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Effect of Penicillin on Bacterial Contamination of Eggs and Tissue Cultures Inoculated with Unfiltered Sputums.

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The isolation of viral agents from the human respiratory tract, by the direct inoculation of tissue cultures or chick embryos with sputum or nasopharyngeal washings, is hindered by various bacteria which are invariably present in the nose and throat, and it is common practice to remove these contaminating microorganisms by filtering the sputum or washings before inoculation. At the same time, however, so much virus may also be lost that the filtrates are non-infective, as Hirst[1] showed very well in the case of influenza virus, and for this reason attention has recently been directed to the use of technics in which filtration is avoided. Rickard, Thigpen and Crowley[2] found that chick embryos inoculated with untreated and unfiltered throat washings from cases of influenza usually survived 48 hours, and that the virus, when present, developed normally in spite of heavy contamination of the egg fluids with a variety of bacteria from the nasopharynx. Eaton, Corey, Van Herick, and Meiklejohn[3] also isolated influenza virus directly in eggs from unfiltered throat washings, and attempted to prevent bacterial contamination of the embryos by preliminary treatment of the washings with Zephiran;[4] but they found that this agent, in concentrations which would not affect the virus, was not consistently effective in removing the microorganisms. More recently, Hirst[5] reported that the inoculation of unfiltered throat washings together with penicillin into the amniotic sac was the most sensitive method of isolating influenza virus in eggs, and that penicillin would satisfactorily control bacterial contamination without interfering with the virus.

In line with these studies we have studied the effect of various antibiotic agents on the bacterial contamination of tissue cultures and chick embryos inoculated with unfiltered sputums, with the purpose of developing a method whereby filtration, with consequent loss of viral agents, could be avoided, but bacterial contamination either prevented or held to a minimum. Preliminary experiments indicated that sulfadiazine, even in high concentrations, was inadequate as a bacteriostatic agent. Zephiran interfered with the development of influenza virus in amounts that controlled bacterial growth. Propamidine and pentamidine were found to be lethal for chick embryos and for tissues in culture. Penicillin, however, gave more favorable results, which form the substance of this report.

Experimental. Fifty-five specimens of sputum from patients with infections of the respiratory tract were examined. Most of these patients had pneumonias of bacterial etiology, or primary atypical pneumonia. A few had an acute catarrhal bronchitis. Two persons exhibited a severe gingivo-stomatitis suspected of being herpetic in origin.

All of the sputums were freshly collected and immediately suspended in equal volumes of physiological saline or nutrient broth by grinding with an abrasive in a mortar, followed by centrifugation at 3000 rpm for 20 minutes in an angle-head centrifuge. The supernatant fluids were then divided into 2 parts, one of which was not treated, while penicillin was added to the other to give a

1 Hirst, G. K., *J. Immunol.*, 1942, **45**, 293.

2 Rickard, E. R., Thigpen, M., and Crowley, J. H., *J. Immunol.*, 1944, **49**, 263.

3 Eaton, M. D., Corey, M., Van Herick, W., and Meiklejohn, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 6.

4 Personnel of U. S. Naval Laboratory Research Unit No. 1, *Science*, 1942, **96**, 53.

5 Hirst, G. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 155.

TABLE I.
Effect of Penicillin on Bacterial Contamination of Tissue Cultures Inoculated with 55 Unfiltered Sputums.

A	
Single tissue cultures inoculated with untreated sputum.	
Sterile	Contaminated
2	53

B		
Paired tissue cultures inoculated with sputum plus penicillin.		
	One sterile	
Both sterile	One contaminated	Both contaminated
31	1	23

TABLE II.
Effect of Penicillin on Bacterial Contamination of Chick Embryos Inoculated with 55 Unfiltered Sputums.
A. Chick Embryos Inoculated with Untreated Sputum (one egg each specimen).

