INVESTIGATION ON GROWTH MEDIA FOR MYCOPLASMA: EVALUATION OF INFUSIONS, PEPTONES, SERA, YEAST EXTRACTS AND OTHER SUPPLEMENTS

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Mycoplasmas are the smallest microorganisms that are cultivable on cell-free media. All of them, except saprophytic strains, have strict growth requirements. They can be reliably recognized and discriminated from similar agents by cultivation on lifeless media first of all. Their exacting cultural requirements, and the slowness, paucity, and irregularity of their growth have been a bottle-neck to the progress of studies of these organisms.

Since the success of cultivation of the agents of bovine contagious pleuropneumonia and ovine contagious agalactia, many workers have been trying to devise a new medium or improve the media previously used. Every improvement led to a success in cultivating some additional members of the mycoplasmal species, and to an expansion of information about the biology of the organisms and also about their relationship to the disease of man and animals.

In early periods, such ordinary meat infusion-peptone media as Martin's broth supplemented by horse serum were used. Then, various types of enzymatic digest of animal tissue were introduced as basal media. SCHOETENSACK²⁹⁾ isolated mycoplasmas from dogs in his "M. P." broth. TURNER and CAMPBELL³⁴⁾ devised a "V. F." medium for the cultivation of the organisms of bovine pleuropneumonia. LAIDLAW and ELFORD¹⁹⁾ isolated saprophytic mycoplasmas from sewage in Hartley's digest broth. These enzyme-digested media were improved later. SHEPARD³⁰⁾ isolated "T" strains from non-gonococcal urethritis of man in a medium that had been prepared from a trypsin-digested concentrate of beef heart muscle. BARBER and FABRICANT³⁾ reported strains which had been obtained only in a single variations of VF medium and other media which had been enriched with horse, cattle, or pig serum and with or without yeast extract.

Most workers have used an ox-heart infusion broth supplemented by one per cent peptone after BENNETT⁴⁾ as a rich basal medium.

KLIENEBERGER¹⁸⁾ and DIENES⁶⁾ recommended the addition of red blood cell suspension to the medium being subsequently boiled and clarified by sedimentation. They isolated mycoplasmas in this transparent "boiled blood medium" from rats and mice suffering from chronic respiratory disease and arthritis, and human genitourinary tracts. Descended from Klieneberger's medium, Hofstad's medium¹⁶⁾ was prepared from an infusion of chicken meat for the isolation of *Mycoplasma gallisepticum*, the agent of CRD in chickens, and Herderschee's medium¹⁵⁾ for *M. pneumoniae*, the agent of atypical pneumonia in man.

Some workers also prepared a medium from an ox-heart infusion-peptone broth, to which they had added some supplements.

EDWARD⁹⁾ described a selective medium prepared from fresh ox-heart infusion-peptone base to which fresh yeast extract had been added. To that medium he added potassium phosphate¹²⁾ and DNA¹¹⁾ which had replaced flakes of the vaginal mucus of cattle and hog gastric mucin. In these media, he isolated mycoplasmas from the respiratory and genitourinary tracts of cattle and dogs.

MORTON *et al.*²¹⁾ compared the nutritional value among eight kinds of infusion from ox, thirteen kinds of peptone and human ascitic fluid, horse and rabbit sera, using seven human strains and one murine strain. Their medium had dehydrated beef heart for infusion and Bacto-peptone as basic ingredients and was enriched with human ascitic fluid or with Bacto-PPLO serum fraction. Their medium is commercially available in such dehydrated form as Bacto PPLO agar or broth (Difco). It has been used widely in America for the isolation of mycoplasmas from various animals and man.

The most widely used medium, developed by HAYFLICK⁵⁾, was a modification of the formula described by EDWARD⁹⁾. This consisted of the basal PPLO medium of MORTON *et al.* Enriched with pooled horse serum and extract of fresh baker's yeast, it was employed in the first successful propagation of *M. pneumoniae* on agar. OLSON *et al.*²⁵⁾ recommended "C" medium for the isolation and production of antigen of mycoplasmas. For the same purposes was also utilized Bacto PPLO medium which had been supplemented by inactivated swine serum, DPN, trypticase, soluble starch, and cysteine-HCl. Those authors²⁵⁾ asserted that theirs was, at present, a choice medium, since *M. pneumoniae* and *M. synoviae*, which are probable the most fastidious mycoplasmas, grew well on it.

