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An enzyme-linked immunosorbent assay was developed to measure specific antibody response in bovine sera to bovine adenovirus type 3 (BA3), an etiologic agent of respiratory disease causing economic losses annualy to the cattle industry. Observed endpoint titers were determined using the intersection point from optical density values of serially diluted sera with a positive-negative threshold. Regression equations were determined from standards with titers ranging from low to high and used to predict ELISA titers from a single-serum dilution. A near-linear relationship existed between the observed and predicted ELISA titers of 118 bovine sera (r=0.9261). Predicted ELISA titers were determined using the single-dilution method for another 76 bovine sera and the correlation between the ELISA titers and serum-virus neutralization titers for these sera indicated a strong linear trend (r=0.8172).

Both the ELISA and serum-virus neutralization titers on the bovine sera tested indicated widespread exposure to several types of bovine adenovirus. Although detection of active infection would still require examination of sera

over time for evidence of a rising titer, the single-dilution ELISA devised should provide a rapid and sensitive method for detection of antibody response to bovine adenovirus type 3.

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE SEROLOGIC DIAGNOSIS OF BOVINE ADENOVIRUS TYPE 3

by

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INTRODUCTION

Bovine respiratory disease (BRD) causes heavy annual losses to the cattle industry worldwide, affecting cattle of all types and ages, tending to recur, and responding poorly to treatment[88]. The most severe respiratory disease is seen in calves under intense systems of management, with the resulting interaction between pathogenic organisms and environmental stress[101]. Multiple etiologic agents have been implicated in BRD, including bovine adenoviruses (BAs), parainfluenza type 3 virus, bovine respiratory syncytial virus, infectious bovine rhinotracheitis virus, coronaviruses, mycoplasma, chlamydia, Pasteurella hemolytica, and Pasteurella multocida. Pneumonia, either chronic or acute, is the most frequent respiratory infection in calves[123]. Since most pneumonias experimentally induced with a single pathogen are mild, the severity of natural outbreaks most probably results from a combination of two or more etiological agents[57].

Vaccines have been developed for some of the most important causes of BRD. However, BAs remain among the viruses for which vaccines have not been produced in the United States. These viruses have been shown to cause

severe economic losses in calf fattening lots[2,8,28,79,116]. Experimental isolations and seroepizootiological studies indicate that bovine adenovirus infection is widespread in the United States[15,71,79], England[66], Europe[8,85,86], Japan[119], and Australia[23,24,126]. Bovine adenoviruses can cause pneumonia, pneumoenteritis, enteritis, conjunctivitis, and kerato-conjunctivitis[64,79] and have been implicated in weak calf syndrome[26,31]. Strains vary in their pathogenicity, and infections may be inapparent or linked to coinfection with another virus or bacterium that is primarily responsible for pathogenicity[64,81]. The multifactorial nature of respiratory tract infections and the ability of adenoviruses to produce latent infections increase the difficulty in assigning an exact etiological role to adenoviruses isolated from animals with respiratory disease, unless accompanied by evidence of seroconversion[64]. However, the frequency of inapparent infections and the difficulty in isolating some of the many bovine adenovirus strains may have led to a previous underestimation of the significance of these viruses as etiologic agents of BRD[64,107]. Generally, mortality from bovine adenovirus infection is low; however, the morbidity may be very high, and survivors may remain unthrifty, adding significantly to economic losses[2].

Bovine adenovirus type 3 is a member of subgroup 1 and serological studies have indicated widespread exposure to this type in the United States[71,79]. This BA type has been

shown to be an etiologic agent of enzootic pneumonia in calves in Europe[116] and Oregon[79]. Comparative calf inoculation studies indicate that BA3 produces more extensive pathology than BA1 or BA2, and may be the most virulent of the subgroup 1 viruses[30,80]. Therefore BA3 was selected as the prototype virus for this investigation.

There are two distinct subgroups of BAs, consisting of nine distinct types. The subgroups differ antigenically and also in ease of propagation in cell culture. The detection of BA infection is complicated by this lack of a group-specific antigen between the two subgroups, as well as the difficulty in propagating some of the subgroup 2 isolates in cell culture. The commonly used serum-virus neutralization (SN) test is specific to a single type, requires good replication and CPE production in cell cultures, and is both time and labor intensive. Immunofluorescence tests and immunodiffusion tests, although less time and labor intensive, are subject to cross-reactivity between some strains, even between subgroup 1 BAs and human, ovine, or porcine adenoviruses[1,32,85].

The difficulty in determining the etiology of BRD would be reduced if the diagnosis of BA infection did not depend either on the type-specific and time-consuming SN test or a lengthy period needed to isolate the viruses, especially the subgroup 2 viruses. The purpose of this study was to develop an enzyme-linked immunosorbent assay (ELISA) for rapid detection of a serologic response to BA3 and possibly

exposure to other subgroup 1 viruses. Because the two BA subgroups possess different major antigens, the cross reaction of sera in an ELISA test procedure with a subgroup 2 strain was also investigated. The subgroup 2 viruses were represented by bovine adenovirus type 4 (BA4). Development of a rapid and sensitive test specific for viruses in each BA subgroup would greatly improve the ability to detect the level of response to BAs in disease outbreaks.

LITERATURE REVIEW

HISTORICAL BACKGROUND:

In 1953, Rowe and collegues[108] discovered a cytopathogenic agent in cell cultures of human tonsils and adenoidal tissue. A short time later, Hilleman and Werner[49] isolated similar cytopathogenic agents from army recruits suffering from an influenza-like epidemic. In retrospect, it appears that acute respiratory disease and epidemic keratoconjunctivitis were caused by adenoviruses for at least 30-100 years before their isolation and identification as the causative agents[53]. Early names for these agents reflected their pathogenicity - 'adenoid degeneration' (AD), 'acute respiratory disease' (ARD), and 'adenoid-pharyngeal conjunctival' (APC) agents[53]. The origin from adenoid tissue was retained in the group name, adenoviruses, proposed in 1956 by Enders and colleagues[33].

HOST RANGE:

Adenoviruses are ubiquitious[44], and the number of types described is increasing. Adenoviruses were first isolated from human adenoid tissue[108] but have since been isolated from many animal species, including cattle, sheep, horses, llamas, pigs, monkeys, dogs, birds, mice and reptiles[5,37,43,62,80,93]. At present there are 41 human types[110], 9 bovine types[12], 6 ovine types[112], 4 porcine, 1 equine, 1 caprine, 1 murine, 14 avian types[92], and many

others described but not as yet accepted.

Adenovirus infections are primarily restricted to the normal host species; however, lambs have succumbed to some bovine adenoviruses, foxes and bears have died from canine adenovirus infections and several bird species can be infected by fowl adenovirus 1 and egg-drop syndrome virus[124]. Asymptomatic infection across species barriers has also been detected by the presence of specific antibody[53]. Serum-virus neutralization tests of human sera has revealed the presence of antibodies to simian, bovine and canine adenoviruses[53]; furthermore, antibodies to human adenovirus 12 have occasionally been detected in simian sera[65].

CHARACTERISTICS OF ADENOVIRUSES:

Physical and Chemical Properties:

Adenoviruses are simple viruses, consisting of 13% deoxyribonucleic acid (DNA) and 87% protein[46]. They are nonenveloped, icosahedral viruses that are 60 to 90 nm in diameter[35,40,42,51]. The protein coat is made up of 252 hollow, cylindrical capsomeres: of these 240 are "hexons", surrounded by 6 identical neighboring structures and 12 are "pentons" at each vertex[41]. Projecting from the penton base is a fiber, 10-37 nm long (depending on the species or subgroup), with a terminal knob, approximately 4 nm in

diameter[51,95]. Four minor proteins are associated with the hexons or pentons and serve to stabilize the capsid, act in virion assembly, and form links with the core proteins[40]. The density of adenoviruses in cesium chloride is 1.328-1.340 g/cm^3 for mammalian adenoviruses and 1.32 - 1.35 g/cm^3 for avian adenoviruses[124].

