JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1975, p. 219-224

Vol. 1, No. 2
Printed in U.S.A.

Use of Electron Microscopy for Detection of Viral and Other Microbial Contaminants in Bovine Sera

C. K. Y. FONG, P. A. GROSS, G. D. HSIUNG,* AND N. S. SWACK

*Departments of Laboratory Medicine and Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510, and Virology Laboratory, Veterans Administration Hospital, West Haven, Connecticut 06516**

Received for publication 18 November 1974

A total of 25 lots of bovine serum samples were pelleted in Beem capsules for thin sectioning and were examined by electron microscopy. These included 17 lots of fetal bovine serum pools and five lots of calf serum pools obtained from commercial sources, and three lots of adult bovine serum from local dairy farms. Virus-like particles, 50 to 300 nm in diameter, were detected in 17 of 25 (68%) of the sera. Five of 25 serum samples showed the presence of mycoplasma-like agents. Incubation of bovine serum at 35 C for 1 or 2 weeks appeared to destroy some of these agents, but in certain instances it enhanced bacteria and bacteriophage contaminants. The advantages of electron microscopy using the thin-sectioning technique for detection of microbial contamination in bovine sera are illustrated.

For several decades, bovine sera have been a major constituent in culture media for the cultivation of cells in vitro. The problems of microbial contamination of bovine sera were not of great concern until recent reports showed that some of these biological products may harbor one or more kinds of endogenous agents. The isolation of animal viruses (11), bacterial viruses (3, 10, 12), and mycoplasma (1) warrants further investigation into techniques for detecting microorganisms in bovine sera.

In the accompanying paper (14), we described different centrifugation methods for the recovery of animal viruses from bovine sera and compared the efficiency of each. When bovine serum contained specific antibodies to certain viruses, the presence of the viruses in the serum could not be detected by routine isolation methods. Electron microscopy appears to offer the advantage of visualizing the virus-antibody complex which otherwise would not be recognized by standard laboratory techniques. In this study, we report a simple, rapid method using electron microscopy for the detection of virus-like and other microbial agents in sera from various sources.

MATERIALS AND METHODS

Source of sera. Seventeen lots of fetal bovine serum (FBS) pools and five lots of calf serum (CS) pools were obtained from seven different commercial sources. They had been filtered through a 0.22-μm membrane filter before being received in our laboratory. In addition, three lots of bovine sera from individual cattle at the University of Rhode Island were included. These were prepared from blood samples aseptically drawn from each animal. One of the latter samples was filtered through a D7 Seitz filter (Hormann Co., Milldale, Conn.). All samples were stored at −20 C.

Methods of concentration of sera. All sera ranging in amounts from 35 to 100 ml were first clarified by low speed centrifugation in a Sorvall centrifuge using a GSA rotor at 5,000 rpm for 20 min at 4 C. The supernatants were then pelleted at 27,000 rpm for 90 min in a Beckman L2-65 ultracentrifuge using an SW27 rotor at 4 C. A small portion of the pellet was used for direct electron microscopy employing the negative staining technique (see below). The remaining pellet was resuspended in 2% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.3 for 20 min. The latter suspension was pelleted into a Beem capsule using an SW65 rotor at 30,000 rpm for 60 min at 4 C. The capsule was placed in a special adapter as originally described by Smith and Gehle (13). By this method, microbial contaminants in the original serum samples were concentrated 100-fold or more.

Electron microscopy. (i) Thin sectioning. The pellets from the Beem capsules were washed overnight with three changes of 0.08 M cacodylate buffer containing 0.18 M sucrose to remove residual glutaraldehyde. This was followed by postfixing with osmium tetroxide in s-collidine buffer at pH 7.4. The methods of dehydration and embedding were the same as described previously (6). Thin sections were made with an LKB Ultrotome and stained with uranyl acetate and lead citrate. Sections were examined with a Phillips EM 300 electron microscope.

