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APPEARANCE OF BLACK PIGMENTS IN THE EMJH SEMISOLID MEDIUM – CULTURAL CONFUSION

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ABSTRACT

Culturing *Leptospira* is the difficult and challenging task for the bacteriologists. But for maintaining leptospiral cultures and to cultivate this spirochete from the infective samples, it is mandatory to prepare the EMJH semisolid medium without any contaminations. There are more chances to get the EMJH medium contaminated. Some major reasons are summarized in this paper. More scientists working in leptospiral research confused themselves by observing the black deposits on the EMJH medium due to some metabolic process of the leptospires and few suggested contamination is the major reason for this issue. This controversy induced us to perform the laboratory test by taking three sets of EMJH semisolid medium. First set of medium inoculated with five leptospiral strains aseptically, second set inoculated with same five leptospiral strains as defined in first set in contaminatable manner and the third set without leptospiral inoculation but induced contamination by artificial open tube method. After incubation, the tubes were analyzed for Dinger's ring formation and black pigments. The uncontaminated set of EMJH cultures showed Dinger's ring only without any black pigmentation. Among the second set of

medium, the tubes inoculated with Javanica, Patoc and Australis showed black pigments to black deposits and black threads found. This may be due to biochemical metabolic reactions by the contaminant mainly due to *Bacillus* sp (a common laboratory contaminant). More analysis required to incubate further, compare with more leptospiral strains and variations in EMJH semisolid medium composition to confirm the issue.

KEYWORDS: *Leptospira,* EMJH semisolid medium, black pigments, contamination.

INTRODUCTION

The bacteria *Leptospira* are grouped under spirochete and considered as obligate aerobes; when cultivated in a suitable aerated medium like Ellinghausen, McCullough, Jansen and Harris (EMJH) at 30^{o}C and an optimal pH of 7.2, their generation time varies from 7 to 12 hours and yields 6-8 x 10^{9} cells/ ml after a week to month incubation.[1] Vitamin B_1, vitamin B_{12} and long chain fatty acids (Tween 80) are the major source for the leptospiral growth. Fatty acids are the main source of energy and carbon required as a source of cellular lipids, since leptospires cannot synthesize fatty acids de nova.

The culture medium used for the isolation, cultivation and maintenance of leptospires has to enrich with bovine serum albumin (BSA) which was introduced instead of using animal serum.[2] Protein free medium have been developed only for use in vaccine production.[3] The usage of long chain fatty acids as a major carbon source and inorganic salts as nitrogen source are mandatory to dispense in the EMJH medium. Leptospires can grow in several media enriched with rabbit serum including Korthof's, Stuart's, Fletchner's, Vervoort's, Noguchi's, Cox's and Stenberg's medium.

Some strains are more fastidious and require the addition of pyruvate or rabbit serum for initial isolation. Later the usage of sodium pyruvate[4] and bovine serum albumin[2] enhance the leptospiral growth. The growth of contaminants from clinical specimens or from the environment can be inhibited by the addition of 1ml of 5% 5-Flurouracil (5-FU) was suggested.[5]

Growth of leptospires on the EMJH semisolid medium is readily visualized as one or more rings of dense growth in approximately one cm below the surface of the medium, although a lack of rings of growth doesnot necessarily means as absence of leptospires. This ring is designated as Dinger's ring which is formed below the air column of the medium due to the

microaerophilic characteristics of leptospires.[1,6] No such rings were observed on the liquid EMJH medium which is very much useful for serological diagnosis – Microscopic agglutination test (MAT)[7,8] and confirmation of isolates – Cross agglutination absorption test (CAAT).[6] Contaminated cultures may be passed through 0.22 or 0.45µm pore sized filter before subculture into fresh medium.[9]

According to the recent confusion among leptospiral scientists regarding the formation of black pigments on leptospiral culture, the previous reports and literature not suggested and supported. Thus a laboratory based analysis was performed to identify any black pigments or deposits or threads on the leptospiral culture medium after incubating appropriately. This study may provide some ideas to leptospirologists and medical scientists to exclude the observation of black pigments as metabolites of leptospires and may include as contaminants.