Adenoviruses lack lipid membranes and thus are stabile to solvents such as ether, ethanol, and chloroform[35,46]. Mammalian adenoviruses are rapidly heat inactivated at 56 C; in contrast, avian adenoviruses are stable at 56 C[124]. The greatest stability is provided in isotonic saline at a pH range of 6 to 9[124].

Adenoviruses contain a single molecule of linear double-stranded DNA, with a molecular weight of 20-25 million daltons, depending on the serotype[47]. The base composition of adenoviruses is one criterion used for classifying human adenoviruses, and varies from 47 - 61% G+C content[102]. Hybridization studies of DNA-DNA and DNA-mRNA have shown that the human adenoviruses within a subgroup share 70 - 95% of their nucleotide sequences, while the different subgroup viruses only have 5 - 20% homology[40].

Replicative Cycle:

Replication of adenoviruses begins with a slow adsorption to cells, followed by a relatively long growth cycle (30-40 hrs) and a slow release into the medium[125]. Adsorption of virus to cells in culture takes several hours

and probably begins when the fiber of an infective adenovirus particle attaches to a specific receptor on the host-cell membrane[75]. The viral particle penetrates the cell and uncoating of the viral DNA begins immediately in the cytoplasmic matrix[40,91]. The capsid stability is reduced by displacement of pentons, the hexons and associated proteins separate, and the naked viral core enters the nucleus, where viral replication occurs[21,75,104]. Two to three hours after infection, early mRNA is produced and translated into early proteins, required for replication of viral DNA[45]. Replication of viral DNA is initiated at either end of the molecule and proceeds via strand displacement[124]. The maximum rate of viral DNA replication occurs 18-20 hours after infection and has almost ceased after 24 hours[40]. At approximately 20 hours post infection, late viral polypeptides are synthesized at their maximum rate; these proteins are virion structural proteins or their precursors[52].

Viral proteins are synthesized on polyribosomes in the host-cell cytoplasm and then transported into the nucleus, where they assemble in several steps, beginning with formation of a procapsid[40,76]. Assembly may be aided by several polypeptides that function as "scaffold proteins" necessary for formation of the capsid but absent from the final structure[74]. Viral DNA is postulated to enter the empty capsid through openings at the vertices, since only small amounts of either penton base or fiber polypeptides

are present until late in assembly[61]. The viral particle is completely assembled after precursor or "scaffold proteins" are cleaved off, or degraded; the configuration tightens, and the particle becomes impervious to nucleases[52].

The biochemistry of adenovirus lytic infection has been much studied in human adenovirus (e.g. type 2) grown in HeLa or KB cell cultures. Adenoviruses are closely cell-associated even after production of new virus is completed[52]. Therefore, concentration of large volumes of virus is possible by low speed centrifugation to sediment cells from a culture suspension[46,52].

Effect on Host Cells:

Synthesis of host cell DNA, RNA and protein is inhibited by infection with most adenovirus serotypes[50,124]. Host cell DNA synthesis gradually shuts down as viral DNA synthesis begins 6 - 9 hours after infection[54]. Synthesis of host cell ribosomes is also severely inhibited by adenovirus infection[103].

The accumulation of unassembled viral components produces characteristic nuclear lesions in adenovirus-infected cells[16,90]. Assembly of adenovirus components is very inefficient, as only 10-15% of the newly synthesized viral DNA and protein becomes incorporated into virions[40]. The epithelial cells are characteristically the site for replication of adenoviruses both in cell culture and <u>in vivo</u>[16,38]. Affected cells will have enlarged nuclei and

characteristic inclusions containing the excess viral protein and DNA, as well as intact virions[77]. The viral particles of some types will form a crystalline lattice in large basophilic inclusion bodies[16,90]. The human subgroup C adenoviruses produce bar-shaped eosinophilic crystals in host cells from the arginine-rich internal viral proteins[40].

The cytopathic effect seen in cultured cells involves the cells rounding, clustering, and becoming refractile with formation of intranuclear inclusions[49,124]. Cells infected with adenovirus increase their glycolysis, thus producing large amounts of acid as the glycolytic byproducts accumulate[36]. Experiments with purified pentons have shown they are directly toxic to cells, causing them to round and detach from the glass surface[99,121]. Horwitz[53] suggests from this evidence, and the demonstration of penton in circulating blood of patients with fatal cases of adenoviral pneumonia[70] that the penton may also be a rare viral toxin in human disease.

Antigenicity:

The hexon, penton, and fiber all have antigenic determinants that can be used in classification of adenoviruses[52,109]. The hexons contain determinants that will cross-react with similar antigens in all Mastadenoviruses[20,39], except the subgroup 2 bovine adenoviruses[7,124]. Complement fixation tests to a hexon genus-reactive antigen, found on the inner surface of assembled

virions[40], can be used to classify adenoviruses at the genus level[124]. Hexons also induce neutralizing antibodies[122] to a type-specific epitope, which is the predominant antigen exposed in assembled virions[40,94]. Classification of adenoviruses as to species depends primarily on the hexon species-specific reactivity determined by neutralization and secondarily on fiber species-specific reactivity determined by hemagglutination inhibition[124].

The fiber serves in attachment to host cells[40], and may contain a neutralizable determinant, similar to the surface of the hexon[122]; however, the greatest neutralizing activity results from anti-hexon sera[122] and antibodies to the fiber reduce viral infectivity only slightly[40]. The hemagglutination patterns of rat or monkey erythrocytes, by a determinant of the fiber polypeptide, has long been used in classification of human adenoviruses[106]. Most mammalian adenoviruses and a few avian adenoviruses are able to hemagglutinate susceptible red blood cells[124].

The penton-base polypeptide provides a soluble antigen[52,98] that is common to all adenoviruses and causes cytopathic effects in cell cultures. The pentons also provide minor antigens of the virions, as well as the family-reactive soluble antigen[40].

Oncogenicity:

In 1962 two human adenovirus types were shown to possess the ability to induce tumors in newborn

rodents[55,56,120]. Much research into the molecular biology of adenoviruses and their role in the etiology of human cancers was initiated because of this oncogenic potential and the ability of adenoviruses to produce latent infections. Animal, as well as human, adenoviruses have since been shown to have the potential to induce tumors in rodents and transform cells in culture systems. Martineq-Palomo and coworkers[77] determined that the DNA of the highly oncogenic human adenovirus serotypes differed considerably from the DNA of less oncogenic or non-oncogenic types. Studies with simian adenovirus type 7 (SA7) have shown that only part of the viral DNA is responsible for tumor induction[84], and that chemical carcinogens enhance SA7 transformation[19]. Bovine adenovirus type 3 has been shown to induce tumors in hamsters, and transform mouse cells in culture[58]. The transforming region of the DNA has been mapped[59]. Although the extensive research into adenoviral oncogenicity has increased the understanding of the molecular level of cell transformation, there is no evidence that human adenoviruses play a significant role in the etiology of human cancers[44].

CLASSIFICATION OF THE FAMILY ADENOVIRIDAE:

The International Committee on Taxonomy of Viruses (ICTV) provisionally accepted species terminology for the

virus family Adenoviridae in 1981[124]. The family Adenoviridae is divided into two genera on the basis of immunological and morphological differences in virions of the two groups: the Aviadenovirus species infect birds and the Mastadenovirus species infect mammals[96]. The two genera differ both morphologically and in the presence or absence of certain polypeptides[124]. The human viruses of the genus Mastadenovirus have been further classified into subgenera, on the basis of length of fibers, hemagglutination ability, molecular weight of internal polypeptides, G+C content, and DNA homology[124]. The adenovirus species is defined by the ICTV on the basis of immunological distinctiveness, determined by quantitative neutralization with animal antisera[124]. The ICTV system for naming adenovirus species involves a letter code to indicate the host genus (h for human, bos for bovine, ovi for ovine, etc.) and a sequential number code within each host grouping[124]. The complexity of host classification has complicated the naming of simian adenoviruses so there are no approved species terms for serotypes isolated from non-human primates[92].