(ii) Negative staining. After centrifugation at 27,000 rpm, a small drop of the concentrated pellet material was applied to Formvar-coated grids. The

excess liquid was removed by touching with the edge of a filter paper. The grids were stained with 2% sodium phosphotungstate and examined under the electron microscope.

RESULTS

Observation of microorganisms in thin sections. (i) Virus-like particles. A total of 25 serum samples were examined and the results are shown in Table 1. Virus-like particles were seen in 17 of 25 sera examined (68%). Virus size ranged from 50 to 300 nm in diameter. Some particles showed the presence of an electron dense core. Many virus particles revealed an internal helical structure suggesting a myxovirus; they were seen in 10 FBS pools, two CS pools, and one individual cattle serum. An example of a large myxo-like virus is illustrated in Fig. 1a. Virus particles ranging from 70 to 125 nm in diameter with spikes on the surface of the envelope were also seen in one of the CS pools (Fig. 1b) and two of the FBS pools (Fig. 1c and d). In one FBS pool, many bacteriophage-like particles, 90 nm in diameter, were seen (Fig. 1e). These particles had a hexagonal shape and contained a relatively large electron dense core enclosed by an outer membrane. Virus-like particles of hexagonal shape with 60 nm diameter were found in another FBS sample (Fig. 2a). Virus-like aggregates containing numerous particles, approximately 50 nm in diameter, without clear structure, were seen in many of the serum samples examined; some of them contained dense cores (Fig. 2b).

(ii) Mycoplasma-like agents. Structures which may be similar to those described for mycoplasma were observed in five of 25 (20%) of the sera (Fig. 2c). They were variable in size and shape; most were spherical and enclosed by a single membrane.

(iii) Bacteria and bacteriophage. Attempts were made to increase the isolation of bacteria, which might be present in very small numbers in the original serum, by incubating aliquots of each of 17 serum pool lots at 35 C for 1 to 2 weeks followed by centrifugation, fixation, and embedding as described above. It was noted that most of the virus- and/or mycoplasma-like agents disappeared after incubation, with two exceptions. The presence of bacterial contamination in one serum sample was detected only after incubation at 35 C for 2 weeks. Figure 3 shows the results obtained before and after incubation. Before incubation, virus-like aggregates, 50 nm in size, and mycoplasma-like agents with poorly delineated structures were seen (Fig. 3A). Numerous virus particles of various size and different morphology were found in samples prepared after incubation (Fig. 3B, C, and D). Virus particles 50, 75, and 90 nm in diameter were observed throughout the samples (Fig. 3B and C). These virus particles were probably bacteriophages since they appeared concurrently with the bacterial growth (Fig. 3D).

(iv) Negative staining method. In a few of the negatively stained preparations examined, virus-like or mycoplasma-like agents were seen. The surface structures of the particles were usually unclear, and it was difficult to identify each organism as well as to obtain a clean and concentrated preparation from the crude serum samples. Therefore, the negative staining technique was only used for preliminary screening of a few samples.

TABLE 1. *Detection by electron microscopy of virus-like particles in bovine sera*

Serum[a]	Source	No. positive/ no. of lots examined	Presumptive identification of virus-like particles	
			Myxo-like	Bacterio-phage and others
Fetal bovine serum	A	3/4	2	2
	B	2/2	1	2
	C	3/5	2	3
	D	2/2	1	1
	E	2/2	2	2
	F	1/1	1	1
	G	1/1	1	1
Calf sera	A	2/5	2	1
Adult bovine sera	H	1/3	1	0

[a] Fetal bovine and calf serum samples represented pooled samples and were purchased commercially; adult bovine sera were obtained from individual cattle locally.

