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Identification of compounds for improved growth of Leptospira in culture and isolation

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Abstract

Leptospirosis is a recurring global disease of severe illness involving pulmonary and renal involvement in cattle and humans; that needs attention to cure. The major challenge in treating leptospirosis disease is the diagnosis of the disease. The culturing of an organism is a gold standard for confirmation of the disease. The growth and optimistic identification of an organism require at least eight to fourteen days, because of its slow growth characteristics. We have investigated various media conditions that are prepared based on the wealth of information obtained from 'omic' studies and report a sustainable Leptospira growth medium comprising of serum equivalent elements and nutrients for pyrimidine biosynthesis; which allows a visible growth of organism within two days.

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Keywords

Leptospira, Serum-free medium, Growth medium, visible growth, 48 hours.

Introduction

Leptospirosis is a zoonotic disease caused by pathogenic serotypes of leptospira, an obligate aerobic, tightly coiled spirochaete [1, 2]. The primary hosts of these organisms are domestic and wild animals. The direct or indirect contact with their urine, which contains virulent leptospira species, is the major mode of transmission to humans. The mechanism by which the leptospira species causes the disease remains yet unresolved. Leptospirosis is a grossly neglected disease, and clinical manifestations are highly variable and nonspecific to be diagnostically useful [1]. Leptospirosis can be treated with penicillin-derived antibiotics. The risk of leptospirosis gets aggravated and spread widely during rainy seasons. During periods of flood, leptospirosis may cause severe outbreaks among individuals exposed to the leptospiral-contaminated water. In addition, during the harvesting period, there can be outbreaks of leptospirosis due to an increased chance of contact with infected rat urine. It is estimated that 20% of rats carry leptospira that can infect humans; this percentage may vary depending on the habitat and geographic area of the carrier [2-6]. Leptospira species are slow growers, requires a minimum of eight days for growth and positive identification, [2] their average generation time is about 12-18 hours [7]. The major challenge, in the diagnostics of leptospirosis, is the culturing of the organism. Currently, the disease is widely diagnosed by the Microscopic Agglutination Test (MAT), whose reliability is poor; isolation of the organisms from blood and urine is considered the gold standard to confirm the diagnosis [1].

Studies on the nutritional requirement of leptospira have been carried out since 1930s. Leptospira showed accelerated growth upon the addition of amino acids such as aspartic acid, arginine, glycine, glutamic acid, lysine, and tryptophan, and reduced growth in the presence of arginine, glycine, and methionine [7-11]. It can be grown in a protein-free medium containing inorganic salts, fatty acids and vitamins [12]. Its growth is promoted by Bovine serum albumin, Tween 80, polysorbates, sodium acetate, beef extract, vitamin B12, thiamine, ammonium chloride, and sodium pyruvate [13-18].Leptospira species can be cultivated using a Noble agar base supplemented with 10% rabbit serum, with an initial incubation at 30°C in 5% CO_2 for two days, prior to continuous culture in the air at 30°C for at least 5-8 days [19]. While a number of methodologies and approaches to detect and confirm the presence of leptospira as the pathogen in

clinical specimens; there are very few studies reported in order to achieve faster growth for detection and confirmation [20-24].

Recent genomic studies of leptospira provide a comprehensive analysis of the genes encoding regulatory system, signal transduction, and methyl-accepting chemotaxis proteins, reflecting the organism's ability to respond to diverse environmental stimuli [25-28]. Genomic sequence analysis also reveals, the presence of a competent transport system with 13 families of genes encoding for major transporters which are necessary for the long-term survival of the organism [27]. The proteomics studies provide insights about the mode of pathogenesis, host-pathogen interaction and immune response by the host [29]. In this study, various media were tested for the growth of leptospira; by incorporating combinations of nutrients for which transporters are present in the organism and the nutrients for which enzymes are differentially regulated in pathogen and the host reservoir, based on the genomic and proteomics information respectively [29, 30]; mode of infection and the host environment. Outcome of this study, we report M12 growth medium for the isolation and culture of leptospira that show visible growth in 40 hours.

