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SEPARATUM

From the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
(Chief: Prof. Dr. AHMED FARID)

Studies on purification and selective isolation of leptospira

AHMED FARID, MAHMOUD HATEM and MOHAMED REFAI

Summary

Purification of leptospiral cultures experimentally contaminated with E. coli, B. subtilis and M. luteus was undertaken using three different methods, namely, animal inoculation, incorporation of antimicrobial agents and filtration.

I. p. inoculation of hamsters and culturing heart blood taken 15—20 minutes post inoculation was effective in purification. Neomycin sulphate and 5-fluorouracil could inhibit the contaminants but not leptospira when added to the medium combined in concentration of 50—100 µg/ml. Filtration, especially of young leptospiral cultures (4 days) through millipore filter with discs of 0.45 µ porosity, was the best method for purification particularly when the cultures were previously centrifuged or coarsly filtered.

Zusammenfassung

Die Reinigung von Leptospira-Kulturen, die experimentell mit E. coli, B. subtilis und M. luteus kontaminiert worden waren, wurde mit drei Methoden durchgeführt. I. p. Applikation an Hamstern ergab gute Ergebnisse, wenn das Herzblut 15—20 Minuten nach der Injektion kultiviert wurde. Neomycinsulfat und 5-Fluorouracil in 50—100 µg/ml hemmten das Wachstum der verunreinigten Bakterien, aber nicht der Leptospiren. Filtration der jungen Leptospiren-Kulturen durch Millipore-Filter (0.45 µ) war die beste und einfachste Methode, besonders wenn die Kulturen vorher zentrifugiert oder mit grobem Filter behandelt worden waren.

Contamination of leptospiral cultures with various types of bacteria is considered as a major problem in any work concerning leptospirosis (BABUDIERI, 1961; GALTON et al., 1962 and ALEXANDER et al., 1970).

Various techniques were used for purification of contaminated leptospiral cultures. Purification by animal inoculation was firstly described by SCHUFFNER (1940). He obtained satisfactory results by using Guinea pigs. On the other hand, ALEXANDER et al. (1970) prefered young hamsters.

Purification by bacterial filters has been described by SEMIBERT (1965) and ALEXANDER et al. (1970). They proved that filtration through bacterial filters was a good and practical method for removal of contaminants from leptospiral cultures contaminated with other bacteria.

Culture media containing antimicrobial substances have been used since long time both for purification and selective isolation of leptospira. STUART (1946) used sulphadiazine and sulphaguanidine. ARMSTRONG (1959) added cycloheximide to the media to suppress growth of contaminating fungi and bacteria in leptospiral cultures. COUSINEAU and MCKIEL (1961) recommended the use of sulphathiazol, neomycin sulphate and actidione in media for selective isolation of leptospira. ALEXANDER et al. (1970) reported that media containing 5-fluorouracil was an excellent selective media. MYERS and VARELADIAZ (1973) used neomycin, and MYERS (1975) found neomycin together with furazolidone to be effective in inhibiting contaminants.

The present investigation was undertaken to evaluate the different methods used for purification of leptospiral cultures contaminated with other bacteria.

Materials and methods

1. Animal inoculation technique

Cultures of *L. ballum*, *L. canicola*, and *L. grippotyphosa* were contaminated with *B. subtilis*, *M. luteus* and *E. coli* respectively. Each of these contaminated leptospiral cultures was injected (1 ml) i. p. into 4 hamsters. Heart blood was obtained 5, 10, 15 and 20 minutes after injection and every blood sample was inoculated into 4 tubes of EMJH medium. All tubes were examined microscopically after 5 days incubation at 30 °C. using the dark ground microscope.

2. Filtration with millipore filter

Millipore filter containing disks of 0.45 µ porosity was used to filter *L. canicola* culture mixed with *E. coli*. The mixture was either centrifuged (10 minutes at 2000 r. p. m.) or cleared by passing through millipore filter paper before filtration to remove the large particles. 0.5 ml of the filtrate was inoculated into each of 4 tubes of EMJH medium and the growth was judged microscopically after 5 days incubation.

3. Incorporation of antimicrobial agents into the medium

Neomycin and 5-fluorouracil were incorporated into EMJH medium at rate of 25, 50, 100, 150 and 200 µg/ml either separately or combined in equal parts of each. These were inoculated with 0.5 ml of 5 days old cultures of *L. grippotyphosa*, *L. ballum* and *L. canicola* at a time, followed by the same amount of 24 hours old cultures of *B. subtilis*, *M. luteus* and *E. coli* respectively. After 24 and 48 hours incubation at 30 °C all tubes were examined by the naked eye for the presence of turbidity indicative of contamination, and a loopful from each tube was streaked onto blood agar plates. On the fifth day the tubes were examined by the dark ground microscope for leptospiral growth.

Results

1. Purification by animal inoculation

Animal inoculation technique using hamsters was found to be effective in purification of leptospiral cultures, especially when blood was taken 15—20 minutes post inoculation. Before and after this period the rate of isolation of leptospira was much reduced (**Table 1**).

2. Purification by filtration

Young cultures (4 days old) of *L. canicola* contaminated with *E. coli* were purified by millipore filter and disks of 0.45 μ porosity. Out of 40 contaminated tubes, 18 tubes could be purified, i. e. a mean of 1.8 out of every 4 tubes. When the contaminated culture was filtered after centrifugation for 10 minutes, or after clearing through ordinary millipore filter paper, the mean of the purified tubes increased to 3.1 and 3.2 out of every 4 tubes respectively (**Table 2**). On the other hand old cultures (14 days old) when treated in the same way were purified with mean values of 0.7, 1.4 and 1.6 respectively.

