Isolation of *Mycoplasma arginini* from Commercial Bovine Sera and Its Implication in Contaminated Cell Cultures (35913)

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Most mycoplasma contaminants of cell cultures have been classified as either human, swine, or bovine strains (1). Since bovine serum is commonly used in cell culture media, the probable source of bovine strains is contaminated bovine sera. However, all previous attempts to isolate mycoplasma from commercial bovine sera have failed (1–9). This report presents the first successful isolation of mycoplasmas from commercial bovine sera. Data presented indicate that contaminated bovine serum is the major source of bovine mycoplasma contamination of cell cultures. Successful isolations were due to changes in the culture procedure which increased the sensitivity of the test. The most important change is the use of a large volume of serum, 25 ml or more, as the inoculum in a broth medium. The initial isolation was made from a final lot of fetal bovine serum by one of us (MFB) followed by isolations from 10 other final lots by the manufacturer (JK). This led to testing each sublot of raw serum before use in manufacture which resulted in a marked decrease in the contamination rate of final serum lots.

Materials and Methods. Sera. A total of 379 lots of sera were examined, including 239 unprocessed sublots of fetal bovine (184) and calf (55) sera (Table I) and 140 final lots of fetal bovine sera (Table II). Unprocessed serum sublots tested by manufacturer E (Table II) were obtained from 4 suppliers, coded A, B, C, and D in Table I, including St. Louis Serum Co., East St. Louis, IL; Riggs Serum Co., Dubuque, IA; Denver Serum Co., Denver, PA; and Stanley Krutulis Co., Bridgeport, NY. Sublots contained from 3 to 170 liters of sera, were shipped frozen to manufacturer E via air express in plastic bottles containing 900 ml and were maintained frozen until used. Each sublot was treated as a unit. For examination, a sample of raw serum was thawed rapidly, passed through a "Miracloth" prefilter (Chicopee Mills, New York, NY) and a 0.45 μ membrane filter (Millipore Filter Corp., Bedford, MA) and cultured for mycoplasma. Sublots were filtered to remove or reduce substantially the bacterial and fungal contamination before the serum could be cultured for mycoplasma. Mycoplasma-free sublots were selected for further quality testing and for the final manufacturing process. The selected sublots were pooled into a final lot, filtered through a Millipore AP pad, 1.2, 0.45, and 0.3 μ filters once, a 0.22 μ filter twice and tested for mycoplasma. During the study, a total of 140 final lots of fetal bovine serum examined were prepared by manufacturer E or purchased from the 4 other manufacturers and were coded as E, F, G, H, and J in Table II, including Flow Laboratories, Inc., Rockville, MD; Grand Island Biological Laboratories, Grand Island, NY; Microbiological Associates, Bethesda, MD; Industrial Biological Laboratories, Rockville, MD, and Hyland Laboratories, Los Angeles, CA.

Cell Cultures. Source and derivation of 10 contaminated cell cultures and one virus pool from which 11 strains of *Mycoplasma arginini* were isolated are given in Table III.

Media. Broth and agar media used for isolation of mycoplasma are described elsewhere (10, 11). In brief, Hayflick's formula (7) was supplemented with the following at the final concentrations indicated: 1% arginine; 1% dextrose; 1:2000 parts thallium acetate; 1000 units/ml of penicillin; and 0.002% phenol red.

Procedure for Mycoplasma Isolation. Se-

TABLE I. Isolation of Mycoplasmas from Sublots of Unprocessed Bovine Sera Obtained from Suppliers[a]

Suppliers	Isolation of Mycoplasma			
	Fetal bovine sera		Calf sera	
A	22/113[b]	(10.6)	NT[c]	
B	1/17	(5.9)	NT	
C	10/54	(18.5)	16/26	(61.5)
D	NT		6/29	(20.7)
Totals	33/184	(17.9)	22/55	(40.0)

[a] Sublot denotes unprocessed sera obtained from supplier. To remove and/or minimize bacteria and fungi, each sublot was filtered through a 0.45 μ membrane filter before it was cultured in mycoplasma broth containing antibacterial agents, *i.e.*, 1:2000 parts thallium acetate and 100 units/ml of penicillin G.