Materials and Methods

Di-sodium hydrogen phosphate dihydrate, ammonium chloride, glycerol, potassium dihydrogen phosphate, potassium chloride purchased from SDFCL. Magnesium chloride, sodium acetate purchased from AMERSCO. Aspartic acid, sodium pyruvate, tryptone type-1, yeast extract, thiamine hydrochloride, ferrous sulfate heptahydrate, zinc sulfate heptahydrate, sodium chloride, bovine serum albumin, polysorbate-80, vitamin B_{12}, fetal calf serum, asparagine, leptospira Basal medium, leptospira enrichment medium are purchased from HI Media Laboratories Pvt. Ltd. Sodium bicarbonate was purchased from Sigma; glucose, and calcium chloride dihydrate purchased from Merck. N-acetyl glutamine was purchased from the National Biochemical Corporation.

Nutrient selection based on Genomic and Proteomics information

The presence of transporters in the leptospira is analyzed from the Metacyc database [30] and the KEGG database. Nutrients were chosen accordingly, the transporter that can take up nutrients into the cell (Table 1). From the proteomics literature, the nutrients were selected and incorporated for which the host metabolic enzymes were found to be down-regulated during host-pathogen interaction (Figure 1).

Cultivation of Leptospira based on Genomic and Proteomics information

Stuart medium was prepared as per standard protocol, the modified Stuart medium was prepared by substituting aspartate instead of asparagine and excluding glycerol. The pH of both media adjusted to 7.2 and autoclaved. To the cooled media, filter sterilized 10% of Fetal bovine serum was added and 4mL of each medium is distributed in various 15mL capped polypropylene tubes. 100mM stocks of each amino acid (ornithine, lysine, arginine, and histidine), carbon source (100mM stock of sodium acetate and 100mM stock of fructose) and 100mM stock of N-acetyl aspartate were prepared separately, adjusted the pH to 7.2, and filter sterilized. Both the media were supplemented in various combinations viz 1) histidine, arginine, lysine, and ornithine (amino acids); 2) fructose and acetate (carbon sources); 3) N-acetyl aspartate;4) amino acids and N-acetyl aspartate;5) carbon sources and N-acetyl aspartate;6) amino acids and carbon sources;7) amino acids, carbon sources and N-acetyl aspartate;8) without any supplementation. Each combination amino acid consists of 100µL of each amino acid (ornithine, lysine, arginine, and histidine), the combination of carbon sources consists of 100µL of each of sodium acetate, fructose. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium was prepared as per manufacturer's instruction and used as a standard control. Apart from standard control, EMJH medium was also supplemented with the individual nutrients and inoculated with pathogenic serovar. All the tubes were inoculated either with an equal quantity (approximately 10^4 Leptospires/mL) of the pathogenic serovar (*L. interrogans* serovar Pomona) or non-pathogenic serovar (*L. biflexa* serovar Patoc). Each of the medium without inoculum was kept as contamination control. The inoculated tubes along with the controls were incubated at 30°C for four days. After the fourth day of inoculation, each of these tubes were analyzed under dark field microscope and quantified with the standard counting (using a Petroff-Hausser counting chamber) protocol.

Growth of Leptospira based on Habitat

Considering the natural habitat, mode of infection and organelle in which leptospira harbours, various media were prepared individually one after the other (not using any basal medium) that consisting of varying concentrations of urea, oxalic acid, succinic acid and salt concentrations as shown in each column of Table 3. Each medium (each column of Table 3) was prepared individually, pH adjusted, autoclaved and tested one after the other against EMJH medium as standard control. The EMJH medium was prepared as per manufacturer's instruction and used as a standard control, for each medium that was tested. After cooling, 4mL of the each medium was dispensed into the sterile 15mL capped polypropylene tubes. To each tube 10µL of one of the nine different serovars of (4 days old, cultured in EMJH medium) leptospira viz., 1) *L. biflexa* serovar Patoc, 2) *L. interrogans* serovar Hardjo, 3) *L. interrogans* serovar Lai, 4) *L. interrogans* serovar Canicola, 5) *L. borgpetersenii* serovar Poi, 6) *L. interrogans* serovar Icterohaemorragiae, 7) *L. interrogans* serovar Australis, 8) *L. interrogans* serovar Pomona, 9) *L. interrogans* serovar Autmanalis; were inoculated in duplicates and incubated at 30°C. The medium without inoculum used as contamination control (in duplicates). After four days of inoculation, each of incubated tubes were examined for visible growth of leptospires and also analyzed under dark field microscope in comparison with standard EMJH medium qualitatively.