Days of incubation	Survived		Died	
	Sterile	Contaminated	Sterile	Contaminated
1	—	—	None	47
2	—	—	—	1
3	—	—	—	—
4	—	—	—	1
5	6	None	—	—

B. Chick Embryos Inoculated with Sputum Plus Penicillin (2 eggs each specimen).

Days of Incubation	Both embryos survived (34 specimens)		One embryo survived, one died (13 spec.)				Both embryos died (8 spec.)	
			Survived		Died			
	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated
1	—	—	—	—	4	4	3	2
2	—	—	—	—	2	1	3	—
3	—	—	—	—	—	—	—	—
4	—	—	—	—	1	—	2	2
5	68	None	13	None	—	1	3	1

concentration of 500 or 1000 Oxford units per milliliter. The dilution of the treated supernates, as the result of adding the solutions of penicillin, was negligible.

Three tissue cultures, prepared from chopped chick embryo and a modified Simms salt-serum solution,[6] were inoculated with each sputum, one with 0.5 ml of the untreated suspension, and 2 with 0.5 ml of the suspension containing penicillin. The cultures were incubated at 37°C and inspected daily; when turbidity and changes in pH occurred, gram-stained smears were examined microscopically and subcultures were made on rabbits' blood agar.

Each sputum was also inoculated to the amniotic sac of three 11-day-old chick embryos, one receiving 0.2 ml of the untreated suspension, and two 0.2 ml of the suspension containing penicillin. The eggs were incubated at 37° C and candled every 24 hours. When embryos appeared to have died the eggs were opened at once and smears and subcultures made of the allantoic fluids. Embryos that survived were held for 5 days after inoculation and then examined in the same manner.

The results with the tissue cultures are given in Table I. Fifty-three (96.3%) of the 55 single tissue cultures inoculated with untreated sputums showed gross bacterial contamination within 24 hours. Only 23 (41.8%) of the paired cultures inoculated with the same sputums plus penicillin became contaminated, while 31 (56.3%) remained

[6] Rose, H. M., Culbertson, J. T., and Molloy, E., *J. Parasitol.* (Suppl.), 1944, **30**, 16.

sterile for 5 days. With one sputum treated with penicillin, one culture was contaminated and the other sterile.

Differences between the results obtained with treated and untreated sputums were more marked in the eggs than in the tissue cultures, as illustrated in Table II. Forty-nine (89.1%) of the 55 single chick embryos inoculated with untreated sputums died as the result of bacterial contamination, 47 within 24 hours, while either one or both embryos of 47 pairs (85.5%) inoculated with sputum and penicillin survived for 5 days. All of the surviving eggs were found to be bacteriologically sterile.

A variety of microorganisms were recovered from the eggs and tissue cultures that showed bacterial contamination despite the addition of penicillin. In the main these proved to be gram negative bacilli of the Proteus or Pseudomonas groups, together with a number of coliform organisms that were not further identified. Several penicillin resistant strains of staphylococci were also encountered.

Discussion. We observed that the inoculation of fresh unfiltered sputums to developing hens' eggs was almost invariably followed by gross bacterial contamination and death of the embryos within 48 hours. The addition of penicillin to the sputums, however, prevented bacterial growth and permitted survival of the great majority of the embryos. The implication of these findings in respect to the isolation of viral agents from the sputum by direct egg inoculation is apparent.

Herpes virus was isolated on first passage in eggs inoculated with penicillin-treated sputum from the 2 cases of gingivo-stomatitis referred to earlier in this paper. This is the first successful isolation of herpes virus directly in chick embryos and will be described in detail in a separate communication. It is of interest that eggs inoculated with the untreated sputums of the same cases died within 24 hours with failure to recover the virus.

Unfortunately, because of the recent dearth of clinical material, we have been able to examine throat washings from only one case of serologically proved influenza by the method described. In this single instance, however, influenza A virus was readily isolated in eggs by allantoic inoculation of the washings treated with penicillin. Subsequent study of this virus strain in egg passage was greatly facilitated by the absence of bacteria contaminating the allantoic fluids.

In contrast to the favorable results obtained in chick embryos, penicillin failed to prevent bacterial contamination in a large proportion of cultures inoculated with unfiltered sputums.

Summary. The addition of penicillin to unfiltered sputums inoculated to chick embryos prevented bacterial contamination and permitted survival of the embryos in the majority of instances. Less favorable results were obtained in tissue cultures.

Reference is made to the isolation of 2 strains of herpes virus by primary egg inoculation of unfiltered sputums treated with penicillin.