However, BARBER and FABRICANT³⁾ and other workers reported that some strains were able to grow on artificial media after they had grown in chicken embryos or tissue cultures, and that some strains were obtained from only one variation of one basal medium. At present, no growth medium is available which supports all strains of mycoplasmas. Medium ingredients have been evaluated only with a few species of human and avian origin.

The present paper gives data on evaluation of medium ingredients for the growth of many standard strains of various animal origins. Edward's medium, which has been modified considerably by many workers, was used as the base for the present work.

MATERIALS AND METHODS

Test organisms: Twenty-four strains of mycoplasmas originated from eight animals and sewage were used in this investigation. Of them, seventeen strains were kindly supplied by Dr. D. G. ff. EDWARD in 1963 and were used at the 3rd to 5th passage performed at the authors' laboratory. Five strains of *M. mycoides* had been maintained on horse serum agar slant for more than twenty years at the authors' laboratory. The remaining two strains were fresh isolates. *M. hyorhinis* P4E derived from swine enzootic pneumonia had been adapted to artificial medium at the authors' laboratory from the culture pools provided by TAKATORI *et al.*³²⁾

The details of the strains are shown in Table 1.

Cultivation and storage of strains: Mycoplasmas were cultivated with modified Edward's solid or semisolid medium, or such fluid medium overlaid on horse blood agar slant as prepared after the RM medium of ADLER²⁾ as shown in Table 8.

All cultures were made in modified Edward's semisolid medium, incubated at 37°C for 3 to 5 days, and stored at -20°C.

Ingredients used: The ingredients used in this investigation are summarized in Table 2. The details concerning the preparation of these ingredients are described below.

Infusions: Five fresh infusions prepared from meat of different animal species and a papain-digest of beef liver were tested. A mixture of equal volumes of distilled water and

Table 1. Details of Strains Examined

Origin	Mycoplasma	Strain	
Cattle	<i>M. mycoides</i> var. <i>mycoides</i>	PG-1**	Contagious pleuropneumonia
		Sancho**	
		Ma**	
		Nigo**	
		K**	
		M**	
Goat	<i>M. bovis genitalium</i>	PG-11	Genital tract
	<i>M. laidlawii</i>	PG-10	
	<i>M. agalactiae</i>	PG-2	Contagious agalactia
	<i>M. mycoides</i> var. <i>capri</i>	PG-3	Contagious pleuropneumonia
Pig	<i>M. hyorhinis</i>	PG-29	Glässer's disease
		P4E***	Enzootic pneumonia (SEP)
Horse	<i>M. laidlawii</i>	Hn2 ⁺	Respiratory tract
Dog	<i>M. spumans</i>	PG-13	Genital tract
	<i>M. canis</i>	PG-14	Respiratory tract
Rodent	<i>M. arthritis</i>	PG-6	Arthritis
	<i>M. pulmonis</i>	PG-22	Infectious catarrh (CRD)
	<i>M. neurolyticum</i>	PG-28	Rolling Disease
Man	<i>M. hominis</i>	PG-21	Genital tract
	<i>M. arthritis</i>	PG-27	
Chicken	<i>M. gallinarum</i>	PG-16	Respiratory tract
	<i>M. iners</i>	PG-30	
	<i>M. gallisepticum</i>	PG-31	Chronic respiratory disease (CRD)

* Seventeen PG-strains were supplied by Dr. D. G. ff. EDWARD in June, 1963.

** These four strains had been subcultured at this laboratory for more than 20 years.

*** Isolated in tissue culture by Dr. TAKATORI et al.

⁺ A fresh isolate at this laboratory.

defatted minced meat of each animal species was placed in a cold room at 4°C overnight, and then heated slowly to boil for 2 hours. After that the mixture was filtered, sterilized, and stored at 4°C. Broth media were prepared from each infusions. One per cent phytone and 0.5 per cent NaCl were added to each of double diluted stock infusions. The broth media were adjusted to pH 8.4, heated for 30 minutes, filtered to get rid of precipitable phosphates after EDWARD⁷⁾, and finally adjusted to pH 8.0. In later experiments, modified broth was employed as recommended and the procedure to get rid of precipitable phosphates was omitted.

As control media, Turner's buffered VF-HS medium and Hayflick's "complete PPLO agar" were used.

Peptones: Eight peptones were tested. Of them, three were mixed peptones, one plant peptone, one casein peptone, and three peptones derived from animal tissue. Since a fresh infusion from ox heart was better than any other infusion. It was used as a basal medium in testing various peptones for their ability to support growth of mycoplasma.

Sera: Sera from four species of animals (horse, cattle, pig, and rabbit) were tested. They were not inactivated, but were Seitz-filtered and pooled.