"Holistic Approach" to Growth of Leptospira

With an all-inclusive approach based on the mode of infection, transporters present and replenishing the anaplerotic gaps, various media components were used for the growth of leptospira (labelled M5-M12, M12Q, and M14; Table 4 and 5). Each medium (as shown in each column of Table 4 and 5) was prepared individually, pH adjusted, autoclaved and tested one after the other against EMJH medium as standard control. The EMJH medium was prepared as per manufacturer's instruction and used as a standard control. After bringing the media to room temperature, in a laminar flow hood, 4mL of each medium is distributed into the various sterile 15mL capped polypropylene tubes. To each tube 10µL of one of the nine different serovars of (4 days old, cultured in EMJH medium) leptospira viz., 1) *L. biflexa* serovar Patoc, 2) *L. interrogans* serovar Hardjo, 3) *L. interrogans* serovar Lai, 4) *L. interrogans* serovar Canicola, 5) *L. borgpetersenii* serovar Poi, 6) *L. interrogans* serovar Icterohaemorragiae, 7) *L. interrogans* serovar Australis, 8) *L. interrogans* serovar Pomona, 9) *L. interrogans* serovar Autmanalis; were

inoculated in duplicates and incubated at 30°C. The medium without inoculum used as contamination control (in duplicates). After four days of inoculation, each of incubated tubes were examined for visible growth of leptospires and also analyzed under dark field microscope in comparison with standard EMJH medium qualitatively. The M8 medium was further optimized for carbon source, nitrogen source and salts (M8-M12, Table 4). In order to compare the growth of leptospires in M12, M12Q, M14 and EMJH media; these media were prepared simultaneously on the same day, examined and the study was repeated several times (Table 5).

PCR based detection of Leptospira

After 40 hours of inoculation, 15µL of each of the active culture media was taken separately and heat-inactivated at 95°C for 20 min. The growth of nine serovars of leptospira was detected by PCR, using pathogenic serovars specific primers (FP 5'GCAAGCATTACCGCTTGTGG3', RP 5'TGTTGGGGAAATCATACGAAC3') [31]. Briefly, 5µL of heat-inactivated sample was mixed with 15µL of PCR reaction reagent, carried out by cyclic incubation at 94°C for 15sec, 56°C for 35sec, and 72°C for 40sec for 45 cycles, after initial denaturation of 94°C for 5min. The PCR products were analyzed using a 1.2 % Agarose gel electrophoresis.

Clinical mimetic experiment

In order to mimic the culturing of leptospires from clinical samples; 4mL of M12 medium is distributed into the various sterile 15mL capped polypropylene tubes. To each tube 10µL of one of the nine serovars of (4 days old, cultured in EMJH medium) leptospires viz., 1) *L. biflexa* serovar Patoc, 2) *L. interrogans* serovar Hardjo, 3) *L. interrogans* serovar Lai, 4) *L. interrogans* serovar Canicola, 5) *L. borgpetersenii* serovar Poi, 6) *L. interrogans* serovar Icterohaemorragiae, 7) *L. interrogans* serovar Australis, 8) *L. interrogans* serovar Pomona, 9) *L. interrogans* serovar Autmanalis; was inoculated in the presence of 100µL of freshly collected urine sample from a normal healthy male volunteer and in presence or absence of 100µL of 5-fluorouracil (500mg/10mL stock concentration) for selection and visualized after 40 hours of incubation at 30°C.