CLINICAL SYNDROMES ASSOCIATED WITH HUMAN ADENOVIRUS INFECTION:

There are now 41 recognized human adenovirus types, but disease is primarily associated with only one third of these types[53]. Adenoviruses replicate and cause pathology

in the respiratory and gastrointestinal tracts, the eye, urinary bladder, and occasionally the central nervous system[22,53,100]. Human disease syndromes associated with adenoviruses have now come to include meningo-encephalitis[22], acute hemorrhagic cystitis[97], and a pertussis-like syndrome[117], as well as the more commonly seen pneumonia and conjunctivitis. The same types that produce only mild respiratory infection in the normal population can cause more severe, even fatal respiratory or diarrheal illness in immunocompromised patients[63,114,127].

DETECTION METHODS FOR HUMAN ADENOVIRUSES:

The many human syndromes associated with adenoviruses are detected by a variety of methods. The complement fixation antibody response, fluorescent antibody test, hemagglutination inhibition and serum neutralization tests are all used for serological diagnosis in human medicine[53]. Specific cytologic changes occur during adenovirus infection. Therefore, Bayon and Drut[11] used cytologic study of tracheal aspirates to diagnose adenovirus broncho-pneumonia. They concluded that a differential diagnosis of adenovirus inclusions from other viral inclusions was possible. Isolation of adenoviruses in cell culture is also an accepted diagnostic method but is not always feasible, especially for fastidious enteric serotypes[110]. Furthermore, isolation may not indicate an active infection, since latent adenoviruses can be isolated from apparently normal tissue[4].

More recent techniques have also been applied to diagnosing adenovirus infections. Killough and coworkers[67] investigated a monoclonal antibody based radio-immune dot-blot technique for diagnosis of ocular adenovirus infections. Monoclonal antibodies were also used in enzyme-linked immunosorbent assay (ELISA) to detect adenovirus antigen in stool samples[48,110]. Allard et al.[3] used polymerase chain reaction (PCR) for detection of adenovirus in stool samples.

BOVINE ADENOVIRUSES:

Classification of Bovine Adenoviruses:

Bovine adenoviruses have been isolated worldwide from apparently normal cattle as well as those suffering from respiratory or enteric diseases[88]. As new isolates arise, they are classified on the basis of serological distinctiveness into subgroups, as proposed by Bartha[7]. The subgroup 1 bovine viruses possess the soluble subgroup-specific antigen common to those of other mammalian types, and thus will cross react with other mastadenoviruses in complement fixation (CF) and agar gel immunodiffusion tests[7,14,18]. Subgroup 1 viruses also replicate in bovine kidney continuous or primary cell lines and produce a single nuclear inclusion body, irregular in shape[7,13]. Subgroup 2 viruses lack the soluble antigen; do not cross react with other mammalian adenovirus in CF tests; do not grow well, if

at all, in bovine kidney cell lines but can be propagated in primary or low passage cultures of calf testicular or thyroid cells; may require several passages for isolation; and form multiple regular intranuclear inclusion bodies[7,13,111]. The members of these two subgroups also differ in their virulence and methods of detection[80].

DNA restriction enzyme analysis confirmed the classification of BAV-1,2,3 and 9 into subgroup 1 and BAV-4,5,6,7, and 8 into subgroup 2[13]. This study showed a significant difference in genome size between the members of subgroup 1 and 2, as well as a difference in the number of nucleotide sequences in the two subgroup members that were recognized using a specific restriction endonuclease (Eco-R1). Benko and coworkers[14] also used Southern blot hybridizations to compare homology of DNA sequences between BA subgroup members and representative human or porcine adenoviruses. From several hybridization experiments, they were able to estimate a genetic relatedness to human adenovirus 2 (HAV-2) with BAV-9 the most similar followed by BAV-3, PAV-3, BAV-2, and BAV-1 the most different. This study would indicate that strains isolated from the same host species may be genetically more distantly related to each other than they are to some strains of different host origin. This evidence, together with the lack of homology between DNA of subgroup 2 viruses to other mammalian adenoviruses, confirms the distinctiveness of subgroup 2 BAVs.

Diseases Associated with Bovine Adenoviruses:

Bovine adenoviruses were first isolated in the United States from the feces of an apparently healthy cow[68]. This strain (#10) was the prototype for BA type 1. It has since been shown to induce mild respiratory and enteric disease signs in calves inoculated intranasally or intratracheally with this virus[89]. Klein and coworkers[69] also isolated a second BA type from the feces of an apparantly normal calf in 1960. This virus represents BA2 and has been demonstrated to cause mild respiratory tract illnes in an inoculation study on colostrum-deprived calves[30]. BA2 has also been associated with natural cases of pneumonia[27] and associated with a recurrent chronic respiratory disease problem in a beef cattle herd[88].

Bovine adenovirus type 9 was isolated in Hungary and has not been available for study in the United States. Although grouped into subgroup 1 by some methods, there is doubt as to the exact origin of this type, a similar strain being initially isolated from swine[80]. Therefore, the exact placement of type 9 bovine adenoviruses will have to wait for accessibility to further comparative studies.

Bovine adenovirus type 3 was also first isolated from an apparently healthy cow, but this time from the conjunctiva[28]. This type was later isolated from conjunctiva, nasal cavity, and tonsillar fossa of newborn calves in a large beef cattle herd showing naturally occuring pneumoenteritis[79], and from feedlot cattle with acute respiratory

tract disease[73,78]. In calf inoculation studies pyrexia, respiratory distress, and nasal and conjunctival discharge were produced in colostrum-deprived calves[29]. Five of the 8 calves inoculated intranasally and intratracheally also developed diarrhea, one calf was dyspnoic, and two calves died. Seroepizootiologic studies indicate that 70-98% of adult cattle possess serum neutralizing antibodies to BA3[71,80]. Of the subgroup 1 bovine adenoviruses, BA3 seems to be the most significant pathogen.

Subgroup 2 adenoviruses, BA4 and BA5, were first isolated in Hungary from calves with enteritis and pneumo-enteritis[9]. These viruses could only be replicated in primary calf testicular cell cultures and required several passages before producing cytopathic effects[6]. Bovine adenovirus type 4 has also been isolated in Japan[83,118], Australia[126], and Oregon[82]. In experimental inoculation studies, mild respiratory tract illness, characterized by nasal discharge, coughing, pyrexia, and respiratory distress, is induced[87]. Although enteric disease is produced naturally, it was not induced experimentally, even though virus was isolated from the intestinal tract[87]. Subgroup 2 viruses (types 5 and 7) may also be one cause of a neonatal-disease syndrome termed "weak calf syndrome"[25,26,31,115]. Serologic studies of calves have shown widespread exposure to subgroup 2 viruses also: 76% of 200 calves from a normal slaughterhouse in Austria showed serum neutralizing antibodies to BA4[17].

Bovine adenoviruses from types 6, 7, and 8 are all included in the subgroup 2 classification of bovine adenoviruses. Bovine adenovirus type 6 was isolated from calf testicular cell cultures, as a latent virus[105]. Calf inoculation studies with BA6 produced infection associated with viremia, mild respiratory disease, and pathologic changes in respiratory and enteric tracts[88]. The Fukuroi strain adenovirus, prototype of BAV type 7, was isolated from blood samples of a cow with respiratory distress, pyrexia, anorexia, and diarrhea[60]. Type 7 bovine adenovirus has also been isolated from neonatal calves with "weak calf syndrome"[87]. Bovine adenovirus type 8 was first isolated by Bartha et al.[10] and, with types 4 and 5, is believed to be the major cause of pneumoenteritis in calves in Hungary[88], although experimental inoculation failed to induce either enteric or respiratory disease[87].