Yeast extracts and other supplements: Three types of yeast extracts, one dried mushroom (*Cortinellus shiitake*) extract and Seitz-filtered bovine rumen fluid, were tested. A fresh yeast extract was prepared by adding 50 g of dry yeast (a product of the Nihon

Table 2. Medium Ingredients Used for Comparison

1. Infusions	
Fresh beef heart infusion	
Fresh horse heart infusion	
Fresh swine heart infusion	
Fresh chicken meat infusion	
Fresh whale meat infusion	
Papain-digest of beef liver (FEINBERG <i>et al.</i> , 1957)	
Control: Bacto PPLO agar (Difco) (CHANOCK <i>et al.</i> , 1962)	
Viande foie (TURNER <i>et al.</i> , 1935)	
2. Peptones	
Bacto peptone (Difco)	} Mixed peptone
Polypeptone (Daigo)	
Bacto tryptose (Difco)	
Phytone (B·B·L)	Papain-digest of soya-bean meal
Trypticase (B·B·L)	Pancreatic digest of casein
Bacto protease peptone No. 3 (Difco)	} Papain-digest of meat
Peptone for toxin production (Daigo)	
Myosate (B·B·L)	Pancreatic digest of heart muscle
3. Sera	
Horse	
Cattle	
Pig	
Rabbit	
4. Yeast extracts and other supplements	
25 % (w/w) fresh yeast (Nitten) extract	
10 % (w/w) yeast hydrolysate (N.B.C.)	
10 % (w/w) yeast extract (Difco)	
10 % (w/w) mushroom (<i>Cortinellus shiitake</i>) extract	
Rumen fluid	

Tensaito Co., Ltd.) to 150 ml of distilled water and boiling the mixture for 30 minutes. After centrifuged at 20,000 G for 20 minutes, it was adjusted to pH 7.8, boiled, centrifuged again, sterilized by Seitz filtration, and stored at -20°C . Two types of dehydrated yeast extracts were dissolved in distilled water at pH 7.8 to make a 10 per cent solution, which was boiled and sterilized by Seitz filtration.

Method of evaluation: The method of evaluation applied is as described below.

Comparison of diluents: To test different diluents, 3-day broth culture of mycoplasmas was diluted in tenfold series in phosphate buffer solution at pH 7.8, Edward's broth, modified Edward's broth, Bacto PPLO broth, and modified Edward's fluid medium. The resultant dilutions were, then, plated on modified Edward's agar medium and Hayflick's agar medium. After incubation at 37°C colonies were counted.

Basis of medium: Modified Edward's agar medium was used as the basis of evaluation for medium ingredient. Namely, when infusions were compared, other ingredients were left, as shown in Table 8. Agar plates were prepared in Petri dishes 9 cm in diameter. They were allowed to stand in the incubator so that the surface of the agar might be dried.

Doses: Each comparison was made between the same lots of stock semisolid cultures used for the 3rd to 5th passage in the authors' laboratory. Overlay medium was inoculated with 10 per cent stock culture and incubated at 37°C for 3 to 5 days. The cultures were diluted in tenfold series in Bacto PPLO broth. In comparing infusions, Edward's broth was used. The method of MILES and MISRA²¹⁾ was applied in which 0.01 ml of each

dilution was placed on the surface of solidified agar.

Conditions of cultivation: The inoculated plates were packed in a polyethylene bag, put in a Petri dish in which wet cotton or gauze had been placed, and incubated at 37°C for 5 to 7 days.

Measurement of growth: Growth was assessed by the colony count method and from colonial size and colonial appearance. Colonies were counted under a dissecting microscope (Olympus type X) at $\times 12.5$ magnification. The number of colony-forming units indicates the power of the respective medium to detect a small number of organisms. Colonial size and appearance were observed under a microscope (Nikon type STR-Ke) at $\times 40$ magnification with $\times 4$ objective lens for metal, by using obliquely transmitted light, and were recorded by photography.

RESULTS

Comparison of diluents: The mean colony counts determined with five diluents are shown in Table 3. In the assay of both *M. hyorhinis* PG-29 and *M. laidlawii* Hn2, where phosphate buffer solution and Edward's broth were used to prepare dilutions of mycoplasmas, unusual counts were encountered. In these assays, one dilution presented thousands of colonies, while the next higher dilution showed a count of zero or very close to zero.