Results

The growth of leptospira was analyzed under dark field microscope (Fig. 2) and observations were documented upon supplementation of nutrients based on its genomic and proteomic data (Table 2). The nonpathogenic (*L. biflexa* serovar Patoc) serovar inoculated in tubes containing various combination of nutrients which are added to both Stuart medium and modified Stuart medium, were analyzed under dark field microscope (Fig. 2 panel a and b). Likewise the pathogenic (*L. interrogans* serovar Pomona) serovar containing tubes were also analyzed under dark field microscope (Fig. 2 panel c and d). On comparison, the modified Stuart medium has given a better count, which is equivalent to that of EMJH medium (Table 2). The EMJH medium also modified to check the effect of individual components added (Fig. 2 panel e). In these experiments we observed slight morphological changes, reduction in motility and aggregation or clumping (Table 2). The growth of leptospira upon supplementation to Stuart media or modified Stuart media based on genomic and proteomic information was not convincing.

We reorganized the media compositions based on habitat and mode of pathogenesis, without using any basal medium. Accordingly, various media were prepared by incorporating urea, oxalic acid and succinic acid (Table 3). Nine serovars of leptospira were grown in each of these media with EMJH medium as standard. The use of urea, oxalic acid and succinic acid did not show any growth, but few organisms detected compared to standard EMJH medium. Notably, the medium in which urea was added, showed no growth or scant noticeable growth; when observed under dark field microscope, the organisms were viable in media with high concentrations of urea, even after four days of inoculation (data not shown). The media components were further optimized for filling the anaplerotic gaps (Table 4).

In M8 medium, the organism showed better growth when examined under dark field microscope (data not shown). The M8 medium was further optimized by reducing the concentration of salts. In M9 medium, removal of pyruvate and acetate affected the growth drastically. In M10 medium re-introduction of pyruvate and removal of only sodium acetate did not produce considerable effect. Though the initial concentrations of salts in the media M5 to M8

was fixed arbitrarily, the concentration of various metal ions namely, Na^+, K^+, Ca^{2+} was fixed to concentrations in serum (M12 and M12Q media; Table 5). In M14 medium growth of the organisms were not satisfactory, M12Q medium did not show any visible growth. In M12 medium, all the nine serovars of leptospira showed visible growth of the ring at the bottom of the tube, after 40 hours of inoculation (Fig. 3). We have also tested its growth in glass tubes, the 15ml polypropylene tubes seem to be better for visualization of the ring at the bottom of the tube. Once the M12 medium showed visible growth, the volume of inoculum is reduced to half (approximately 10^3 Leptospires/mL); still it showed the visible growth between 39-40 hours (Data not shown). The minimal inoculum necessary for ring formation still needs to be determined.

The amplification of a 262bp segment of Hap1 gene confirmed the growth of leptospira in M12 medium (Fig. 4). The M12Q medium did not show any visible growth, but it showed a better amplification of the Hap1 gene compared to the EMJH medium. The removal of bicarbonate or sodium acetate or aspartate affected the growth of leptospira (data not shown). The M14 medium (with reduction in composition of media components from M12) is not ideal for the growth of leptospira.

Discussion

Stuart medium constitutes complex ingredients, it also has asparagine. It is a convenient medium to add or remove components for culture studies. The original rationale to use asparagine may be based on its conversion to aspartate by asparaginase. However, the gene for asparaginase enzyme is not present in the leptospira [32] genome (KEGG genome database); thus the rationale for aspartate incorporation in the medium.

Initially, we used urea concentration broadly in the range of urine urea concentrations as organism harbours the kidney. Later, we used urea concentration that is equivalent to that of normal serum urea concentrations or twice the serum urea concentrations in these media (Table 3) as the organism survives first in the blood. These experiments were not successful; the post facto analysis of the leptospira genome indicates the gene encoding urease enzyme was absent

(KEGG genome database). The survival of these organisms in high concentration urea may be due to presence of one or more amido-hydrolase that might show urease like activity [33, 34]. However, the studies prior to 'genomics era', has reported the presence of urease activity in various serovars of leptospira [35].