MATERIALS AND METHODS

CELL CULTURES:

An embryonic bovine kidney cell line (K699) maintained in the Oregon State University Veterinary Diagnostic Laboratory, Virology Section, was used to propagate stocks of bovine adenovirus, type 3 (BA3) and to perform SN assays. The bovine spleen cell line (Sp699) was used to perform SN assays with bovine adenovirus types 1,2, and 4. Stock BA4 was propagated in primary or secondary cultures of bovine testiclular cells. Cells were propagated in Minimum Essential Medium (MEM) with Earles salts, supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc, Logan, UT), 0.5% lactalbumin hydrolysate, 0.11% sodium pyruvate, and 0.15% sodium bicarbonate. The medium was also supplemented with penicillin (100 units per ml) and streptomycin sulfate (100 ug per ml). The cells were grown to a confluent monolayer in 150 sq. cm., plastic, cell culture flasks (Corning Laboratory Sciences Co., Corning, NY) at 37 C. The cell cultures were determined to be free of contaminating bovine viral diarrhea virus using direct immunofluorescence.

VIRUS PROPAGATION:

The BA3 virus (subgroup 1 prototype virus) selected for this study was strain 5C, isolated from the conjunctiva of a 10 day old calf with diarrhea and excessive lacrimal

discharge[79]. The subgroup 2 BA selected was BA4 strain 7T, which was isolated from a week old calf with pneumo-enteritis[82]. The other viruses used for SN assays were Strain 10, for BA1 and Strain 19, for BA2. The BA3 5C antigen was propagated in fetal bovine kidney cells (K699) while BA4 was propagated in primary bovine testicular cell cultures. For preparation of virus pools, the stock virus was adsorbed onto the cell monolayer for 6-8 hours at 37 C. After adsorption, 75 ml MEM supplemented with 5% horse serum (Hyclone) was added to the flask. The flasks were incubated at 37 C and examined daily for the appearance of cytopathic effect (CPE). When cells showed CPE over approximately 25% of the cell sheet, the medium was decanted, cells were rinsed once with 10-15 ml of serum-free MEM, and 15 ml of serum-free MEM was added to each flask. Approximately 24 hours after 100% CPE was reached, the virus was harvested (usually 6 - 10 days). The cell monolayer was scraped from the flask surface and titurated into an even suspension. The cell suspension was then either subjected to two freeze-thaw cycles at -20 C to release virus, clarified by centrifugation and frozen in aliquots for SN tests; or purified for ELISA antigen.

ANTIGEN PREPARATION:

The virus suspension was centrifuged at 1800 x g for 20 min and the supernatant fluid was discarded. The pellet was resuspended in calcium-magnesium-free, phosphate buffered

saline (CMF-PBS, pH 7.2) and centrifuged again at 1800 x g for 20 minutes. The supernatant fluid was discarded, the pellet resuspended in a 10mM Tris, 1mM EDTA buffer (TE buffer, pH 7.6) to approximately one tenth of the original volume and frozen at -20 C.

The concentrated supernatant from several antigen preparations was thawed, pooled, then sonicated for 1-2 min, at 4 C, using a microtip (Heat System Ultrasonics, Inc.) at output level No. 5. The virus suspension was extracted with an equal volume of 1,1,2-trichloro-1,2,2-trifluorethane[72] (Uvasol, EM Laboratories, Inc. Elmsford, NY), and stored at -20 C. Cells were also propagated under the same conditions, centrifuged, concentrated and Uvasol extracted to serve as cell antigen control (CAG) in ELISA testing.

VIRUS PURIFICATION:

A discontinous cesium chloride (CsCl) gradient was prepared by layering 6 ml of 1.2 g/cc CsCl over 8 ml of 1.4 g/cc CsCl in 29x89 mm Ultra-Clear centrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA). Both CsCl dilutions were prepared in TE buffer. Approximately 25 ml of the virus suspension was carefully layered on the CsCl gradient and centrifuged at 25K for 90-120 minutes at 4 C, using an SW28 swinging bucket rotor. The resulting band was removed by puncturing the side of the tube with a needle and syringe and withdrawing the light scattering band. The refractive index of the suspension was measured and determined to be

consistent with the density expected for bovine adenoviruses (1.34 -1.35 g/cc).

The virus suspension was dialyzed for 24 hours at 4 C with 2 changes of TE buffer, in dialysis tubing with a molecular weight cutoff of 12-14,000 (Spectrum Medical Industries Inc., Los Angeles, CA). Protein concentration was determined using the Coomassie Blue dye-binding assay (Bio-Rad, Richmond, VA). The color reaction was assayed at a wavelength of 600 nm. A total protein standard (Sigma Diagnostics, St. Louis, MO) and known-positive bovine gamma-globulin were included to ensure consistency between assays.

SERA TESTED:

Sera tested in the study included 128 routine, clinical specimens submitted to the OSU Veterinary Diagnostic Laboratory for respiratory disease screening and samples collected from 18 adult cows from a small, local dairy. Serum was also obtained from five calves which were inoculated with an experimental vaccine for BA3 strain 5C, supplied by Dr. Lucy Chang of Fort Dodge Laboratories. These calves were sampled prior to vaccination and at weekly intervals from prechallenge to 28 days post inoculation. Sources of other sera tested included 23 calves experimentally inoculated with different adenovirus strains in previous research. Each sample was divided, one part being heat-inactivated at 56 C for 30 min for use in the SN assay, and the other part for ELISA assay.

SERUM-VIRUS NEUTRALIZATION:

The virus titer was determined by endpoint dilution assay and the median tissue culture infectious dose ($TCID_{50}$) was calculated using the Reed-Muench method. Diluent for the test was MEM, supplemented with gentamycin, fungizone, and 10% FBS. One hundred median tissue culture infectious doses of virus were added to serial two-fold dilutions of test sera in 96-well microtiter plates. After a 60 min incubation at room temperature, approximatly $1x10^6$ K699 cells per milliliter were added to all wells. Sterile mineral oil (100-150 ul) was added to each well to prevent dehydration, and the plates were incubated 10-14 days at 37 C with a humidified atmosphere of 2.5% CO_2. Cultures were then examined for inhibition of CPE by test sera, and the serum end point was the last point in the dilution sequence which inhibited expression of CPE.

ELISA PROCEDURE:

Antigen Coating:

The basic ELISA procedure was based on the method of Engvall and Perlman**34**, as modified by Thiel and colleagues**119**, to detect antibodies to adenoviruses in human sera. The optimal antigen concentration was determined by performing two-fold serial dilutions beginning at protein concentrations of 100 ug per ml. The viral antigen and CAG

were adsorbed onto Falcon Pro-Bind immunoassay plates (Becton Dickinson Labware, Lincoln Park, NJ) in a pH 8.0 carbonate-bicarbonate buffer, in alternating vertical columns of wells. The plates were sealed with plastic film and the antigens allowed to adsorb at 4 C for 4-7 days before use. Plates were then washed 3-5 times in a 0.01 M phosphate buffered saline with 0.05% Tween 20 (Polyoxyethylene-sorbitan monolaurate, Sigma) (PBS-Tween) and dried by incubating 30 - 60 min at 37 C, then sealed with plastic film and stored at -20 C until required. Before use, the plates were washed once with PBS-Tween. Plates treated in this manner remained satisfactory for more than 6 months.

General ELISA Procedure:

For the general ELISA procedure, the sera were diluted in a Tris-EDTA-NaCl buffer (0.15M NaCl, 0.001M EDTA, 0.05M Tris base, pH 7.4) with 3% normal rabbit serum and 0.05% Tween 20 (TEN-TR). One hundred microliters of diluted serum was added to each test well. Plates were incubated for 1 hour at 37 C on a rotating platform, then washed 4 times in PBS-Tween. One hundred microliters of conjugate [affinity purified goat anti-bovine IgG (H+L)(Fab')2 fragment conjugated to horseradish peroxidase (Jackson Immuno-Research, Avondale, PA) diluted in TEN-TR] was added to each test well. Plates were placed on a rotating platform, and conjugate was allowed to adsorb for 30 min at 37 C. All

wells were then washed 4 times with PBS-Tween. Freshly prepared ABTS substrate [0.30mM 2,2'-azinobis (3-ethyl-benzthiazolinesulfonic Acid) and 2.2mM hydrogen peroxide in 0.01 M citrate buffer, pH 4.0] was added to the washed plate, 150 ul per well. The plate was rotated to mix the reagents. The optical density (OD) of the reaction was determined at 405 nm using a Dynatech MicroELISA Reader (Dynatech, Williamsburg, VA). All sample OD readings were determined when reference control serum (1:180 dilution) from a calf hyperimmunized to BA3 reached an OD value of 1.5. A substrate control, incubated with dilution buffer and substrate only, as well as a conjugate control, incubated sequentially with dilution buffer, conjugate and substrate were included, with the reference positive to standardize the plates, ensuring reproducibility of results.