Table 3. Toxic Effects of Diluents for Mycoplasma

Strain	Diluent	Medium*	Mean plate count/0.01 ml**						
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
<i>M. laidlawii</i> Hn2	PBS	A	—	T	T	500	1.6	0	0
		C	—	T	T	250	0.8	0	0
	Edward's broth	A	T	6.8	0	0	0	0	0
		C	T	10.8	0	0	0	0	0
	Modified broth	A	—	T	T	T	134	13.4	1.4
		C	—	T	T	T	125	14.4	1.6
	PPLO broth	A	—	T	T	500	172	19.0	2.4
		C	—	T	T	300	157	24.4	2.6
	Modified Edward's liquid medium	A	—	T	T	T	250	16.2	4.5
		C	—	T	T	T	180	15.8	1.2
<i>M. hyorhinis</i> PG-29	PBS	A	—	600	2.2	0	0	0	0
		C	—	73	0	0	0	0	0
	Edward's broth	A	T	0	0	0	0	0	0
		C	200	0	0	0	0	0	0
	Modified broth	A	—	T	300	82	7.6	0.8	0
		C	—	3.8	0	0	0	0	0
	PPLO broth	A	—	T	T	180	5.2	0.4	0
		C	—	220	12	0.4	0	0	0
	Modified Edward's liquid medium	A	—	T	T	163	15.8	1	0
		C	—	200	20.2	1	0	0	0

* A: Modified Edward's agar medium, C: Hayflick's agar medium.

** Mean plate counts: Average of five plates of modified Edward's medium and Hayflick's medium using five different diluents.

T: Too numerous to count, —: Not tested.

This was also seen in the assay of *M. mycoides* PG-1, Sancho, Ma, Nigo, K, and M, *M. agalactiae* PG-2, *M. gallisepticum* PG-31, *M. neurolyticum* PG-28, and *M. canis* PG-14. Modified Edward's broth, Bacto PPLO broth, and modified Edward's liquid medium were found to have no toxic activity.

M. hyorhinis PG-29 grew better on modified Edward's medium than on Hayflick's medium in this experiment.

Infusions: Table 4 gives the results obtained. Fresh infusion from ox heart was as effective as Hayflick's medium in supporting growth of all mycoplasmas. *M. hominis* PG-21 did not grow on horse heart infusion medium or on chicken meat infusion medium. *M. bovis genitalium* PG-11 did not grow on whale meat infusion medium. *M. bovis genitalium* PG-11 and *M. hominis* PG-21 did not grow on pig heart infusion medium. On liver digest medium, *M. bovis genitalium* PG-11, *M. hyorhinis* P 4 E, or *M. hominis* PG-21 did not grow, but *M. agalactiae* PG-2, *M. hyorhinis* PG-29, *M. spumans* PG-13, *M. neurolyticum* PG-28, and *M. gallisepticum* PG-31 grew poorly. The colonial size observed on fresh beef heart infusion medium, namely modified Edward's medium, was slightly larger than that on Hayflick's medium. Whale meat infusion, liver-digest medium, and VF medium revealed the smallest colonial size.

From these results, fresh beef heart infusion was regarded as the best for supporting growth of mycoplasmas.

Peptones: Table 5 shows that fifteen out of nineteen strains revealed almost the same

Table 4. Values of Various Types of Infusions

Strain \ Infusion							Control	
	Beef heart infusion	Horse heart infusion	Pig heart infusion	Chicken meat infusion	Whale meat infusion	Liver digest	Hayflick's medium	Viande foie
PG-1	6.0*	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Sancho	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Ma	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Nigo	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
K	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
M	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
PG-11	5.0	4.4	0	4.6	0	0	3.8	0
PG-2	6.0	5.0	6.0	5.0	6.0	4.0	6.0	6.0
PG-3	6.5	6.1	6.1	5.9	6.5	6.3	6.0	6.1
PG-29	5.8	6.0	6.0	6.2	6.2	4.0	6.2	0
P4E	4.3	4.5	5.0	5.2	4.7	0	5.1	0
PG-13	3.3	3.3	3.5	3.5	3.3	2.5	3.5	3.2
PG-14	6.0	6.0	6.0	6.0	6.0	5.8	5.8	5.8
PG-6	7.7	7.3	7.5	8.0	7.8	7.4	7.7	7.4
PG-28	7.0	5.0	6.0	6.0	6.0	6.0	6.0	6.0
PG-21	2.8	0	0	0	2.5	0	2.8	0
PG-27	6.6	7.0	7.6	7.0	7.4	6.7	7.3	7.5
PG-16	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
PG-30	5.0	6.0	5.0	5.0	6.0	5.0	5.0	5.0
PG-31	5.0	5.0	5.0	5.0	5.0	4.0	5.0	0
PG-8	6.9	6.7	6.7	6.3	7.0	7.0	6.9	6.0
PG-10	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Hn-2	6.0	6.0	6.0	6.0	6.0	6.0	5.0	6.0

* -log No. of CFU/ml.

