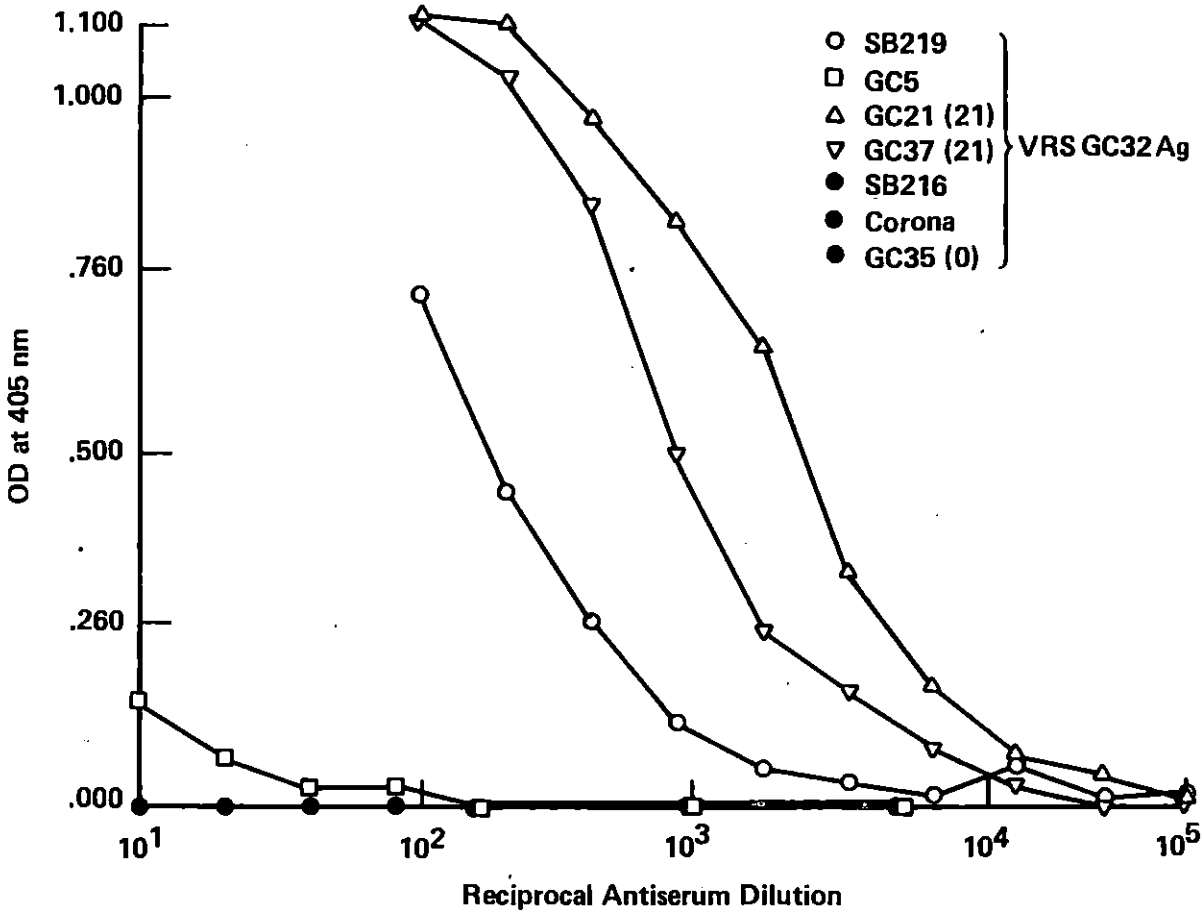

1.100
1.000
.760
.500
.260
.000
OD at 405 nm
10^1
10^2
10^3
10^4
10^5
Reciprocal Antiserum Dilution
SB219
GC5
GC21 (21)
GC37 (21)
SB216
Corona
GC35 (0)
VRS GC32 Ag

Table 16. Homologous and heterologous titers of experimental bovine antisera to GC2 ("Breda" virus 1) and to GC32 ("Breda" virus 2) antigens by the indirect ELISA method

Antiserum	Antigen	
	Breda virus I (GC2)	Breda virus 2 (GC32)
Breda virus I		
SB216	<10	<10
SB219	965 ± 232.3	170
Breda virus 2		
GC21(21)	230	2,200.0 ± 509.9
GC37(21)	120	617.5 ± 54.4
GC35(0)	<10	<10
Rotavirus		
GC5	<10	<10
Coronavirus		
Corona	<100	<100

Discussion

The ELISA confirmed the data obtained from the HAHI test that "Breda" virus isolates 1 and 2 are antigenically different and can be considered to be different serotypes. In contrast to the rotavirus results, sera obtained from animals convalescent to either "Breda" virus 1 or 2 show no cross HAHI reaction and possess significant differences by the ELISA. The homologous versus the heterologous differences were 5.7 and 5.1-9.5 with Breda 1 and 2 antigens respectively. The error of the tests was less than 2-fold. The ELISA also confirmed the IF test that Breda virus 1 and 2 share antigens. By ELISA as well as HAHI and IF, there was no antigenic sharing with bovine coronavirus when this antigen was obtained as semipurified virus from tissue culture source or from feces of an infected calf.