Optimal Conjugate Concentration:

The optimal conjugate concentration was determined by performing the general ELISA procedure with known negative, high positive, and low positive sera, using dilutions of the conjugate ranging from 1:1000 to 1:10,000. The conjugate dilution selected showed an absence of reaction in diluent control wells, a color change visible in the low positive, wells, and a strong reaction in the high positive wells, suggesting a linear trend.

Determination of Positive-Negative Threshold:

A positive-negative threshold was determined by calculating the mean OD plus 3 standard deviation units of 10 sera, negative for BA3 antibodies by SN. The resulting single value was plotted as a horizontal line on log-log graph paper.

Determination of Observed ELISA Titers:

Each test serum was diluted in TEN-TR, beginning with 1:20, and then in three-fold dilutions to 1:14,580, in Falcon U-bottom microtiter plates. These dilutions were transferred to the washed ELISA plate using a 12 channel pipettor. The general ELISA procedure was followed, and the corrected absorbence value was obtained by subtracting the OD of the CAG from the OD of the same test sample with viral antigen. The corrected absorbence values for the serial dilutions of each test serum was plotted and the point of intersection with the horizontal positive-negative threshold was taken as the endpoint dilution of the test serum[113]. This value was referred to as the "observed" ELISA titer. The linear trend was evaluated by regression analysis of "observed" ELISA to SN values and the correlation coefficient (r) was determined.

Determination of Predicted ELISA Titers:

Four serums were chosen as low, medium-low, medium-high, and high ELISA standards to make standard curves. Using a

modification of the double-regression method described by Snyder and coworkers[113] (1982), a regression analysis was performed between the optical densities of the 4 standards and their previously determined "observed" ELISA titers, at each dilution. Using these regression equations, a predicted titer was calculated for each test serum. Another regression was performed to compare the observed titer, to the predicted value at each dilution. From these comparisons, the single dilution that provided the best fit of observed to predicted titers was determined.

The Single Dilution ELISA Procedure:

For single dilution ELISA testing, the general ELISA procedure was followed, using the one dilution calculated to give the best correlation between observed and predicted titers. Using this single dilution, instead of serial three fold dilutions, the predicted ELISA titers for 76 previously untested calf sera were obtained. A regression analysis was performed comparing the single dilution titers, calculated from the regression equation, with the SN titer for each serum.

RESULTS

SERUM-VIRUS NEUTRALIZATION:

The SN test results indicated widespread antibody response to bovine adenoviruses among the populations sampled. Only fetal bovine sera or sera from very young calves showed no antibody response to at least one type, and most animals showed response to several types. Calves which were experimentally vaccinated with BA3 showed a four-fold or greater increase in titer by 14 days post-vaccination, and this increase persisted or dropped only slightly during the 28 day test period (Table 1). These calves had either negative or very low titers to BA1 and low to moderate titers to BA2 and BA4, that generally did not vary by more than one dilution step during the test period.

ELISA TITRATION RESULTS:

The general ELISA procedure was optimized using a viral and CAG concentration of 12.5 ug protein per well, a conjugate concentration of 1:5000, and an excess of substrate (150 ul per well). The optical density of negative sera was similar to controls with no serum; high positives had values of 1.0 to 2.0 by 15 min and low positives had values of 0.5 to 1.0 by 15 min (Table 2). When assays were standardized with the reference positive, a few positive samples had OD values greater than 2, but the majority had mid range values.

Table 1: Serum-Virus Neutralization Titers of Five Calves Vaccinated Against Bovine Adenovirus Type 3

Sample*	BA1	BA2	BA3	BA4
439-0	N	16	N	16
439-7	N	32	N	16
439-14	N	16	16	16
439-21	N	16	16	16
439-28	N	32	32	32
449-0	N	4	N	16
449-7	N	4	2	16
449-14	4	16	256	16
449-21	2	16	128	16
449-28	N	16	128	16
451-0	N	64	N	64
451-7	N	32	N	128
451-14	4	32	128	64
451-21	2	64	128	64
451-28	4	128	128	128
458-0	N	16	N	64
458-7	N	16	4	64
458-14	2	64	128	64
458-21	N	32	64	64
458-28	N	64	128	64
460-0	N	8	N	16
460-7	N	8	N	16
460-14	N	16	64	16
460-21	N	4	64	16
460-28	N	8	32	32

* Each calf was sampled at 0, 7, 14, 21, and 28 days post inoculation.

N = Negative at a 1:2 dilution.

Table 2: Determination of Optimal Antigen Concentration for Bovine Adenovirus Type 3 Enzyme-Linked Immunosorbent Assay.

Sample:		High Positive	Low Positive	Negative Control	Diluent Control
100 ug/ml	BA3	1.339	0.717	0.043	0.040
"	CAG	0.212	0.163	0.043	0.067
50 ug/ml	BA3	1.474	0.927	0.038	0.022
"	CAG	0.275	0.189	0.037	0.023
25 ug/ml	BA3	1.497	0.979	0.031	0.030
"	CAG	0.336	0.187	0.024	0.021
12.5ug/ml	BA3	1.590	1.108	0.065	0.021
"	CAG	0.252	0.141	0.042	0.015
6.25ug/ml	BA3	1.185	0.681	0.023	0.015
"	CAG	0.312	0.352	0.020	0.020
3.13ug/ml	BA3	0.811	0.335	0.018	0.020
"	CAG	0.125	0.132	0.036	0.022

BA3 = purified viral antigen
CAG = cell control antigen
Negative Control = Fetal bovine serum
Diluent Control = conjugate and substrate with no serum

POSITIVE-NEGATIVE THRESHOLD:

Of approximately 150 bovine sera tested for BA antibodies by SN, only fetal sera or sera from very young calves were negative for all BA types. Ten sera, determined to be negative at a 1:2 dilution for BA3 and negative or having only low titers to BA1, BA2, and BA4, were used for the positive-negative threshold. The OD readings for these 10 sera were determined at dilutions ranging from 1:60 to 1:14580 and the mean and standard deviation values determined (Table 3). This mean plus 3 standard deviations (0.062), was plotted and used to determine the observed ELISA titer for each serum (Fig. 1).

OBSERVED ELISA TITERS:

The OD values of 118 bovine sera were plotted on log-log graph paper and the observed titer determined as the intersection point with the positive-negative threshold. Fetal bovine sera, that were negative in SN tests to all bovine adenovirus types, showed only baseline reaction on ELISA, equivalent to control samples with no bovine serum. The linear trend was evaluated by regression analysis of observed ELISA titer to SN titers (Fig. 2). The correlation coefficient of 0.7204 indicated a moderate correlation between ELISA and SN titers.