Table 5. Values of Various Types of Peptones

Strain \ Peptone	Bacto peptone (Difco)	Polypeptone (Daigo)	Phytone (B-B-L)	Bacto tryptose (Difco)	Trypticase (B-B-L)	Bacto proteose peptone No. 3	Peptone (Daigo)	Myosate (B-B-L)
PG-1	5.4*	5.7	5.6	5.8	5.8	5.5	5.4	NT
Sancho	9.2	9.3	9.5	9.3	9.3	9.3	9.3	9.3
Ma	9.5	9.3	9.5	9.3	9.3	9.3	9.3	9.5
Nigo	8.1	8.7	8.3	8.4	8.3	9.3	9.4	9.4
K	9.6	9.5	9.7	9.6	9.4	9.4	9.6	9.5
M	9.2	9.2	9.4	9.4	9.3	9.4	9.4	9.4
PG-2	9.2	9.1	9.3	9.0	9.0	9.0	9.0	NT
PG-3	8.9	8.7	8.7	8.5	8.0	8.6	8.7	9.9
PG-29	5.5	5.5	5.5	5.4	5.2	5.4	5.4	5.2
PG-14	7.9	7.8	7.8	8.0	7.9	7.8	7.9	NT
PG-6	7.0	8.0	8.0	8.0	8.0	8.2	7.8	8.1
PG-21	5.5	0	5.5	5.4	5.3	3.9	5.5	4.1
PG-27	8.4	6.0	8.0	8.3	6.0	8.0	8.2	NT
PG-16	3.7	3.3	5.3	7.2	5.6	3.6	0	8.0
PG-30	7.0	6.6	6.9	6.8	6.8	6.8	6.5	6.8
PG-31	7.6	7.7	7.9	7.6	7.6	7.6	7.6	7.7
PG-8	8.2	8.2	9.3	8.3	6.0	8.0	8.3	NT
PG-10	6.5	6.5	6.3	6.5	6.3	6.4	6.2	6.7
Hn2	8.3	7.9	8.3	7.0	7.9	8.2	7.6	7.9

* -log No. of CFU/ml.

growth on all the peptone media used. *M. hominis* PG-21 did not grow on polypeptone media. Growth of *M. gallisepticum* PG-31 was completely inhibited on Daigo peptone medium, slightly on medium containing trypticase or phytone, and markedly on Bacto peptone, Bacto proteose peptone No. 3, and polypeptone. *M. arthritis* PG-27 was inhibited by polypeptone and trypticase, and *M. laidlawii* PG-8 by trypticase.

M. agalactiae PG-2 produced "film and spots" on phytone, tryptose, and myosate media, and "spots" alone on trypticase and proteose peptone media.

These results show that Bacto tryptose, Bacto myosate and phytone (B-B-L) were the best.

Sera: Table 6 gives the results obtained. Eleven out of twenty strains revealed almost the same growth on all the serum media used. Growth of the other nine strains was suppressed on some media. All the strains grew well on media containing horse and pig serum. When pig serum was used, only *M. canis* PG-14 grew poorly. When bovine serum was used, *M. mycoides* Sancho and Nigo, *M. pulmonis* PG-22, *M. gallinarum* PG-16, or *M. hominis* PG-21 did not grow, and *M. mycoides* M and *M. gallisepticum* PG-31 grew poorly. The colony size of mycoplasmas, especially *M. mycoides* Ma, *M. hominis* PG-21, and *M. gallisepticum* PG-31, was small on medium containing bovine serum. *M. hominis* PG-21 was the only one, the growth of which was not supported by rabbit serum. On a medium containing rabbit serum, the colony size of *M. mycoides* Ma and *M. gallinarum* PG-16 was slightly small.

M. agalactiae PG-2, *M. bovis genitalium* PG-11, *M. pulmonis* PG-22, and *M. gallinarum* PG-16 produced "film and spots" only on a medium containing horse serum.