The rationale for the usage of oxalic acid and succinic acid was to replenish the anaplerotic gaps, so that the organism will be driven towards nucleic acid biosynthesis and subsequently towards cell division. Since the results were not promising; first the carbon source and nitrogen source were fixed by using multiple carbon source (Glucose, sodium acetate and sodium pyruvate) and nitrogen source (casein and yeast extract). The important key to move from one experiment (one medium) to the next experiment (one medium) is to make sure that proper carbon source, nitrogen source and salts are supplied and look for components that can enhance growth (any visible growth). However, such study may not represent an optimal approach, in fact in our approach, after fixing one core reagent, it was necessary to vary the already fixed nutrient composition to optimize growth. Further, we used the salts which will be equivalent in concentration to the serum composition. The organism has several ammonia transporters that take up ammonia into the cell [30]. We have included ammonium chloride in the medium. Most importantly, it needs carbon dioxide for its survival [19], so we included bicarbonate in the medium. Furthermore, the leptospires are actively motile organism; suggesting that the organism has to invest lots of its energy for mobility and the energy metabolism are not the limiting factor for its growth. We have included sodium acetate in the medium that can compensate the energy component without the need for the detoxifying effect of bovine serum albumin.

The fact that an organism is resistant to 5-fluorouracil [36], suggests that leptospires are solely dependent on the de-novo pyrimidine synthesis. Interestingly, the aspartate transcarbamylase enzyme (the first enzyme of de-novo pyrimidine biosynthetic pathway) is secreted by Leptospira into the medium along with other exoproteins [37]. On decreasing the amount of bicarbonate in the M12 medium resulted in a drastic reduction in the growth of leptospira (data not shown); together with the fact that organism down-regulates aspartate-specific metabolic enzymes of the reservoir host. We hypothesize that leptospira's growth is

enhanced by introducing supplements of aspartate and bicarbonate in growth medium, which is necessary for de-novo pyrimidine biosynthetic pathway.

It is interesting observation that even though M12Q medium is identical to that of M12 medium except for N-acetyl glutamine; it is yet unclear why the ring does not appear upon addition of N-acetyl-Glutamine to the medium. Whether ring formation is due to biofilm formation [38] of leptospires, and whether N-acetyl glutamine has role in this needs to be investigated. Further studies are needed on ring formation and the utilization of “acyl amino acids” by leptospires at the biochemical level.

In order to check the growth of leptospira serovar from urine samples in M12 medium; we mimicked the condition by inoculating with Leptospires in the presence and absence of urine obtained from healthy individuals and 5-fluorouracil used as a selective agent. After 40 hours of incubation at 30^{0}C the tubes inoculated with leptospira showed a visible growth ring at the bottom of the tube; visible growth ring with fuzzy appearance was seen in tubes containing 5-fluorouracil and the tubes inoculated with only urine did not show any visible ring formation (a representative of results of nine serovars are shown in Supplementary Figure). The visible ring formation by leptospires is well documented in the literature; but after eight to nine days of initial inoculation in the semisolid medium [2]. In principle, the formation of the ring is an indicator of the profuse growth of leptospires [2]; it is still a challenging task in terms of diagnosis because it requires 4-6 weeks for confirmation of the disease by culture [39]. In the present study the M12 medium reported here shows visible growth (ring) of leptospira by 40 hours of inoculation, which might be helpful in the early diagnosis of Leptospirosis in the future.

Future studies are needed to address the growth of leptospires from the blood samples of patients with active disease. The sensitivity of M12 medium has to be established with different type of serovars at different concentrations and subjected to rigorous appropriate statistical analysis to use it for diagnostics purpose.