[b] Number positive per number tested; percentage given in parentheses.

[c] None tested.

TABLE II. Isolation of Mycoplasma from Final Processed Lots of Commercial Fetal Bovine Sera.

Manufacturer	Isolation of Mycoplasma	
E	16/131[a]	(12.2%)
F	2/2[b]	
G	0/3	
H	0/3	
J	0/1	

[a] Number positive per number tested.

[b] Lots from this manufacturer were not selected at random and were tested when cell cultures fed with these lots became contaminated. The cell cultures and bovine sera contaminants were identified as *M. arginini* in each case.

rum (25 ml) was inoculated into 100 ml of broth medium. Occasionally, larger inocula were used, *i.e.*, 50 or 100 ml of sera were inoculated into 200 or 400 ml of broth media, respectively. Broth cultures were incubated at 36 $\pm$ 1° and then were subcultured (approx. 0.1 ml) to agar media at 4 to 5 and at 14 days and, occasionally at 21 days. Agar cultures were incubated in a 5% CO_2–95% nitrogen atmosphere (12) and were examined for at least 7 days. Positive broth cultures were subcultured to a series of agar plates for serologic identification tests.

Identification of Mycoplasma. *Mycoplasma* species were determined by serologic tests using the growth inhibition (GIT) (13) and plate immunofluorescence (FA) (14, 15) procedures as described elsewhere. Some modifications were made: serum content of culture media was reduced from 20% to 2–5% or was replaced with 1 to 2% Serum Fraction (Difco) and each antiserum was tested against 3 tenfold dilutions of antigen. The antisera reagents prepared against 39 well-established *Mycoplasma* species and serotypes and used in these studies are given in Table IV.

Results. Serum Sublots and Final Lots. Mycoplasma isolations were made initially from final lots of manufactured sera and this led to testing unprocessed serum sublots obtained from suppliers. Each of 239 unprocessed serum sublots tested were contaminated with bacteria, containing from 10^2 to 10^7 colonies/ml; fungi were present also. Mycoplasmas were isolated from 55 of 239 (23%) sublots, including 33 of 184 (17.9%) fetal bovine serum sublots from 3 suppliers and 22 of 55 (40%) calf serum sublots from 2 suppliers (Table I). The data show that some sublots obtained from each of the 4 suppliers were contaminated. Calf serum sublots (40%) were contaminated more frequently than were fetal serum sublots (18%) (Table I). Of 55 mycoplasma strains isolated, 13 were examined for identity. The 42 remaining strains were not available for study. Of 13 strains examined, 11 were identified as *M. arginini* and 2 strains (DBS-792 and DBS-898) were unrelated to 39 well-established *Mycoplasma* species (Table IV) and may represent new species. *M. arginini* was isolated from fetal bovine and calf sera and from sublots obtained from each of 4 suppliers, indicating widespread distribution for this mycoplasma. Eighteen of 140 (12.8%) final lots of fetal bovine sera from 2 of 5 manufacturers were positive (Table II). Of 18 strains isolated, 17 were identified as *M. arginini* and strain DBS-803 was unrelated to 39 well-established *Mycoplasma* species and may represent a new species. On reexamination, mycoplasma were reisolated from 17 of the 18 positive final lots of fetal bovine sera using the new culture procedure

TABLE III. Isolation of *M. arginini* from Uninoculated Cell Cultures.[a]

M. arginini strains	Cell cultures	
	Derivation	Obtained from
DBS-547	HA subline, human amnion	S.H. Singer, Bethesda, MD
DBS-583	AL-3 subline, African lymphoma	B.G. Young, College Park, MD
DBS-585	EB-2 subline, Burkitt lymphoma	B.G. Young, College Park, MD
DBS-586	SKL-2 subline, lymphoblastic leukemia	B.G. Young, College Park, MD
DBS-587	SKL-3 subline, myelomonocytic leukemia	B.G. Young, College Park, MD
DBS-713	Human thyroid medillary carcinoma	N.A. Wivel, Bethesda, MD
DBS-733	Mouse neuroblastoma	J. Tumilowicz, Camden, NJ
DBS-740	Sendai virus, diluted in medium containing fetal bovine serum, passed in embryonated eggs	A.S. Rabson, Bethesda, MD
DBS-69	BSC-1 subline, African green monkey kidney	H.C. Orr, Bethesda, MD
I-92	MDCK subline, canine kidney, (Madin and Darby)	Lot BP2410, Flow Labs., Inc. Rockville, MD
I-108	HEK subline, human embryonic kidney	H.M. Yamashiroya, Chicago, IL