These results suggest that horse and pig sera were the most effective for promoting

Table 6. Values of Various Types of Sera

Strain \ Serum	Horse	Cattle	Pig	Rabbit
PG-1	8.5*	8.7	8.7	7.9
Sancho	9.2	0	9.5	8.5
Ma	9.4	9.3	9.4	9.5
Nigo	9.6	0	9.6	8.5
K	9.6	9.7	9.6	9.3
M	9.7	6.3	9.6	8.1
PG-2	9.2	9.3	9.3	9.0
PG-3	9.2	9.0	9.0	9.2
PG-29	5.2	5.4	5.4	5.2
PG-14	8.3	8.2	5.1	8.4
PG-6	8.0	7.0	8.3	8.4
PG-22	5.1	0	5.3	5.3
PG-21	4.5	0	4.4	0
PG-27	7.1	6.3	7.0	7.0
PG-16	6.2	0	6.5	6.5
PG-30	7.3	6.8	7.0	7.4
PG-31	6.2	3.0	5.7	6.3
PG-8	7.4	7.4	7.6	7.4
PG-10	9.2	9.0	9.2	9.0
Hn2	8.7	8.7	8.2	6.7

* -log No. of CFU/ml.

growth of mycoplasmas.

Yeast extracts and other supplements: Table 7 gives the results obtained. All the strains generally revealed the same growth on all the media used. *M. canis* PG-14 and *M. laidlawii* Hn2 grew relatively well on a medium containing no yeast extract, and *M. neurolyticum* PG-28 on media with fresh yeast extract and with yeast hydrolysate. In general, the colonial size of mycoplasmas was small on media containing rumen fluid, but no yeast extract, and large on media with fresh yeast extract and with yeast hydrolysate. It seems that rumen fluid inhibited the film production. Spots were observed mostly on medium containing no yeast extract.

These results show that fresh yeast extract and yeast hydrolysate (N.B.C.) were better supplements for all mycoplasmas.

Throughout the experiments, it was frequently observed that there were differences in colonial appearance and such metabolic products as "film and spots" among medium regardless of the colony count on the medium. It is noteworthy that colonies were small and embedded in agar without any peripheral zone when the medium was poor or when the organism had been inoculated densely. "Film and spots" on horse serum medium appeared well in an area where colonies were fairly dense.

Some examples of the colonial appearance are shown in Plates I and II. Plate I shows the colonies of *M. agalactiae* PG-2. Two types of colonies and spots were seen on media containing no yeast extract. A double peripheral zone appeared on the medium containing mushroom extract. "Film" was poorly produced on the media containing rumen fluid but no yeast extract. Plate II shows the colonies of *M. laidlawii* Hn2. Typical colonies were seen on the medium containing fresh yeast extract. Colonies were small and coarse on the media containing Bacto yeast extract and rumen fluid but no yeast extract.

In conclusion, a fresh beef heart infusion-phytone medium with horse serum and fresh

Table 7. Values of Various Types of Yeast Extracts and Other Supplements

Strain	Yeast extract	Without yeast extract	Fresh yeast extract	Yeast hydrolysate	Bacto yeast extract	Mushroom extract	Rumen fluid
PG-1.		8.3*	8.3	8.3	8.3	8.8	8.3
Sancho		9.6	9.6	9.6	9.5	9.6	9.6
Ma		9.7	9.6	9.3	9.5	9.5	9.6
Nigo		9.5	9.5	9.5	9.5	9.5	9.6
K		9.6	9.6	9.7	9.6	9.6	9.6
M		9.4	9.4	9.6	9.5	9.5	9.6
PG-11		7.9	7.8	7.9	7.8	7.8	7.6
PG-2		9.0	9.1	9.0	9.1	9.1	9.1
PG-3		8.7	8.7	8.4	8.7	8.6	8.7
PG-29		7.5	7.7	7.6	7.3	7.6	7.6
PG-13		7.6	7.5	7.5	7.5	7.5	7.4
PG-14		7.7	6.6	6.7	6.7	6.5	6.8
PG-6		8.3	7.6	8.3	8.4	8.4	8.4
PG-28		6.8	7.9	7.8	7.0	6.8	7.0
PG-21		7.7	7.7	7.6	7.8	7.7	7.7
PG-27		8.0	8.0	8.0	8.0	8.0	8.0
PG-16		8.6	8.0	8.6	8.5	8.6	8.5
PG-30		6.6	6.7	6.7	6.7	6.5	7.2
PG-31		8.7	8.7	8.7	8.7	7.8	8.7
PG-8		8.3	8.3	8.3	8.3	8.3	8.3
PG-10		6.2	6.3	6.3	6.1	6.3	6.2
Hn2		9.8	8.8	9.1	8.8	8.8	8.8

* $-\log$ No. of CFU/ml.

yeast extract was recommended. The formula of the recommended medium is shown in Table 8.