Conclusion

We have developed a M12 medium for leptospire visible growth in 40 hours. The M12 medium reported in this manuscript was our modest attempt to develop a culture medium to support the rapid and selective growth of leptospira. The key optimization parameters are obtained from genomic and proteomic literatures, organism's natural habitat and the mode of pathogenesis. This methodology of combining existing proteomics, genomics, natural habitat and pathogenesis information can be used to other slow-growing pathogenic organisms that solely depend on culture method as a gold standard for its disease diagnosis.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Legends

Fig. 1. Host enzymes down-regulated in rats infected with Leptospira (based on proteomic studies [29]).

Fig 2. Dark field microscopy of fourth day culture of Leptospira. a) *L. biflexa* serovar Patoc, Non Pathogenic (NP) organism inoculated in Stuart's (S) medium supplemented with various combinations of nutrients. b) *L. biflexa* serovar Patoc, Non Pathogenic (NP) organism inoculated in Modified (M) Stuart medium supplemented with various combination of nutrients c) *L. interrogans* serovar Pomona, Pathogenic (P) organism inoculated in Stuart's (S) medium supplemented with various combination of nutrients. d) *L. interrogans* serovar Pomona, Pathogenic (P) organism inoculated in Modified (M) Stuart medium supplemented with various combination of nutrients. e) *L. interrogans* serovar Pomona, a Pathogenic (P) organism inoculated in EMJH (E) medium supplemented with individual nutrients.

Fig. 3. The Leptospira cultures in EMJH medium and M12 medium after 40 hours of inoculation. In the M12 medium ring like white layer formed at the bottom neck of the tube. 1) *L. biflexa* serovar Patoc, 2) *L. interrogans* serovar Hardjo, 3) *L. interrogans* serovar Lai, 4) *L. interrogans* serovar Canicola, 5) *L. borgpetersenii* serovar Poi, 6) *L. interrogans* serovar Icterohaemorragiae, 7) *L. interrogans* serovar Australis, 8) *L. interrogans* serovar Pomona, 9) *L. interrogans* serovar Autmanalis and 10) uninoculated media control.

Fig. 4. Detection Leptospira serovars by PCR amplification of Hap1 gene (product size of 260 bp). The lane 1-10, 11-20, 21-30, and 31-40 represents the amplification of Hap1 gene of various Leptospira serovar grown in EMJH, M12, M12Q, and M14 media respectively. The 50bp ladder is labelled as 'L'. The *L. biflexa* serovar Patoc (lanes 1, 11, 21 and 31) was used as cross contamination control. Various serovars tested for the amplification of Hap1 gene are
Lane 1, 11, 21, 31 = *L. biflexa* serovar Patoc
Lane 2, 12 22, 32= *L. interrogans* serovar Hardjo
Lane 3, 13, 23, 33 = *L. interrogans* serovar Lai
Lane 4, 14, 24, 34= *L. interrogans* serovar Canicola
Lane 5, 15, 25, 35= *L. borgpetersenii* serovar Poi
Lane 6, 16, 26, 36= *L. interrogans* serovar Icterohaemorragiae
Lane 7, 17, 27, 37= *L. interrogans* serovar Australis
Lane 8, 18, 28, 38= *L. interrogans* serovar Pomona
Lane 9, 19, 29, 39= *L. interrogans* serovar Autmanalis
Lane 10, 20, 30, 40= Media Control

Supplementary Fig. The representative of results of growth of nine serovars (after 40 hours) in M12 medium inoculated along with urine and in the presence or absence of 5-Fluorouracil.

Table 1. List of transporters, those take up the substrate inside the Leptospira (Metacyc Database).

Substrate uptake: Compounds transported into the cytosol	***L. interrogans*** **serovrs Copenhageni str. 2006007831**
Compounds transported into the cytosol	*(RS)*-Malate
	Bile acid
	Fatty acid
	Heme
	Sugar
	Ammonia
	Amino acid
	Chloride
	D-glucose
	Glutamate
	Proton (H^+)
	Potasssium ion (K^+)
	L-alanine
	Magnesium ion (Mg^{2+})
	Sodium ion (Na^+)
	Phosphate
	Sodium sulfate
	Sucrose
	Sulfate
	Thiosulfate

Table 2. Quantification of Non Pathogenic serovar (*L. biflexa* serovar Patoc) and Pathogenic serovar (*L. interrogans* or serovar Pomona) after the fourth day of inoculation; leptospira grown in EMJH medium, Stuart's medium, modified Stuart medium (Stuart medium devoid of glycerol and consisting of aspartate instead of asparagine); supplemented with combination of nutrients.