MATERIAL AND METHODS

The isolation of leptospires from clinical specimens is the backbone of diagnosis and confirms the clinical diagnosis of the disease. Further maintenance of the known leptospiral strains in the laboratory helping us to prepare in-house genus specific Enzyme linked immune sorbent assay (ELISA) microtitre plates[6] and performing serovar specific MAT. Even though varied culture media available to cultivate leptospires, in this study, we prepared and standardized EMJH semisolid medium to analyze the presence of black pigments after appropriate incubation. Leptospires need semisolid medium comprised carbon, nitrogen, certain growth factors, inorganic salts and selective agent for better growth (Table 1) and in this study the same was impregnated.

Table 1: Chemical requirements of leptospiral growth

Ingredients	Concentration	Application
Carbon and Nitrogen sources		
Tween 80[10]	0.05%	Carbon source (enhance the growth – stretching of spirochetal nature by utilizing long chain fatty acids)
Bovine serum albumin (BSA)[11]	1%	Nitrogen source for the cell multiplication and respiration
Growth factors		
Vitamin B_1 (Thiamine)[12]	5mg in 10ml	Metabolic role in the cellular production of energy
Vitamin B_{12} (Cyanocobalamine)[13]	1mg in 1ml	Increase the metabolic rate of flagella and enhance the motility in spirochete
Vitamin B_3 (Nicotinic acid)[14]	1mg in 1ml	Act to suppress the growth of other microorganisms; utilization of

		fermentable carbon sources
Inorganic salts		
Zinc sulphate[15]	0.4%	Membrane stabilization and growth of cells
Copper sulphate[15]	0.3%	
Calcium chloride[16]	1%	Virulent cells resond to higher levels of Mg. Increased levels of Ca did not replace the requirements for higher levels of Mg
Magnesium chloride[17]	1%	
Sodium pyruvate[16]	20%	Reducing the lag periods seen with lower inoculum
Selective agent		
5-fluorouracil[5]	100mg in 5ml	Selective agent; Uracil is a pyrimidine, pyrimidine is lethal to various micro-organisms, but not to leptospires
Agar-agar[18]	0.1%	Solidifying agent; long term maintenance of the cultures

Preparation of 3 sets of EMJH medium

Total of 150ml of EMJH semisolid medium was prepared. The EMJH base and mineral salts' solution were sterilized by autoclaving; on the other hand BSA, vitamin solution and selective agent (5FU) were sterilized by filtration using membrane filter whose pore size is 0.45µm. Then the EMJH base medium, BSA, vitamin solutions and mineral salts' mixture were mixed aseptically and 10ml of this nutrient mixture was dispensed in the sterile screw capped test tubes. The selective agent (5FU) solution was mixed only in the first set of tubes. All the tubes were kept at room temperature for 24 to 48 hours to check the sterility.

The sterility of the medium was analyzed by direct wet mount microscopy and inoculated in the nutrient agar plates. After confirming the medium free from contamination, then the procedure of this work was performed. The 15 screw capped tubes included in this study were divided into 3 sets – each 5 tubes. First set of tubes were used for inoculating selected five leptospiral serovars in aseptic condition and the strains were obtained from Regional Medical Research Centre (ICMR), Andaman and Nicobar islands, INDIA (Table 2).