DISCUSSION

The undesirable effect of microbial contamination introduced into culture media through the use of bovine sera has been a major concern (2, 4, 5). Therefore it is necessary to search for a rapid, efficient method of detecting these agents in bovine sera. The method of centrifugation of bovine sera into Beem capsules and examination by electron microscopy described in this study greatly facilitates the recognition of microbial contamination. The technique is simple and efficient. To preserve the structure of these agents, glutaraldehyde fixation immediately after concentration by high speed centrifugation may be necessary. Although final identification of these agents cannot be made until they are grown in cell cultures or other media, the recognition of their presence is important since

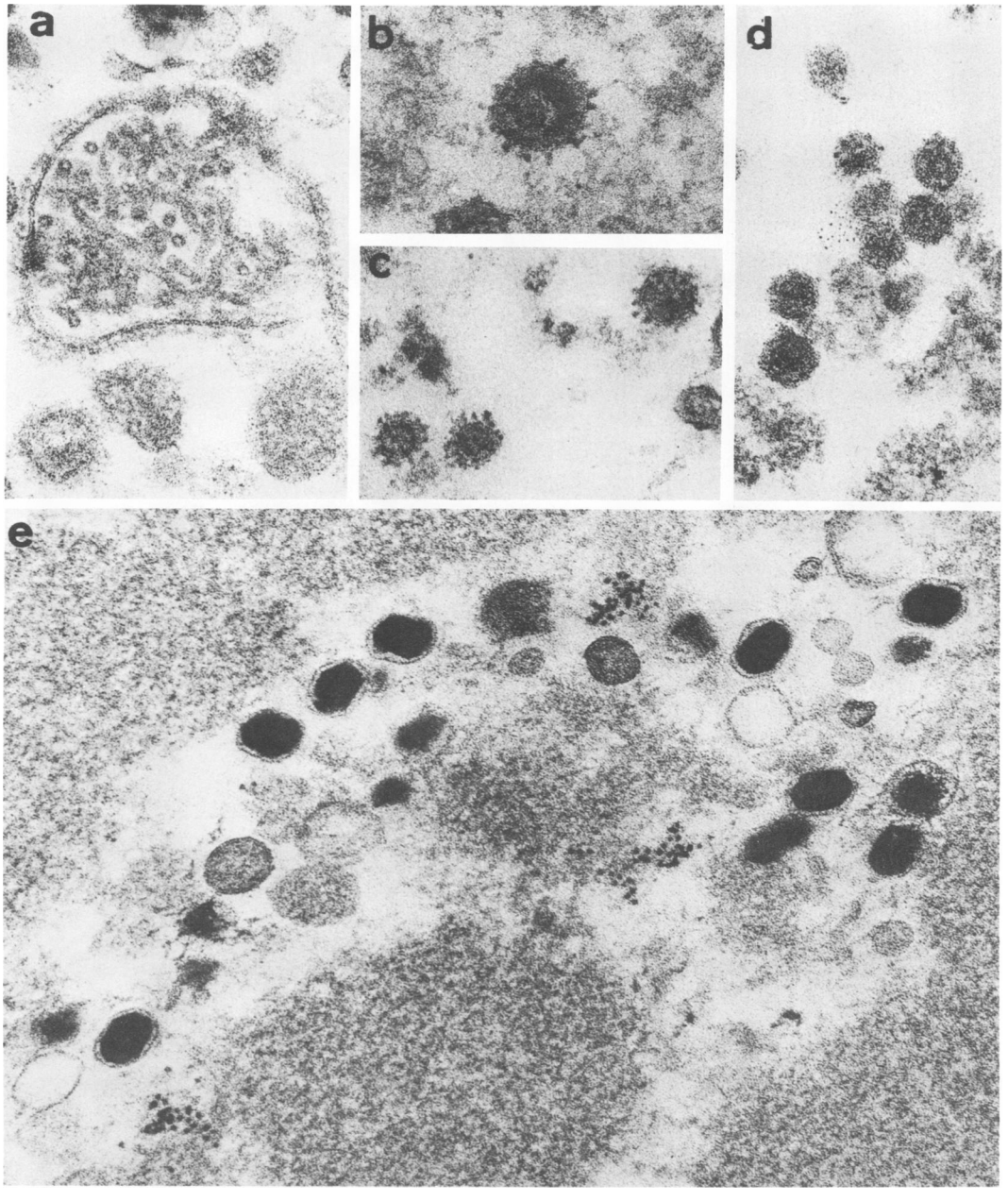

FIG. 1. *(a) Virus-like particles with internal structure resembling myxovirus seen in one fetal bovine serum sample. ×100,800. Virus-like particle showing spike-like structure on the surface of the envelope seen in (b) one of the calf serum pools and (c and d) from two separate FBS pools. ×100,800. (e) Many bacteriophage-like particles, 90 nm in diameter, seen in one of the fetal bovine serum pools. × 100,800.*

many of them may be extremely fastidious and cannot be cultivated by the methods available at this time.

Negative staining techniques have been used for the recognition and characterization of viral agents in purified preparations. This method is of little value in the examination of serum samples which usually contain heavy debris that obscures the surface structure of virus particles. The Beem capsule thin-sectioning technique described in this study overcame this difficulty and offers advantages for the easy