Tube Name	**Media**	**Media supplement**	**Serovar inoculated**	**4th day**	
				count	**observation**
S1NP	Stuart	Amino acids (AA)	Nonpathogenic	450	
S2NP	Stuart	Carbon sources (CS)	Nonpathogenic	500	
S3NP	Stuart	N-acetyl aspartate (NAA)	Nonpathogenic	450	
S4NP	Stuart	AA and NAA	Nonpathogenic	200	
S5NP	Stuart	CS and NAA	Nonpathogenic	450	Lengthy
S6NP	Stuart	AA and CS	Nonpathogenic	350	Agglutination
S7NP	Stuart	AA,CS and NAA	Nonpathogenic	615	
S8NP	Stuart	No supplement	Nonpathogenic	240	
M1NP	Modified Stuart	Amino acids (AA)	Nonpathogenic	350	
M2NP	Modified Stuart	Carbon sources (CS)	Nonpathogenic	350	Sluggish, uneven length
M3NP	Modified Stuart	N-acetyl aspartate (NAA)	Nonpathogenic	500	Agglutination
M4NP	Modified Stuart	AA and NAA	Nonpathogenic	400	
M5NP	Modified Stuart	CS and NAA	Nonpathogenic	550	
M6NP	Modified Stuart	AA and CS	Nonpathogenic	400	Agglutination
M7NP	Modified Stuart	AA,CS and NAA	Nonpathogenic	400	Agglutination
M8NP	Modified Stuart	No supplement	Nonpathogenic	750	
S1P	Stuart	Amino acids (AA)	Pathogenic	80	
S2P	Stuart	Carbon sources (CS)	Pathogenic	100	
S3P	Stuart	N-acetyl aspartate (NAA)	Pathogenic	100	
S4P	Stuart	AA and NAA	Pathogenic	60	
S5P	Stuart	CS and NAA	Pathogenic	180	Sluggish
S6P	Stuart	AA and CS	Pathogenic	200	Agglutination
S7P	Stuart	AA,CS and NAA	Pathogenic	100	Sluggish
S8P	Stuart	No supplement	Pathogenic	120	
M1P	Modified Stuart	Amino acids (AA)	Pathogenic	80	
M2P	Modified Stuart	Carbon sources (CS)	Pathogenic	200	
M3P	Modified Stuart	N-acetyl aspartate (NAA)	Pathogenic	200	
M4P	Modified Stuart	AA and NAA	Pathogenic	100	

M5P	Modified Stuart	CS and NAA	Pathogenic	200	Sluggish
M6P	Modified Stuart	AA and CS	Pathogenic	90	Sluggish
M7P	Modified Stuart	AA,CS and NAA	Pathogenic	100	Sluggish
M8P	Modified Stuart	No supplement	Pathogenic	280	
EMJH His	EMJH	Histidine	Pathogenic	100	
EMJH Orn	EMJH	ornithine	Pathogenic	400	
EMJH Arg	EMJH	Arginine	pathogenic	300	
EMJH Lys	EMJH	Lysine	pathogenic	-	No growth
EMJH acetate	EMJH	Sodium acetate	Pathogenic	400	
EMJH NAA	EMJH	N-acetyl aspartate	Pathogenic	350	
EMJH fructose	EMJH	fructose	pathogenic	450	
EMJH	EMJH	-	pathogenic	430	
EMJH	EMJH	-	Nonpathogenic	800	

Table 3. Media components (in mg) used for the growth of Leptospira based on host (Kidney) and natural habitat. The each medium (each column) is prepared individually and tested in duplicates against EMJH medium as standard control.