3. Purification by selective antimicrobial agents

The incorporation of neomycin sulphate in EMJH medium in a concentration of 100 μg/ml resulted in inhibition of the growth of *E. coli*, but not *B. subtilis* and *M. luteus*. The latters were inhibited at concentrations of 150 and 200 μg/ml respectively. In the meantime all three types of the used leptospira showed unrestricted growth at a concentration of 150 μg/ml. In tubes containing 200 μg/ml no contaminating

Table 1: Purification of contaminated leptospiral cultures by i. p. inoculation of hamsters

Group	Organisms	Number and % of isolates of Leptospira at different intervals after i. p. inoculation of hamsters with contaminated cultures			
		5 minutes	10 minutes	15 minutes	20 minutes
1	L. ballum + B. subtilis	+ 25 %	++ 50 %	++++100 %	+++ 75 %
2	L. canicola + M. luteus	+ 25 %	++ 50 %	+++ 75 %	+++ 75 %
3	L. grippo-typhosa + E. coli	— 0 %	++ 50 %	++ 50 %	+++ 75 %

+ to ++++: number of isolates

Table 2: Purification of L. canicola cultures experimentally contaminated with E. coli using millipore filter and disks (0.45 μ)

Serial number of tubes	Filtration of 4 days old cultures			serial number of tubes	Filtration of 14 days old cultures		
	centri-fuged	coarsly filtered	unprep.		centri-fuged	coarsly filtered	unprep.
1	+++	++++	++	11	+	+	—
2	++++	+++	++	12	++	+	—
3	+++	++	+++	13	++	++	+
4	++	++++	—	14	+	+++	—
5	++++	++++	++	15	—	++	++
6	++++	+++	+	16	+++	—	—
7	+++	+++	+++	17	++	++	+
8	++	+++	++	18	++	++	—
9	+++	++++	+	19	+	+	++
10	+++	++	++	20	—	++	+
Total	31	32	18	Total	14	16	7
Mean	3.1	3.2	1.8	Means	1.4	1.6	0.7

+ to ++++: number of tubes with pure leptospira
Total: total number of tubes with leptospira
Means: means of purified tubes out of 4 tubes in 10 trials

Table 3: Decontamination of leptospiral cultures using different antimicrobial agents

Organisms	Antimicrobial agents incorporated in EMJH-medium in μg/ml														
	Neomycin sulphat					5-fluorouracil					Neomycin + 5-fluorouracil				
	25	50	100	150	200	25	50	100	150	200	25	50	100	150	200 μg/ml
L. grippotyphosa	c	c	c	+	—	c	c	+	+	+	c	c	+	+	—
B. subtilis	+	+	+	—	—	+	+	—	—	—	+	+	—	—	—
L. ballum	c	c	c	c	+	c	+	+	+	+	+	+	+	+	+
M. luteus	+	+	+	+	—	+	—	—	—	—	—	—	—	—	—
L. canicola	c	c	+	+	—	c	c	c	+	+	c	+	+	+	—
E. coli	+	+	—	—	—	+	+	+	—	—	+	—	—	—	—

c: contamination
+: growth
—: no growth

bacteria could grow, however, also *L. grippotyphosa* and *L. canicola* were inhibited at this concentration.

The pyrimidine analogue 5-fluorouracil could inhibit *M. luteus* at concentration as low as 50 µg/ml and the other two bacteria at concentration of 100 µg/ml. On the other hand all three types of leptospira could grow unrestrictly up to concentration of 200 µg/ml.

A combined concentration of 50—100 µg/ml from neomycin and 5-fluorouracil suppressed the growth of all contaminating bacteria (**Table 3**).

Discussion

Comparing the three methods of purification applied in this work, it can be concluded that filtration is the best method for purification, especially when it is preceeded by centrifugation at 2000 r. p. m. for 10 minutes or clearing through coarse millipore filter paper. This substantiate the results obtained by Semibert (1965) and Alexander et al. (1970).

The incorporation of antimicrobial agents into the medium was found to be a good and practical method for decontamination of leptospiral cultures. However, it was shown that the different leptospiral serotypes and contaminating bacteria varied in their sensitivity to the various concentrations of these agents. In the present work neomycin was found to inhibit *E. coli*, *B. subtilis* and *M. luteus* at concentrations of 100, 150 and 200 µg per ml respectively. The last concentration was inhibitory also to leptospira, especially in case of *L. grippotyphosa*.

This finding is slightly different from that of Myers and Varela-Diaz (1973) who reported that neomycin was not inhibitory to leptospira serotypes, including *L. grippotyphosa*, up to concentration of 300 µg/ml.

The results obtained with 5-fluorouracil were satisfactory and conform to those of Alexander et al. (1970). However, better results were obtained by using a combined mixture of 5-fluorouracil and neomycin.

Finally the use of laboratory animals is also efficient in purification of contaminated leptospiral cultures, and although it is coupled with some difficulties in application yet it is recommended in case of weak leptospiral cultures.

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Address for reprints:
Prof. Dr. M. Refai,
FAO Food Contamination
Control project, UNDP P.O.B. 30218,
Nairobi, Kenya.