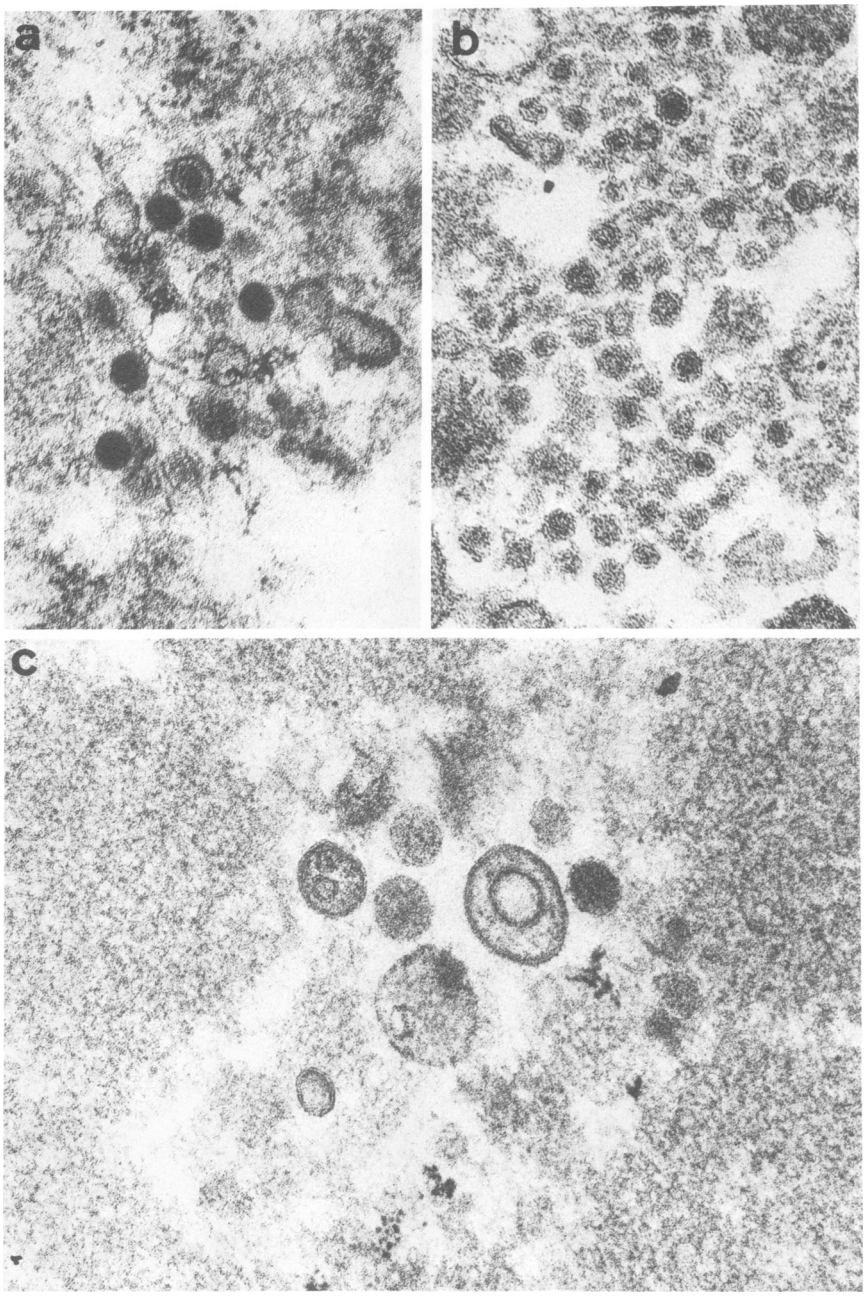

FIG. 2. *(a) Virus-like particles with hexagonal shape, 60 nm in diameter, seen in one of the FBS pools. × 100,800. (b) Virus-like particles with unclear structure, approximately 50 nm in diameter, seen in many of the serum pools. × 100,800. (c) Mycoplasma-like agents seen in one of the fetal bovine serum pools. × 100,800.*

recognition of viral structure.

In the present report, the high rate of virus-like agents detected in bovine sera by electron microscopy was notable. Not only had most of the pooled sera from commercial sources been contaminated by virus, but sera from individual cattle drawn aseptically also contained virus-like agents. Morphologically, some of the virus-like particles seen in many of the sera resembled virus from the paramyxovirus group. Parainfluenza type 3 has been isolated from cattle (7) and antibody to this virus type is common in commercial bovine sera (14.). However, because of the high titers of antibody to parainfluenza type

