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Research Article

In vitro determination of 5-flurouracil as a selective agent for leptospiral culture medium

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Abstract: The bacteria *Leptospira* is a slender delicate spirochete that will grow in the enriched culture medium comprised of rich nitrogen, vitamin and mineral complex. The main objective of this study is to find out the effect of 5-fluorouracil (5FU) on culturing and maintaining leptospires without contamination. Eight groups of culture media with and without 5FU (six test groups and 2 controls) were prepared and inoculated with leptospires. The results highlighted that culture medium with 5FU in screw capped tubes of both initial 30 minutes opening and not showed any contamination. Very less contamination found in the screw capped tubes without 5FU and cotton plugged tubes with 5FU. But the cotton plugged tubes and screw capped tubes that open in the initial 30 minutes without 5FU showed complete contamination. Thus this study proved the need and importance of 5FU as a selective agent in the isolation and maintenance of leptospires in the laboratory conditions.

Keywords: Leptospires, EMJH, 5FU, contamination

Introduction

Leptospira is a thin delegate spirochete that is actively motile in suitable conditions. It mainly requires carbon, nitrogen, long chain fatty acid, vitamin supplements and minerals. Bovine serum albumin (1%) provide wide growth environment whereas Tween 80 act as fatty acid base, vitamins including thiamine hydrochloride, nicotinic acid and cyanocobalamine enhances the viability and motility[1].

Ellinghausen, McCullough, Johnson and Harris (EMJH), commercially available leptospiral culture medium base comprising the major biochemicals of monosodium phosphate, disodium hydrogen orthophosphate, sodium chloride, ammonium chloride and thiamine. Further this base medium is supplemented with BSA and vitamin solution complex. Some studies highlighted the importance of adding 3% L-asparagin, 1% calcium chloride, 1% magnesium chloride and 1% pyruvate sodium as a supplement for some special studies like isolation of leptospires from bovine semen[2]. In addition 0.2% agar provides semisolid nature of the medium, thereby the growth, motility and viability of leptospires[3]. For serological, biochemical and *in vitro* antileptospiral activity studies, EMJH liquid medium (without agar) is used[4].

The maintenance of *Leptospira* species in culture medium is time consuming and has lot of contamination chances during routine subculturing and observing under dark field microscopy[5]. Liquid culture media is the standard for leptospiral growth mainly useful for diagnosis and antileptospiral studies. Semisolid medium develop the leptospiral growth under the surface (due to microaerophilic nature) called Dinger's ring[6] useful for preservation and research purposes. In solid leptospiral culture media, dense colonies are observing under the surface but extremely slow.

In general, due to the supplementation of this culture medium with BSA and vitamins, the chance of getting contamination is likely to be more than other bacterial culture medium due to the rich nutritional package. To overcome this, a selective agent 5-flurouracil (5-FU) is added with leptospiral culture medium[7,8]. In order to prove the compulsory requirement of 5-FU, the present study initiated to determine the role of 5-FU as a selective agent for leptospiral culture medium in *in vitro* condition.

Materials and Methods

Culture media

EMJH media base (Himedia, India) was prepared as instructions given in the manual. Then BSA and vitamin

solutions were prepared and sterilized by filtration using 0.22µ pore sized filter. The base was added with Tween 80 and agar-agar and sterilized by autoclaving. After sterilization, both base and supplements were added appropriately under aseptic conditions.

Selective agent

The solution of 5 FU (100µg/ ml) was prepared and sterilized by autoclaving. Further it was stored at low temperature until use.

Grouping of test media

After preparation without addition of selective agent, the culture media was divided into 8 groups and described in detail in table 1.

Table 1: Grouping of test media

Group (Six tubes each)	Description
Group 1	Set of culture media with 5 FU in screw capped test tubes
Group 2	Set of culture media without 5 FU in screw capped tubes
Group 3	Set of culture media with 5 FU in cotton plugged test tubes
Group 4	Set of culture media without 5 FU in cotton plugged test tubes
Group 5	Set of culture media with 5 FU in open test tubes
Group 6	Set of culture media without 5 FU in open test tubes
Group 7	dia control
Group 8	ture control with 5 FU

Test serovars and inoculation

Leptospira interrogans serovar australis (pathogenic) and *L. biflexa* serovar patoc (non-pathogenic) were included for inoculation in the culture media groups. Aseptically, all the tubes were inoculated with appropriate serovars. Both the test serovars were microscopically confirmed for its livelity before inoculation to the culture media.

Incubation and growth

All the groups were incubated at 30^{o}C for 4 to 7 days in dark condition. From 4th day onwards the tubes were determined for the growth of leptospires and contaminations. The contaminated tubes were also maintained upto the test days completed. The Dinger's ring is referred as the confirmation of leptospiral growth. The growth and contamination in all tubes were further determined by three methods

1. Observation of Dinger's ring
2. Direct dark field microscopy and
3. Inoculation and observation of any growth in nutrient and Saboroud's Dextrose agar medium.