PREDICTED ELISA TITERS:

Four sera, with levels of antibody ranging from low to

Table 3: ELISA Absorbance Values for Serial Dilutions of Ten Bovine Sera Negative for Bovine Adenovirus Type 3

Sample	1:60	1:180	1:540	1:1620	1:4860	1:14580
#45	0.075	0.050	0.018	0.012	0.003	0
#53	0	0.003	0.005	0	0.001	0.003
#5-85	0.022	0.003	0.004	0.001	0.005	0
#163	0.031	0.016	0.002	0	0.001	0.004
#167	0.025	0.029	0.025	0.015	0.006	0.003
#168	0.037	0.012	0.007	0.003	0.007	0.009
#175	0.087	0.032	0.009	0.006	0	0.006
#183	0.008	0.009	0.001	0.004	0.010	0
K693	0.014	0.007	0	0.004	0.004	0.005
80174	0.014	0.005	0.004	0.004	0	0

Mean = 0.011 standard deviation = 0.017

Mean + 3 st. dev. = 0.062

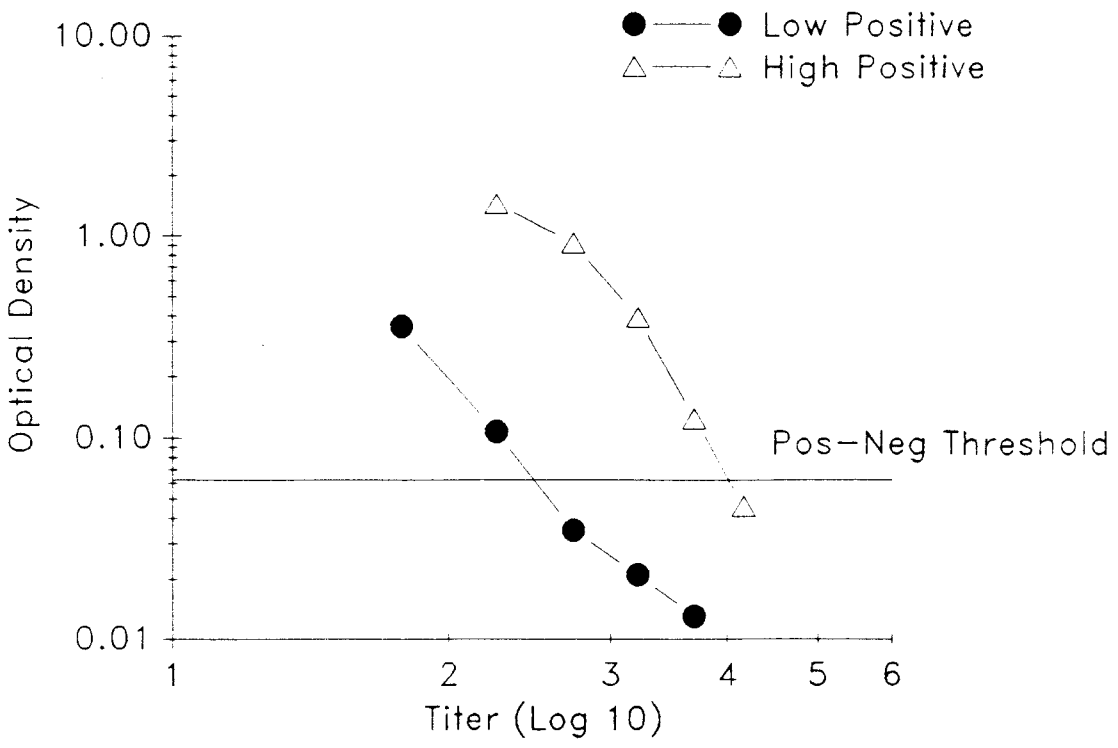

Fig. 1: Use of a positive-negative threshold for determination of ELISA antibody titer for bovine adenovirus type 3.

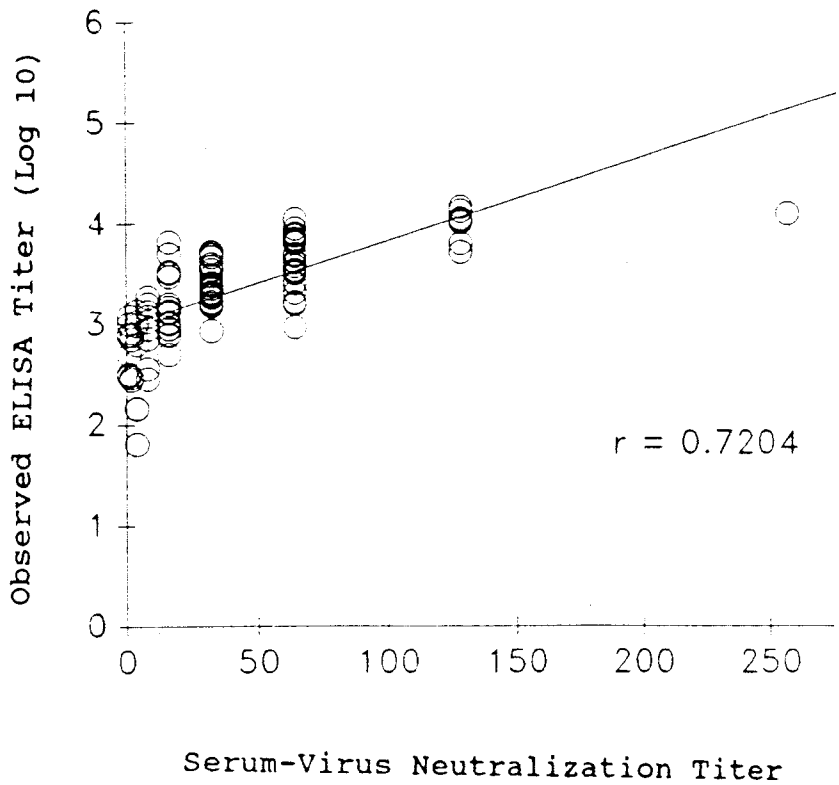

Fig. 2: Comparison of bovine adenovirus type 3 antibody levels of 118 bovine sera determined by ELISA and serum-virus neutralization assays.

high, were selected to make standard curves. The regression equations were determined for these standards at dilutions from 1:60 to 1:4860. Predicted ELISA titers were calculated using the regression equations for the 3 dilutions with the highest correlation coefficients (Fig. 3). These predicted values were compared, via regression analysis, to the observed titers for the same sera (Fig. 4). The 1:180 dilution had the best fit (Table 4), so this dilution and regression equation were used in the single dilution ELISA test.

SINGLE-DILUTION ELISA TEST:

A single dilution ELISA test for serologic detection of antibodies to BA3 was performed on previously untested serums, at the 1:180 dilution. The sera with highest SN titers had the highest ELISA titers, and the negative samples by SN had the lowest ELISA titers (Table 5). The linear trend indicated was evaluated by performing regression analysis between the single dilution ELISA titers and the SN titers. Figure 5 shows the scatter plot, regression line and r-value for the single dilution assay. There was a strong correlation between titers by the different methods, thus the single dilution method should provide a good screening test for exposure to BA3 antibodies.

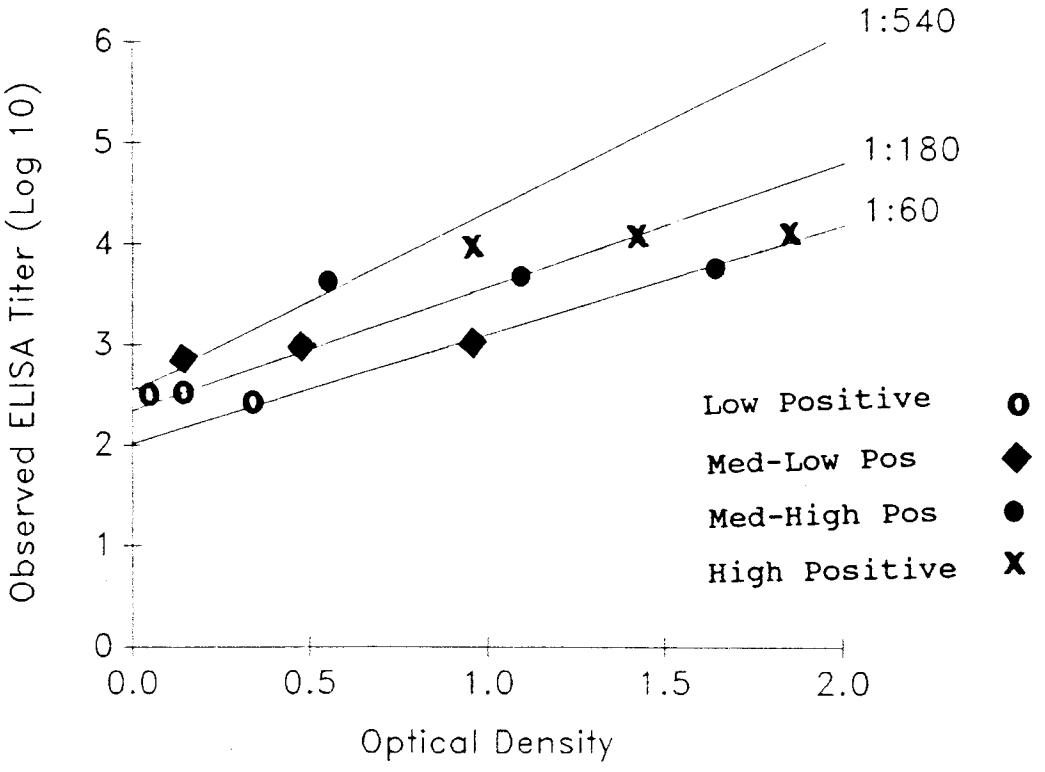

Fig. 3: Observed ELISA titers of four sera at different dilutions. This procedure was designed to determine the optimal dilution of sera for predicted ELISA titers.