When mycoplasmas were grown in the overlay medium, the blood color of the solid portion of slant was maintained or became more reddish, the fluid portion became greenish by the action of hemolytic strains, and turbidity was more apparent than in simple broth.

DISCUSSION

In this investigation, medium ingredients were evaluated systematically by using twenty-four *Mycoplasma* strains of seventeen species originated from eight different animal species and sewage.

MORTON *et al.*²¹⁾, FABRICANT^{11,12)}, SASAKI *et al.*²³⁾, and ADLER and BERG²⁾ compared medium ingredients using only several strains of human, murine, and avian origin. No systematic experiments have been made on other strains of *Mycoplasma*.

MORTON *et al.*²¹⁾ recommended fresh beef heart infusion, Bacto beef heart infusion, and Bacto brain heart infusion. In the present investigation similar conclusions were reached that beef heart infusion was the best for all the mycoplasma strains used. It must be noted that horse and pig meat infusions, chicken and whale meat infusions, bovine liverdigest medium and VF medium could not generally be used, although they were suitable for certain strains.

MORTON *et al.*²¹⁾, comparing thirteen peptones, recommended three mixed peptones; that is, Bacto peptone, bacteriologic peptone (Parke Davis) and Bacto tryptose. They concluded that Bacto peptone was the best. Of three kinds of mixed peptones which had been tested by the authors, Bacto tryptose was the best one. Bacto peptone

Table 8. Solid, Semisolid, Liquid, and Blood Overlay Media

Modified Edward's media, pH 7.8 (AM-1, SM-1, LM-1)*		
Fresh beef heart infusion agar/broth, pH 8.0		70 ml
Phytone (B-B-L)	1.0%	
NaCl	0.5%	
Bacto agar	1.7% (AM-1)	0.3% (SM-1)
Seitz-filtered unactivated horse serum		20 ml
25% (w/w) fresh yeast extract		10 ml
0.2% DNA from herring sperm		1 ml
M-K ₂ HPO ₄		2 ml
2.5% thallium acetate		1 ml
100,000 U/ml penicillin G		1 ml
Modified Adler's medium (OV-1)*		
Modified Edward's liquid medium (LM-1)		2 ml
5% horse blood beef heart infusion agar slant		1 ml

* AM: agar medium, SM: semisolid medium, LM: liquid medium, and OV: overlay medium.

was toxic for some strains. Polypeptone was the worst. Phytone, that was not recommended by them²¹⁾, and myosate were among the best peptones that were found in the present investigation. SASAKI and NAIDE²⁸⁾ reported that peptones originated from animal meat were superior to casein peptone. Of the three peptones originated from animal meat, myosate was the best one. Bacto proteose peptone No. 3 was toxic for some strains. Daigo peptone was the worst. Casein peptone, trypticase, was not recommended. It is assumed that there is room for improvement in the preparation of commercial peptones.

TANG *et al.*³³⁾, EDWARD⁸⁾ and MORTON *et al.*²¹⁾ reported that guinea-pig, steer, and cow sera were inferior and that rabbit, sheep, and horse sera and human plasma, in appropriate concentrations, were satisfactory. FABRICANT¹²⁾, LECCE²⁰⁾ and OLSON *et al.*²⁵⁾ stated that swine serum was the best for certain swine and avian mycoplasmas. The results of the present investigation indicate that horse and swine sera are the best for all mycoplasmas, and that horse sera have never inhibited the growth of these organisms at 20 per cent concentration. Using rabbit serum medium, the authors could easily cultivate *M. mycoides* and *M. agalactiae*, which had been said to grow poorly on this medium. Since growth on rabbit serum medium is one of the important criteria of identification, it is necessary to re-examine this criterion.

It has been shown that there are marked differences in colonial appearance among the media used, and that production of "film and spots" are influenced by such substance as peptone and yeast extract in addition to serum. These points must be studied further, taking growth phase and speed into consideration.

ADLER and BERG²⁾ and BARBER and FABRICANT³⁾ asserted that different media should be used for isolation, because there was difference in the best ingredient between strains. The present study shows that some strains can grow on all media, while others do not grow on certain media. The latter include *M. mycoides*, *M. gallinarum*, *M. gallisepticum*, *M. canis*, *M. arthritis*, *M. hominis* and *M. laidlawii*.