Table 2: List of leptospiral strains included in the first set of tubes

Serogroup	Serovar	Strains
Australis	Australis	Ballico
Canicola	Canicola	Hond Utrech IV
Javanica	Poi	Poi
Sejroe	Hardjo	Hardji Prajitno
Semaranga	Patoc	Patoc I

All the listed leptospiral cultures were confirmed by CAAT and the cultures were maintained by periodic subculturing in EMJH semisolid medium and sterility were checking then and there. The second sets of tubes were inoculated with the same leptospiral strains as inoculated in first set; but in this set, the inoculation done in a contaminatable manner (inoculation done in open place of the laboratory not in laminar air flow hood). The third sets of tubes were not inoculated with leptospiral cultures but the artificial induction of contamination done. This was performed by keeping the EMJH semisolid medium open (without screw capping) in the laboratory for one hour and then all tubes were incubated at room temperature for leptospiral growth and detecting any black pigment produced or not. Periodic observation was made by observing the tubes for Dinger's ring formation in leptospiral inoculated tubes and other contaminants in all tubes.

The first and second sets of tubes were frequently observed under high power illumination of dark field microscopy for the presence of leptospires alone whereas all the second and third sets of tubes were performed with wet mounting and Gram staining for observing contaminants. A correlation of the contaminants with black pigments was also done by contaminating the variations of components in EMJH combinations. The combinations used in this study were described in table 3.

Table 3: Various combinations of EMJH medium preparations for determining black pigments

Category	Combinations
A	EMJH base alone
B	EMJH base + BSA
C	EMJH base + Tween 80
D	EMJH base + Vitamin solutions
E	EMJH base + Mineral mixture
F	EMJH base + 5FU

This preliminary study was further determined for the metabolic analyses that have been performed to confirm the black pigments were produced by the contaminants not by leptospiral strains.

RESULTS AND DISCUSSION

All the sets of tubes were observed for the leptospiral growth and contaminations. It was found that there was no contamination found in all tubes of set A due to the maintenance of strict aseptic condition while processing the inoculation. In our study, except Patoc and

Javanica, the growth of all strains appeared as Dinger's ring. By direct observation of the tubes, no visual contamination found, further confirmed with no growth on nutrient agar and no bacterial contaminations observed in direct dark field microscopy. And after 7th, 10th, 21st, 28th and 35th days on incubation, no such contaminations found and the tubes were in aseptic condition.

On the 28th and 35th days of incubation, bacterial contamination found on the Canicola and Sejroe tubes where the leptospiral movements and concentrations are reduced if further incubation due to depletion of nutrients (Table 4). But none of the tubes supported the presence of black pigments in this set.

Table 4: Periodic growth determination in set A tubes (EMJH medium without any induction of contamination

Leptospiral serovars	Periodic Observation of growth in EMJH tubes upto 35 days														
	Day 1			Day 14			Day 21			Day 28			Day 35		
	LC	Cont	BP	LC	Cont	BP	LC	Cont	BP	LC	Cont	BP	LC	Cont	BP
Australis	+	NC	NBP	++	NC	NBP	+++	NC	NBP	+	NC	NBP	SC	NC	NBP
Canicola	+	NC	NBP	++	NC	NBP	+++	NC	NBP	+	CF	NBP	NL	CF	NBP
Javanica	+	NC	NBP	+++	NC	NBP	++	NC	NBP	+	NC	NBP	SC	NC	NBP
Sejroe	+	NC	NBP	+++	NC	NBP	++	NC	NBP	+	CF	NBP	NL	CF	NBP
Semaranga (Patoc)	+	NC	NBP	++	NC	NBP	+++	NC	NBP	+	NC	NBP	NL	CF	NBP

[EMJH – Ellinghausen, McCullough, Jansen and Harris medium; LC – Leptospiral concentrations; Cont. – Contamination; BP - Black pigments

+ - leptospires with 4 – 5 cells per dark field microscopy field; ++ - leptospires with 10 – 12 cells per dark field microscopy field; +++ - leptospires in full field; NC – no contamination found; NBP – No black pigments; SC – growth in scanty; NL – absence of leptospires]

In the second sets of tubes, among the five leptospiral strains inoculated, three tubes inoculated the tubes inoculated with Australis, Javanica and Patoc showed more contaminants and also supported the presence of black pigment. In the tubes inoculated with Australis and Javanica showed no even single leptospiral cells, meanwhile dominated with contaminants. In the Patoc tube, one or more leptospirals found in scanty manner with predominated with contaminants.