[a] A total of 21 strains of *M. arginini* were isolated at DBS from uninoculated cell cultures, including those reported here and elsewhere (16).

described. However, mycoplasma was not isolated from these same lots using the old standard procedure of inoculating 0.1 ml on agar medium.

Cell cultures. Eleven strains of *M. arginini* isolated from 10 uninoculated cell cultures and one virus pool are given in Table III. In several cases, every cell culture line maintained by the laboratory was contaminated with *M. arginini.*

Discussion. Data are presented on the first successful isolations of mycoplasmas from commercial bovine sera. Successful isolations were due, in part, to changes made in the culture procedure which increased the sensitivity of the test. These changes include the use of large volumes, 25 ml or more, of serum as inocula (generally a 0.1 to 1.0 ml inoculum is used) and the testing of unprocessed serum sublots obtained directly from the serum suppliers. Serum harvested by each of the four suppliers were contaminated. Calf serum sublots were contaminated more frequently than were fetal bovine serum sublots. The rate of contamination of unprocessed sera varied with the supplier and ranged from 6 to 61% contamination of sublots tested. Successful isolations were made early in the study from final lots of manufactured serum and 16 final lots of contaminated serum were withdrawn from release. The contamination rate decreased markedly when unprocessed sublots were tested before serum was pooled into a final lot.

Data presented indicate that the culture procedure described is an effective and useful method for detecting mycoplasmas in commercial bovine serum. It is a simple and economical test and should be used before the final lot can be considered as, and in fact should be labeled as, sterile. In addition, this procedure may have general application for isolation of low-order mycoplasma populations in contaminated or infected biologic materials.

A total of 73 mycoplasma strains were isolated from bovine sera. Of these, 31 strains were examined for identity and 28 were identified as *M. arginini.* Strain DBS-792, DBS-803, and DBS-898 were unrelated to 39 well-established *Mycoplasma* species and serotypes by both growth inhibition and immunofluorescence procedures and may represent new species. The remaining 42 strains were

TABLE IV. Mycoplasma Antisera Employed for Testing DBS Strains 792, 803, and 898.

Species	Strain	Species	Strain
Primate		Canine	
M. hominis	PG-21, Botte	*M. spumans*	PG-13
M. fermentans	PG-18	*M. canis*	PG-14
M. salivarium	PG-20, B3	*M. maculosum*	PG-15
M. pneumoniae	FH	*M. edwardii*	PG-24
M. orale 1	CH19299, 274	*M. sp.*	689
M. orale 2	CH20247	Feline	
M. orale 3	DC333	*M. felis*	27
M. lipophilum	Ma By	*M. gateae*	Mart
M. primatum	Simian 292	*M. feliminutum*	Ben
M. sp.	Simian 291	Avian	
Bovine		*M. gallisepticum*	PG-31, H1344
M. mycoides var. *mycoides*[a]	Gladysdale	*M. gallinarum*	PG-16
M. bovigenitalium	PG-11	*M. iners*	PG-30
M. bovirhinis	PG-43	*M. anatis*	1340
M. sp.	Calf 188	*M. meleagridis*	529
Caprine and ovine		Murine	
M. mycoides var. *capri*[a]	PG-3	*M. neurolyticum*	Type A
M. agalactiae[a]	Agalactia	*M. pulmonis*	PG-34, Negroni
M. arginini	G-230, BBL-88	*M. arthriditis*	PG-6, PG-27
M. sp.	Goat 189	Acholeplasma	
M. sp.	BBL-G145	*A. laidlawii*	PG-8, PG-9
Swine		*A. granularum*	BTS-39
M. hyorhinis	GDL	*A. axanthum*	S-743

[a]These antisera were obtained from the Research Resources Branch, NIAID, NIH, Bethesda, MD and were prepared under contracts NIH 69-59 and PH 43-68-1516.

not available for study.