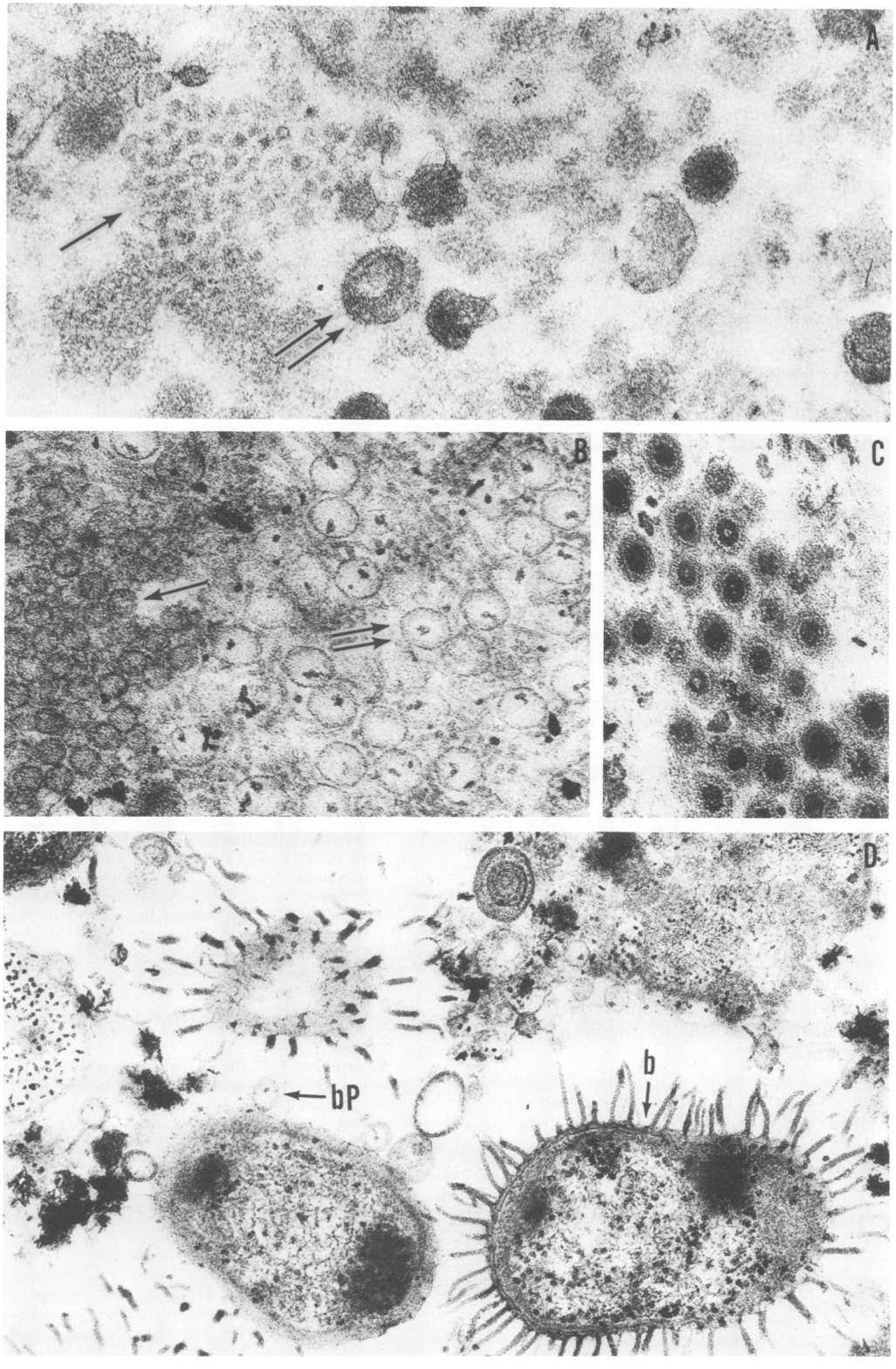

FIG. 3. *Electron micrographs of a fetal bovine serum pellet sample (A) taken before incubation and (B, C, and D) after 2 weeks incubation at 35 C. (A) Virus-like aggregates (arrow) and mycoplasma-like agents (double arrows) with unclear structures. ×38,400. (B) Presence of two different sizes of virus-like particles, 50 nm (arrow) and 90 nm (double arrows) in diameter. ×79,200. (C) The third type of virus-like particles, 75 nm in diameter. × 100,800. (D) Presence of bacteria (b) and bacteriophage (bp). ×48,000.*

3, isolation of this virus from bovine sera is usually difficult, as indicated in the accompanying paper (14). In this case, recognition of the presence of virus was accomplished only by morphological observation.

The virus structures illustrated in Fig. 1b-d were within the medium size range, 70 to 125 nm. These particles resemble the ribonucleic acid-containing enveloped viruses, including the oncorna- and coronavirus groups. The small (50 nm) virus-like particles in aggregates (Fig. 2b) appeared to be another common contaminant in bovine serum. Although the structure of these virus-like particles was usually not very clear, they were within the size range of the bovine virus diarrhea group. Since the presence of bovine virus diarrhea virus in bovine sera has been reported by other investigators (8, 9), further identification and characterization of these small virus particles is of particular significance.

Other types of virus such as bacteriophages appeared to be frequent contaminants, especially in serum pools of commercial products (3, 10, 12). It is apparent that most of the bacterial contaminants acquired during serum collection were removed by filtration, but a small or undetectable amount of bacteria can be increased by incubation as occurred in one of the FBS pools examined. The propagation of the bacterial population enhanced the replication of the bacteriophages, resulting in the large number and variety of virus particles seen in this sample (Fig. 3B, C, and D) as compared to the same samples before incubation (Fig. 3A). However, the presence of disintegrated virus particles seen in serum samples after incubation would indicate that some mycoplasma-like and certain viral agents may have been inactivated. Therefore, the incubation procedures for bovine sera was of little value for the detection of viral agents in sera.