Further all the contaminated tubes were subjected to Gram's staining to determine the type bacterial contaminants found that to analyze the relationship with the black pigment formation. The Gram's staining and other confirmatory results supported the contaminant as

Bacillus sp, a common laboratory bacterial contaminant. The detailed observations of the sets of tubes with the type of contaminant found were depicted in table 5.

Table 5: Black pigment to black threads observation among leptospiral inoculated and uninoculated EMJH tubes.

Tubes	Detection of contaminants that produce black pigments							
	II set of tubes (EMJH medium with leptospiral inoculation & contaminants)				III set of tubes (EMJH medium without leptospiral inoculation)			
	Gram Staining	Spore Staining	Colonies in NA	Black color detection	Gram Staining	Spore Staining	Colonies in NA	Black color detection
1	G+ bacilli	Have spores	Irregular form & undulate margin	Black pigments	G+ bacilli	Have spores	Irregular form & undulate margin	Black threads
2	-	-	-	-	G+ bacilli	Have spores	Irregular form & undulate margin	Black pigments
3	-	-	-	-	-	-	-	-
4	G+ bacilli	Have spores	Irregular form & undulate margin	Black pigments	-	-	-	-
5	G+ bacilli	Have spores	Irregular form & undulate margin	Black pigments	G+ bacilli	Have spores	Irregular form & undulate margin	Black deposits

[NA- Nutrient agar; G+ - Gram positive]

The third sets of tubes without leptospiral inoculation also subjected for Gram's staining to analyze the types of contaminants. Among uninoculated tubes, 3 tubes supported with black pigments; black deposits and black threads were found among one tube each respectively (Table 5). The comparative description of the table 5 partially described the role of contaminants in the black pigment formation in the EMJH semisolid medium. The predominant contaminant identified in this study is *Bacillus* sp., in all tubes except one tube that supported with *Pseudomonas* and *Staphylococcus*. Further it was noticed that there was no observation of black pigments on the *Pseudomonas* and *Staphylococcus* contaminated tube. Thus this paper identified and supported to some extend that the Gram positive bacilli – *Bacillus* sp., may be responsible for the formation of black pigments. The ranges of black pigments to black threads were observed in the tubes (Table 5). After preliminary confirmation of the contaminants as *Bacillus*, further the isolate inoculated tubes with various combinations of EMJH medium preparations were analyzed for the presence of black pigments to threads. The culturing leptospires are rarely used in the clinical settings because of the need for prolonged culture and the low diagnostic sensitivity, but this technique has an important role in the study of outbreaks and global epidemiology and provides a crucial pool

of clinical strains for studies of pathogenesis.[19] Currently we are doing filtration sterilization for heat labile substances like vitamins and bovine serum albumin solution. But previously some studies highlighted tyndallizing the culture tubes by placing them further successfully in a 56^{o}C water bath for one hour.[20] The best possibility to detect and identify the pathogenic agents to the serovar levels in domestic, wild animals and humans references the cultural method as successful to understand the epidemiology of leptospirosis.[21] No study so far identified that the leptospires produced black pigments to black threads. We forwarded a first report related to black pigment formation in the EMJH medium contaminated with *Bacillus* sp – a common laboratory contaminant and no black pigments observed in any EMJH medium inoculated with leptospiral serovars. The direct dark field microscopy of leptospires showed up against brown background and must be remembered, however, that some of the tissue fibers may turn black so that they may be mistaken for leptospires. Only this dark field microscopy related black color observation was identified and so far no reports found that the black pigments is because of leptospiral metabolites.[20] To analyze this issue completely, further an extension study required by involving leptospiral serovars and to prepare variations in the EMJH combinations and identify whether any metabolic products of *Leptospira* may be resulted in black pigments to black threads.

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