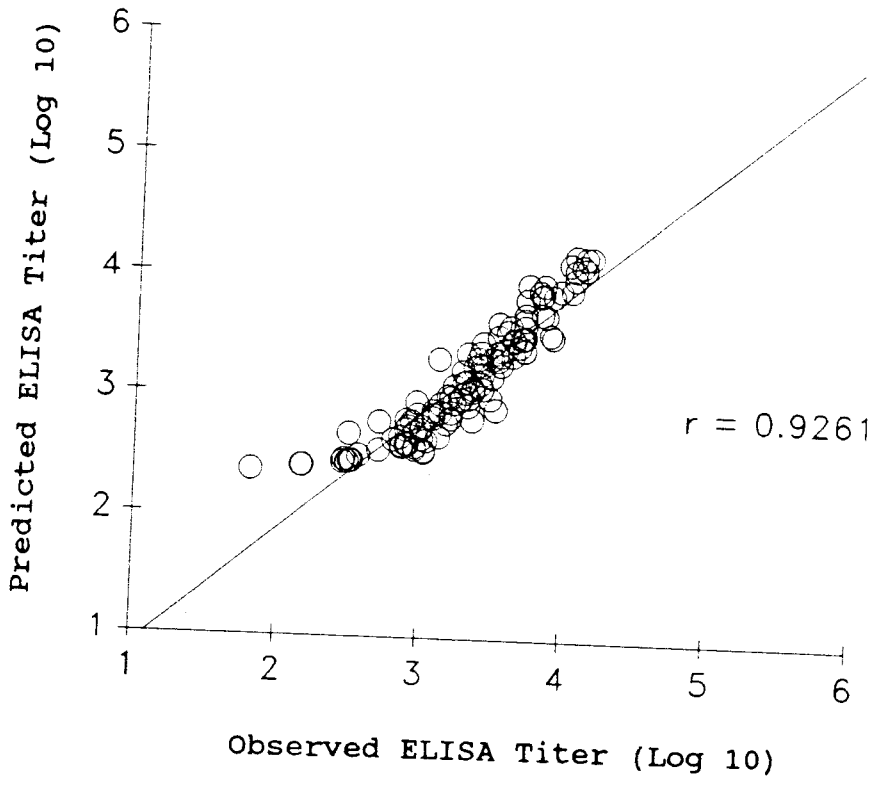

Fig. 4: Comparison of observed and predicted ELISA titers of bovine adenovirus type 3 antibody at a 1:180 dilution.

Table 4a: Absorbance Values of Four Bovine Sera used to Determine Standard Curves in the Bovine Adenovirus Type 3 (BA3) ELISA.

	Dilution				
Sample O.D. at:	1:60	1:180	1:540	1:1620	1:4860
Low Positive	.356	.107	.035	.021	.013
Medium-Low Positive	.949	.490	.189	.081	.029
Medium-High Positive	1.658	1.091	.541	.187	.064
High Positive	1.788	1.439	.943	.543	.205

Table 4b: Regression Equations and Correlation Coefficients of BA3 ELISA Standard Curves at Different Dilutions.

Regression Equation and Correlation Coefficient (r):		
1:60 Dilution	y = 1.0886x + 2.012	r = 0.9862
1:180 Dilution	y = 1.2317x + 2.3421	r = 0.9998
1:540 Dilution	y = 1.7742x + 2.5474	r = 0.9750
1:1620 Dilution	y = 2.8081x + 2.7209	r = 0.89251
1:4860 Dilution	y = 7.2880x + 2.7384	r = 0.86716

O.D. = Optical Density

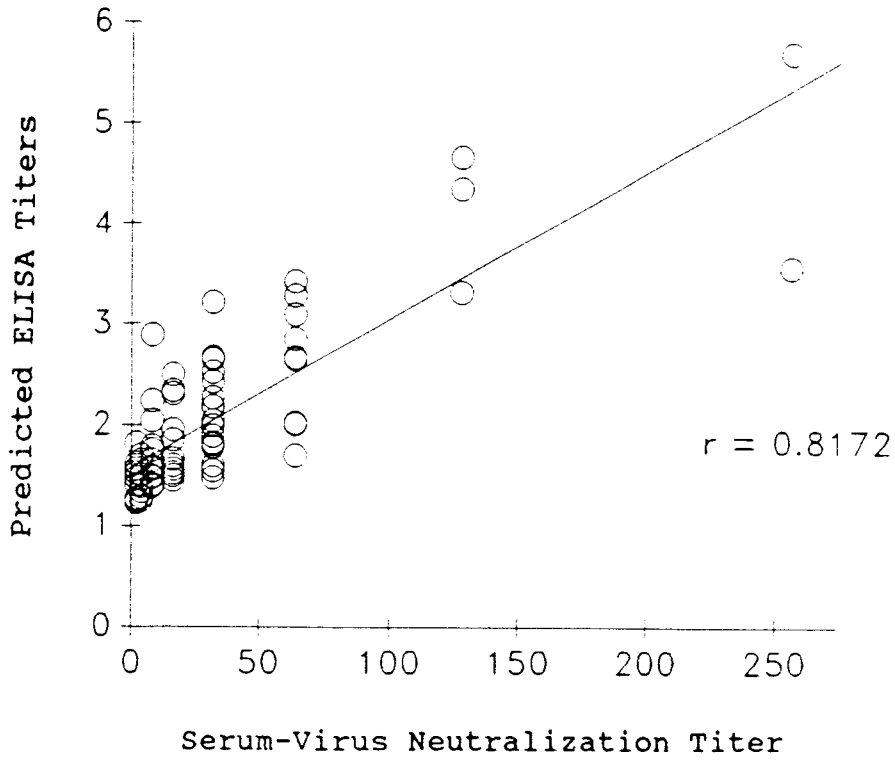

Fig. 5: Bovine adenovirus type 3 antibody titers of 76 bovine sera determined by single-dilution ELISA and serum-virus neutralization assay.

Table 5: Determination of Antibody Titers to Bovine Adenovirus Type 3 using a Single-Dilution ELISA Method and Serum-Virus Neutralization (SN) Assay for 76 Bovine Sera.

SN	# of Samples	ELISA-Avg.	St. Dev	Range
Neg	7	26.4	18.0	17 - 66
2	4	34.3	6.7	26 - 42
4	8	29.6	11.9	18 - 50
8	14	104.2	197.8	25 - 776
16	12	95.8	96.0	28 - 316
32	17	265.5	382.7	30 - 1622
64	9	849.4	902.0	50 - 2630
128	3	23,734	22,396	2042 - 46,774
256	2	246,705	-	3631 - 489,779

Titers are expressed as reciprocals, e.g. dilution of 1:32 would be expressed as 32.

DETERMINATION OF CROSS REACTION BETWEEN SUBGROUPS:

An ELISA to BA4 was also developed in an attempt to determine the cross reaction between the SN and ELISA titers of the subgroup 1 and subgroup 2 viruses (Fig.6). However, there was poor correlation between serum neutralization and ELISA titers, so further work to identify cross-reacting antigens is necessary.