The authors isolated mycoplasmas at a high frequency from various animals²²⁾ and tissue cultures²³⁾, using the recommended media in combination with agar and overlay medium. They further employed more complex media, which were supplemented by 0.01 per cent DPN and 0.5 per cent cysteine-HCl, for a higher yield of mycoplasmas²⁴⁾.

Toxic activity of saline was reported by KELTON¹⁷⁾, in whose experiment broth was not to be toxic. In the present experiment, however, broth that had excluded the precipitable phosphate exhibit similar activity. The mechanism of this toxicity must be

a subject of further research.

SUMMARY

Various types of infusions, peptones, sera, yeast extracts, and other supplements were evaluated by using 24 strains of 17 species of mycoplasmas originated from 8 different species of animals and sewage.

A fresh beef heart infusion was proved to be the best of the infusions prepared from various animal tissues.

Phytone, Bacto tryptose, and myosate were the best peptones.

Horse and pig sera were the best ones.

Fresh yeast extract and yeast hydrolysate (N.B.C.) were better supplements for all mycoplasmas.

Finally, fresh beef heart infusion-phytone media with horse serum and fresh yeast extract, which were the modification of Edward's medium, were recommended.

For liquid cultivation an overlay medium was recommended. When mycoplasmas were grown in the overlay medium, the blood color of the portion of slant was maintained or became more reddish, and the fluid portion became greenish in the case of hemolytic strains. In this medium, turbidity was more apparent than in simple broth.

Some strains could be grown easily on all the media tested, but others could not on certain media.

The colonial appearance and such metabolic products as the "film and spots" differed markedly with media. Further studies are necessary on colonial morphology and variation taking growth phase and speed into consideration.

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Mycoplasma の 培 地 の 研 究

肉水・ペプトン・血清・イーストエキス類の検討

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Mycoplasma は無細胞培地に發育できる最小微生物で、その認識の第一段階として、人工培地に發育させることが最も必要である。培地については、多くの研究があるが、限られた菌株のみで行なわれている。また、組織培養や孵化鶏卵を使わなければ分離できぬものもあり、分離培地によって、検出される Mycoplasma が異なることが報告されている。現在、一般には、一定種類の培地を使用している場合がほとんどである。すべての Mycoplasma に満足される培地はいまだ得られておらず、Mycoplasma 菌株間の比較は、ほとんど行なわれていない。そこで、まず従来用いられている培地の共通素材の種類によって、各種動物由来の菌株間にいかなる差異がみられるかを検討した。

使用したのは、8 動物種および下水由来の 17 菌株 24 株である。比較した培地素材は、肉水 8 種類、

ペプトン 8 種類、血清 4 種類、イーストエキス類 5 種類である。比較には、固型培地を使用し、集落計算による定量と、透過斜光線照明による顕微鏡写真での集落形態の観察を併用した。実験結果を要約すると、つぎの通りである。

肉水は、新鮮ウシ心筋浸出液がすぐれていた。

ペプトンは、phytone, Bacto tryptose, myosate がすぐれていた。

血清では、ウマ血清がすぐれ、ブタ血清におきかえることもできた。

イーストエキス類では、新鮮イーストエキスおよび yeast hydrolysate (N.B.C.) が良好であった。

Mycoplasma 全般の培地として、ウシ心筋浸出液、phytone よりなる基礎培地に、ウマ血清、新鮮イーストエキスを添加した変法 Edward 培地が推奨される。

Overlay 培地では、発育に伴い、血液寒天斜面部は赤色を維持し、液体部は、溶血性の菌株では緑色を帯び、普通のブイヨン培養より濁りの程度が大で、増殖の確認に便利である。

Mycoplasma には、培地の組成を変えることによって、発育に差があるものと、変わらぬものが認め

られた。

菌株によっては、培地の変化により、集落の形態やフィルム・スポットなどの代謝産物の産生能に著しく差がみられた。集落の形態および変異は、培地、Mycoplasma の発育相、発育速度と関連して、さらに検討されねばならない。

EXPLANATION OF PLATES

PLATE I. Colonies of *Mycoplasma agalactiae* PG-2.

PLATE II. Colonies of *Mycoplasma laidlawii* Hn2.

Figs. 1 and 7. Colonies on media without yeast extract.

Figs. 2 and 8. Colonies on media with fresh yeast extract.

Figs. 3 and 9. Colonies on media with yeast hydrolysate (N.B.C.).

Figs. 4 and 10. Colonies on media with Bacto yeast extract.

Figs. 5 and 11. Colonies on media with mushroom extract.

Figs. 6 and 12. Colonies on media with rumen fluid.

1mm

1mm