Mycoplasma arginini has been isolated from various normal and infected bovine tissues from animals in this country (16) and in Europe (17). In addition, *M. arginini* has been isolated from 21 cell cultures sent to us for examination since 1968 by 13 different investigators from separate and distant laboratories, including those reported here and elsewhere (16). In 2 cases, *M. arginini* contamination spread to every cell culture line in the laboratory. Thus, *M. arginini* is a major source of bovine mycoplasma contamination of cell cultures. Since mycoplasmas can alter cell culture morphology (18–20) and metabolism (21–24) and can influence interferon activity (21) and virus propagation and yields (25–28), investigators must examine bovine serum and cell cultures for mycoplasma in order to interpret properly results of study. To reduce the risk of bovine mycoplasma contamination of cell cultures, some investigators routinely process bovine serum before use. Two procedures are used with varying degrees of success: (a) serum is heated at 56° for 30 min to inactivate mycoplasmas and (b) serum is heated at 45° for 1 hr and then filtered through a 0.22 μ membrane filter. Heat inactivation was most successful with small volumes of serum, *i.e.*, 100 ml or less.

Data presented indicate that *M. arginini* is a common contaminant of commercial bovine sera. Our findings indicate that *M. arginini* is a common contaminant of cell culture (16). Twenty-one of 60 (35%) of the mycoplasma strains isolated from contaminated cell cultures since 1968 by one of us (MFB) were identified as *M. arginini*. However, our data do not explain: (a) the relatively recent isolation of *M. arginini* from cell cultures (16) vis-a-vis the use of fetal bovine and calf sera as a "universal" ingredient of tissue culture media for more than a decade; and

(b) why some investigators do not experience *M. arginini* contamination. A partial explanation may be found in changes made in the processing of commercial bovine sera during the past decade resulting from increased demands for large volumes of this product. These demands may have been responsible for changes in methods used for collection, harvest and processing of serum at the abattoir, by the supplier and the commercial processing company. Earlier studies (1, 5) have shown that mycoplasma contamination of cell cultures depends upon numerous factors involving the investigator, the nature of study, and the type of cell culture used. The lowest contamination rates were found among investigators who use (a) primary cells vs continuous cells, and (b) who do not use antibiotics or serum (and heat-inactivate the serum if used), or mouth pipettes, or large volumes of cells or large numbers of containers. Investigators should examine their source of bovine serum and, if necessary, they should test serum before use.

Summary. Mycoplasmas were isolated from 73 of 379 (19%) commercial bovine serum lots examined, including 55 of 239 (23%) unprocessed fetal bovine and calf serum sublots received from 4 suppliers and 18 of 140 (13%) final lots obtained from 2 of 5 manufacturers. Of 31 mycoplasma strains examined for identity, 28 were identified as *M. arginini* and 3 strains were unrelated to 39 well established *Mycoplasma* species and serotypes and may represent new species. *Mycoplasma arginini* was isolated from 10 uninoculated cell cultures and one virus pool also. Data presented indicate that *M. arginini* is a common contaminant of commercial bovine sera and of cell cultures and that bovine serum is a major source of bovine mycoplasma contamination of cell cultures.

Addendum. Since *Acholeplasma laidlawii* is a common cell culture contaminant (1) of bovine origin, attempts to isolate this agent from commercial bovine serum were continued. The culture procedure was modified as follows: the horse serum component was deleted from the broth medium and cultures were incubated for 6 weeks at 32° with weekly subcultures to agar medium. Using this procedure, *M. arginini* and, in addition, *A. laidlawii* were isolated from 3 of the 16 positive final lots of fetal bovine serum listed in Table II. These findings indicate that both *M. arginini* and *A. laidlawii* are present in some final lots of commercial bovine serum and that the source of bovine mycoplasma contamination of cell cultures is commercial bovine serum.

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