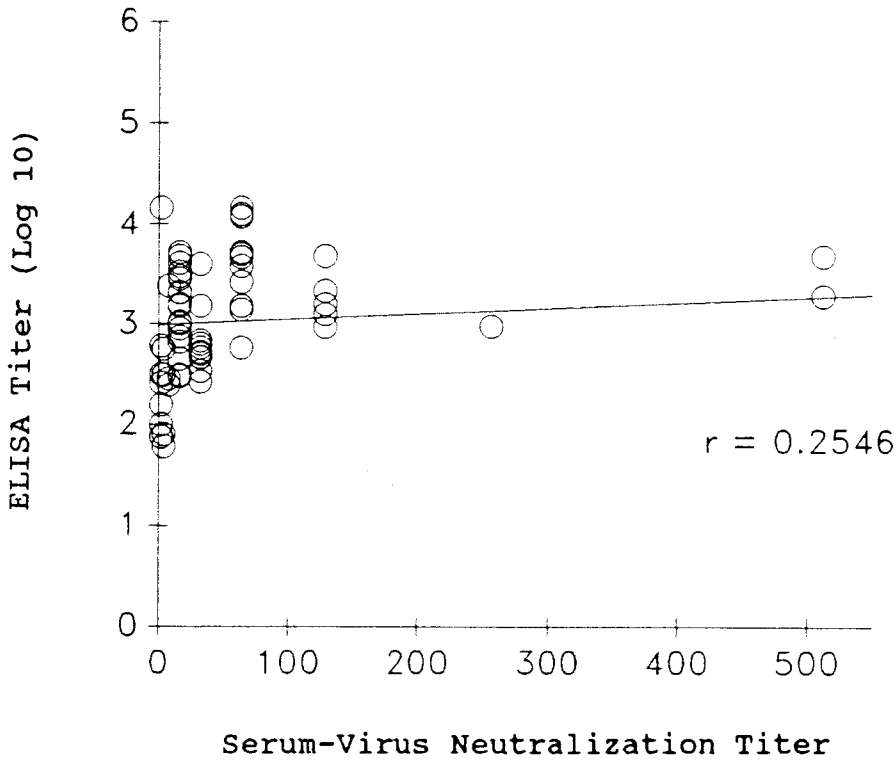

Fig. 6: Bovine adenovirus type 4 antibody levels of 65 bovine sera determined by ELISA and serum-virus neutralization assays.

DISCUSSION

The use of ELISA as a diagnostic tool has gained widespread acceptance since the early investigations of the 1970's. A variety of ELISA protocols are now in common usage; therefore, problems with standardization of technique and interpretation of results need to be resolved for each specific application.

Variability of results may occur from differences in antigen lots, different background absorbance from plate to plate, temperature variation affecting the rate of reaction, dilution error, and many other factors. To reduce inter-assay variance, care was taken in this study to ensure thorough washing of plates, careful titration, thorough mixing of reagents, and incubation at 37 C, rather than ambient temperature. The two antigen lots used in this study both had the same optimal antigen concentration. The inherent variability of absorbance values in different antigen lots and control reagents was also minimized by standardizing each plate with a reference positive serum. Correcting each test-serum absorbance reduces background noise, increasing the stability of serum titers, similar to the "Correction Factor method of Snyder and colleagues[113].

Snyder et al.[113] also addressed the difficulty in interpretation of ELISA results by transforming raw absorbance data into ELISA antibody titers. Establishing a

positive-negative threshold allows for a numerical evaluation of the antibody level in test sera. To further evaluate the significance of the BA3 ELISA titers determined using Snyder's method, the ELISA titers were compared to SN titers for the same samples. Although the correlation was not perfect, there was good indication that the two procedures both measured similar trends in exposure to BA3.

Thiele and colleagues[119] found poor correlation between ELISA titers to human adenovirus and the corresponding complement fixation titers for the same sera. Because the same samples were positive by both methods, they concluded that the tests were detecting some of the same epitopes, despite the variation in the total antibody populations measured.

The complement fixing antigen, common to all mammalian adenoviruses (except the subgroup 2 bovine adenoviruses), is a property of the soluble hexon capsomere and is oriented toward the virion core[53]. The hexon also carries the type-specific antigenic epitope for neutralizing antibodies, which is present at the virion surface[53]. Possibly the difference in orientation of the CF and SN antigenic epitopes would explain the better correlation of ELISA to SN titers obtained in this study in comparison to the poor correlation to CF titers obtained by Thiele et al[119].

Measurement of IgM antibody levels provides a tool for detection of early disease and might also decrease confusion in interpretation of ELISA titers. Thiele and coworkers[119]

measured IgM levels by comparing results using two affinity-purified, goat anti-human IgG conjugates: the H+L to detect both IgG and IgM, and the Fc5 fraction specific for IgG. For three samples from patients undergoing active adenovirus infections, their ELISA system was able to detect an increase in IgG titer and a decrease in IgM titer between acute and convalescent sera. The detection of IgM response would be particularly useful for evaluation of antibody levels to the ubiquitious bovine adenoviruses, therefore, an investigation utilizing Thiele's two conjugate method for BA3 ELISA testing should prove interesting.

Further investigation might also involve use of unpurified antigen to decrease the time and labor involved in antigen preparation. Because the K699 cells used for propagation of the virus showed a very low background absorbance, it should be possible to use Uvasol-extracted antigen, omitting the density gradient purification. As long as the absorbance of cell antigen control remained low, the test should remain sensitive to low positive samples.

A preliminary study was undertaken to determine the extent of cross reaction between the subgroup 1 and subgroup 2 viruses. Although cross-reactions do occur between serotypes within a subgroup, the antigenic determinants for BA subgroup 1 viruses are distinctly different from the BA subgroup 2 viruses, and thus, should not cross-react to any significant extent. Bovine adenovirus type 4 was selected as prototype virus for subgroup 2, and an ELISA detecting

antibodies to BA4 was developed. However, there was poor correlation between the SN titers and the ELISA titers. This might be explained by greater cross-reaction between the different viruses within the subgroup, as it becomes very difficult to find animals without exposure to several different types.

Determination of cross-reactivity between viruses within a subgroup was hindered in this study by the broad exposure of the test animals to multiple types of both subgroup 1 and 2 viruses. Often when samples did not appear to correlate exactly between BA3 SN and ELISA titers, the possibility of cross-reaction to either BA2 (a subgroup 1 type) and BA4 (a subgroup 2 type) could not be eliminated. To determine the exact specificity of the BA3 ELISA, it would be necessary to have sera from animals that were reared in isolation from exposure to multiple types and specifically inoculated to a particular type. However, this is prohibitively expensive and not expressive of the situation that normally exists in modern cattle rearing practices.

The widespread exposure to bovine adenoviruses indicated by the SN titers for the sera tested in this study, correlates well with other serological surveys[15,71,79]. The ubiquitious nature of adenoviruses is seen also in human populations where most adults show previous exposure to adenoviruses. The ubiquitous exposure to bovine adenoviruses makes determination of the specific

BA type involved in an outbreak of respiratory or enteric disease, extremely difficult. The development of a rapid diagnostic assay for determination of antibody response to specific serotypes would be a marked improvement on the time and labor involved in performing multiple SN assays.

The ELISA procedure, developed to detect antibodies to BA3, produced titers that correlated well to SN values, using either three-fold serial dilutions or single dilution values. The ability to accurately predict antibody titer from a single serum dilution is more economic of both labor and materials than the serial titration method. Results of this study indicate that a single-dilution method would provide rapid and accurate indication of infection to BA3 when used to sample a proportion of affected calves. With adult animals, recent infection could be distinguished from past exposure if paired samples were compared, following a time interval, to determine rising antibody levels.

The value of ELISA in determination of antibody levels is often criticized because it provides an absorbance reading that is difficult to interpret in relation to positive or negative responses. The use of a positive-negative threshold to establish titers and use of a procedure that provides good correlation between SN and ELISA titers should minimize the difficulty in titer interpretation. Further reduction of this difficulty could be achieved by evaluation of <u>in vivo</u> protection to ELISA antibody titer. In this way, a single-dilution ELISA for

antibodies to BA3 could prove an effective tool for veterinary diagnostic laboratories.

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