To improve the serotype differences, cross-absorption of the two antisera with the alternate virus should produce antibodies only reacting with serotype specific antigens. Alternatively, attempts to purify envelope antigens by rocket immunoelectrophoresis and then production of specific antibody to each of the precipitin arcs should produce serotype specific antibody.

It was fortunate that the calves did not produce antibodies that would react with tissue antigens nor with antigens of nutritional origin. This was shown by lack of cross-reaction between "Breda" virus antisera and coronavirus. It is not possible, particularly with the methods employed, to purify enveloped viruses free of cell antigens. This lack of foreign antigens other than virion antigens

must be due to the fact that cow's milk made up the dietary component and the virus replicated in bovine tissues. In contrast, in other studies (to be published), human sera were shown to react by this ELISA with purified fecal antigen obtained from the calf prior to inoculation with "Breda" virus and these sera also reacted with the "Breda" virus antigen. Also in other studies (to be published), this ELISA has been utilized for the detection of "Breda" virus antibodies in calf and cow sera. Over 90% of these sera reacted, but there was little reaction with the control uninfected gnotobiotic fecal sample. Setting up controls for ELISA reactions for use with conventional sera require much careful thought and the use of uninfected material from the same source is probably the best negative control.

GENERAL DISCUSSION

Although enzyme-immunoassays offer a number of advantages including sensitivity, convenience and low cost, they were not suitable for rotavirus serotyping without previous heterotypic cross-absorption of typing antisera, which was not the case with "Breda" virus 1 and 2.

Concerning rotaviruses, Holmes (1983) reported on the probability of both serotype-specific and some shared antigenic determinants to be present in the major outer-shell glycoprotein (gp34) responsible, per se, for the best neutralizing response. This fact may explain, besides the cross-reaction in rotavirus neutralization test, the different degree of cross-reactivity obtained by the different ELISAs employed here since they measure, primarily, the common rotavirus antigens. Heterologous cross-absorption of the rotavirus antisera was necessary to remove the antibodies common to the antigens in order to produce serotype specific sera. If the results of this study are confirmed, there are differences amongst the rotaviruses in the degree of shared antigens and multiple cross-absorption may be necessary. In practice, this is too expensive and the more easily and cheaply performed neutralization test would remain the test of choice for serotyping.

Serotyping of "Breda" virus 1 and 2 by the ELISA remains promising. Although a difference of 10-fold between the homologous and the heterologous reactions was not obtained, the variability of

the test was low and the homologous reaction was always higher than the heterologous one. This ELISA measures both the common (as also detected by IF test) and the specific (as detected by HI test) antigens but the antibodies to the specific antigens are in higher concentration facilitating the serotyping by this enzyme-immunoassay. Specificity of the "Breda" antisera could be improved by cross-absorption which may result in antisera that only react with serotype specific antigens.

Parenteral immunization always has the risk of inducing antibodies to extraneous antigens unrelated to the infecting virus specific antigens. Convalescent rotavirus antisera usually do not show serotype specific responses and it was necessary to use hyperimmune guinea pig rotavirus antisera for rotavirus serotyping, which increased the possibility of obtaining non-specific reactions. However, the hyperimmune sera prepared in guinea pigs were produced by inoculation of a variety of rotaviruses all grown in the same tissue culture. Thus, the response of the guinea pigs was to rotavirus specific antigens, at least at a high dilution. The use of convalescent sera removes or reduces the risk of introducing non-viral antigens. There is no explanation why the convalescent sera carry serotype specific antibodies of low titer for rotavirus but high titer (when compared with antibodies to common antigens) for "Breda" virus. However, "early antibody" usually shows greater antigenic specificity so one can conclude that "Breda" virus immunology is closer to the expected than rotavirus immunology.

In conclusion, it may be said that the ELISA would appear to be best suited for the detection of the common rotavirus antigens (Yolken et al., 1978a) and for the detection of antibody to the common rotavirus antigens (Thouless et al., 1982) leaving the neutralization test for the serotyping of rotavirus isolates (Woode et al., 1976; Thouless et al., 1977; Gaul et al., 1982). In contrast, the ELISA would appear to be suitable for detection of common and serotype specific "Breda" virus antigens and for common and serotype specific antibodies.

SUMMARY

In this study, different ELISAs for rotavirus serotyping and one for "Breda" virus serotyping were examined.

Rotavirus serotyping was achieved by the indirect ELISA method with previous heterologous cross-absorption of the rotavirus antisera removing the antibodies common to the antigens and obtaining serotype specific antisera. This method is too expensive and the more easily and cheaply performed neutralization test remains the test of choice for rotavirus serotyping.

Serotyping of "Breda" virus 1 and 2 by the indirect ELISA test remains promising based on the low variability of the test and the constant higher reaction obtained with the homologous antigen-antibody system when compared with the heterologous one.

ELISA is suitable for the detection of common rotavirus antigens as well as antibodies, and for the detection of common and serotype specific "Breda" virus antigens and antibodies.

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