Since the recognition of microorganisms in bovine sera is of practical importance, the simple, rapid method described in the present study is highly recommended when facilities are available. However, final identification cannot be made until the microorganism is isolated and cultivated. Therefore, electron microscopy can only serve as an ancillary technique for detection of microbial contaminants in bovine serum and cannot be substituted for cell cultivation methods.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service research contract NIH-DBS-72-2105 and FDA 233-74-1035 from the Department of Health, Education and Welfare, and research grant AI-08648 from the National Institute of Allergy and Infectious Diseases. The adult bovine sera were obtained through the courtesy of P. W. Chang, University of Rhode Island.

We wish to express our appreciation to Barbara Meek, JoEllyn Bradley, JoAnne Larsen, and David Potocik for excellent technical assistance.

ADDENDUM

While this manuscript was in preparation, a paper entitled "Brief communication: small, virus-like particles detected in bovine sera by electron microscopy" appeared in the Journal of the National Cancer Institute (**52**:1931-1933, 1974) which supports our findings as described in this manuscript.

LITERATURE CITED

1. Barile, M. F., and J. Kern. 1971. Isolation of *mycoplasma arginini* from commercial bovine sera and its implication in contaminated cell cultures. Proc. Soc. Exp. Biol. Med. **138**:432-437.
2. Boone, C. W., N. Mantel, T. D. Caruso, Jr., E. Kazam, and R. E. Stevenson. 1972. Quality control studies on fetal bovine serum used in tissue culture. In Vitro **7**:174-189.
3. Chu, F. C., J. B. Johnson, H. C. Orr, P. G. Probst, and J. C. Petricciani. 1973. Bacterial virus contamination of fetal bovine sera. In Vitro **9**:31-34.
4. Fedoroff, S., V. J. Evans, H. E. Hopps, K. K. Sanford, and C. W. Boone. 1972. Summary of proceedings of a workshop on serum for tissue culture purpose. In Vitro **7**:161-167.
5. Fogh, J., N. B. Holmgren, and P. P. Ludovici. 1971. A review of cell culture contaminations. In Vitro **7**:26-41.
6. Fong, C. K. Y., and G. D. Hsiung. 1972. Development of an equine herpesvirus in two cell culture systems: light and electron microscopy. Infect. Immun. **6**:865-876.
7. Kniazeff, A. J. 1968. Viruses infecting cattle and their role as endogenous contaminants of cell cultures. Natl. Cancer Inst. Monogr. **29**:123-132.
8. Kniazeff, A. J. 1973. Endogenous virus contaminants in fetal bovine serum and their role in tissue culture contamination, p. 233-242. *In* J. Fogh (ed.), Contamination in tissue culture. Academic Press Inc., New York.
9. Kniazeff, A. J., V. Rimer, and L. Gaeta. 1967. γ-Globulin in fetal bovine sera: significance in virology. Nature (London) **214**:805-806.
10. Merril, C. R., T. B. Friedman, A. F. Attallah, M. R. Geier, K. Krell, and R. Yarkin. 1972. Isolation of bacteriophages from commercial sera. In Vitro **8**:91-93.
11. Molander, C. W., A. J. Kniazeff, C. W. Boone, A. Paley, and D. T. Imagawa. 1972. Isolation and characterization of viruses from fetal calf serum. In Vitro **7**:168-173.
12. Petricciani, J. C., F. C. Chu, and J. B. Johnson. 1973. Bacteriophages in live virus vaccines. Proc. Soc. Exp. Biol. Med. **144**:789-792.
13. Smith, K. O., and W. D. Gehle. 1965. Pelleting viruses and virus-infected cells for thin-section electron microscopy. Proc. Soc. Exp. Biol. Med. **130**:1117-1119.
14. Swack, N. S., C. K. Y. Fong, G. D. Hsiung, and P. A. Gross. 1975. Methods for the detection of viruses in bovine serum. J. Clin. Microbiol. **1**:212-218.