Results

All the inoculated tubes were observed initially for the presence of Dinger's ring or any other contaminations. The detailed results related to macroscopic determination were depicted in table 2.

Table 2: Macroscopic observation of leptospiral growth

Group	Description	Observation of leptospiral growth				
		Australis (n=3)		Patoc (n=3)		Percentage
		Lepto.	Cont.	Lepto.	Cont.	
Group 1	EMJH with 5FU in screw capped tubes	3	0	3	0	100
Group 2	EMJH without 5FU in screw capped tubes	2	1	2	1	66.7
Group 3	EMJH with 5FU in cotton plugged tubes	1	2	1	2	33.3
Group 4	EMJH without 5FU in cotton plugged tubes	0	3	1	2	16.7
Group 5	EMJH with 5FU in screw capped tubes opened initially for 30 minutes after inoculation	3	0	3	0	100
Group 6	EMJH without 5FU in screw capped tubes opened initially for 30 minutes after inoculation	0	3	0	3	0
Group 7	Media control	0	0	0	0	0
Group 8	Culture control with 5 FU	3	0	3	0	100

[Lepto. - *Leptospira*; Cont. – Contamination]

The formation of Dinger's ring in all groups was determined. The screw capped tubes showed clear ring whereas others showed scanty and mostly no rings. This may be due to the contamination that dominated in the EMJH medium with and without 5 FU. The dark field microscopy (DFM) observation of all the tubes denoted the presence of contaminants due to the sensitive nature of the culture medium. Except the screw capped tube with 5 FU, all others have contamination while observed under DFM.

Eventhough, some leptospiral cells were observed with contaminants, the observation of Dinger's ring on that tubes are totally absent. At the same time, the tubes without contamination have clear ring. This is clearly noted that contaminants do not allow the leptospires to form Dinger's ring. Thus, this study clearly analyzed the need and importance of 5FU as a selective agent in culturing leptospiral serovars in EMJH semisolid medium.

The DFM revealed that all the inoculated tubes have contamination ie, growth of other microorganism along with or without leptospires. It was clearly noted that the high contaminated culture tubes (without any covering) suppressed the growth of leptospires completed than less contaminated tubes (with cotton plug and with screw capped tubes added with 5FU). Scanty leptospiral cells are observed in screw capped tubes without 5FU whereas the screw capped tubes with 5FU showed no contamination. This also suggested that the need of 5FU in EMJH medium for culturing and subculturing the leptospiral serovars.

The inoculated NA and SDA plates showed wide bacterial and fungal growth after appropriate incubation respectively. Group1,5,7,8 showed no bacterial and fungal growth due to

the presence of 5FU and closed environment (avoid the entry of microbial sources from the environment). Eventhough the tubes of group 5 is opened for first 30 minutes of incubation (very less exposure of microbial entry), the 5FU controls and prevent the growth of entered microorganisms. Thus, this study clearly suggested that the need of 5FU in the leptospiral medium for avoiding the growth of contamination from the environment and commensals during sample processing.

Discussion

The growth of leptospiral strains in various chemical and environmental conditions were well studied in this investigation. The role of selective agent – 5FU that control the growth of other microorganisms other than leptospires is proved. The *in vitro* antimicrobial activity of various antineoplastic agents was analyzed by combination with antimicrobial drugs might be more profitable synergistic effects (5FU with beta lactum antibiotics)[9,10,11] but it is the first study to analyze the 5FU alone for antileptospiral activity. Few studies also suggested that the direct antibacterial effects of most antitumour agents are not of sufficient magnitude to alter patterns of infection by themselves in compromised hosts or to interfere with recovery of bacteria by standard culture methods[12].

Various studies highlighted the role of interference of 5FU in different microorganisms. The study analyzed the importance of 5FU that metabolized through fluorouridine monophosphate (FUMP) and affects multiple cellular pathways of *Mycobacterium tuberculosis* (Mtb), mainly the cell wall biosynthesis[13]. The mechanisms of action of 5FU as an antimicrobial may be more complex than solely that of inhibiting DNA synthesis by creating a thymine deficiency in rapidly growing bacterial cells[14]. Even 5 FU can possibly carry by the nanocontainers for drug delivery purposes and also supported for achieving anticancer synergism[15].

The findings of another study showed that the tested leptospiral strains found to be highly resistant to various antibiotics and 5-fluorouracil[16] and the same were proved in this study. Further, the study will be extended to determine the lowest concentration of 5FU that will inhibit the microbial contaminants also to understand the mechanism behind the antimicrobial entity and how the leptospires survive in the 5FU environment.

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