Components	**M1A**	**M1B**	**M1C**	**M1D**	**M3**	**M4A**	**M4B**	**M4C**
NH_4Cl	-	270	25	25	-	-	-	50
Urea	-	255	-	100	50	50	50	50
Aspartatic acid	15	12	12	12	-	-	-	200
Sodium acetate	15	-	-	-	200	150	200	60
Sodium Pyruvate	100	300	50	50	60	-	60	200
Succinic acid	150	-	65	50	200	250	200	200
Oxalic acid	325	-	50	50	200	250	200	200
Glucose	-	300	-	100	200	100	200	-
Fructose	-	-	-	-	-	100	-	-
Glycerol	5mL	-	-	-	-	-	-	-
Benzoic acid	10	-	-	-	-	-	-	20
$KHCO_3$	10	25	-	-	20	-	20	-
NaCl	-	-	100	100	-	-	-	-
$MgCl_2$ anhydrous	10	-	5	5	-	-	-	10
$ZnSO_4.7H_2O$	-	-	10	10	-	-	10	20
$FeSO_4.7H_2O$	20	-	10	10	10	15	20	10
$CoCl_2$ $2H_2O$	15	35	20	20	5	10	10	-
$CuCl_2$	-	-	-	10	-	-	-	-
$MnCl_2$	3-5	-	-	-	-	-	-	-
$CaCl_2.2H_2O$	-	-	10	10	-	-	-	-
KH_2PO_4	150	-	125	125	200	150	200	200
Na_2S	1	-	-	-	-	1	-	-
Final pH at 25^0C	7.2 +/- 0.2	7.2 +/- 0.2	7.2 +/- 0.2	7.2 +/- 0.2	7.2 +/- 0.2	7.2 +/- 0.2	7.2 +/- 0.2	7.2 +/- 0.2
MilliQ water	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL

Table 4. Media components (in mg) used for the growth of Leptospira based on; mode of infection, transporters present and replenishing the anaplerotic gaps. The each medium (each column) is prepared individually and tested in duplicates against EMJH medium as standard control.

Components	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**	**M11**	**M12**
NH_4Cl	25	85	25	15	15	15	15	15
Aspartatic acid	12	133	133	40	40	40	15	15
Asparagine	10	-	-	-	-	-	-	-
Casein	-	-	-	-	140	100	100	100
Glucose	-	125	120	120	120	120	100	100
Sodium acetate	25	80	-	30	35	-	-	35
Sodium Pyruvate	50	50	50	60	-	60	6	6
Succinic acid	65	65	65	150	-	-	-	-
Oxalic acid	50	50	50	-	-	-	-	-
Benzoic acid	-	12	-	-	-	-	-	-
$KHCO_3$	15	250	250	-	-	-	-	-
$NaHCO_3$	-	-	-	100	100	100	40	40
Yeast extract	-	-	-	-	125	50	50	50
Thiamine HCl	-	2	2	2	2	2	2	2
NaCl	100	75	100	80	80	80	-	-
$MgCl_2$ (anhydrous)	-	5	5	17	17	10	15	15
$CaCl_2.2H_2O$	-	-	-	-	-	-	13	13
$CoCl_2.\ 2H_2O$	50	5	5	-	-	-	-	-
$CuCl_2$	7	4	5	-	-	-	-	-
$MnCl_2$	2	2	2	-	-	-	-	-
KCl	-	30	30	-	25	20	20	20
KH_2PO_4	125	-	-	-	-	24	10	10
$Na_2HPO_4.\ 2H_2O$	-	89	89	50	10	144	100	100
$Na_2MoO_4.2H_2O$	5	5	5	-	-	-	-	-
Na_2S	1	1	1	1	1	1	1	-
Final pH at 25^0C	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
MilliQ water	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL

Table 5. Composition (in mg) of EMJH, M12, M12Q and M14 media.

Components	EMJH	M12	M12Q	M14
$Na_2HPO_4.\ 2H_2O$	100	100	100	100
KH_2PO_4	30	10	10	10
Tryptone	-	100	100	100
Yeast Extract	-	50	50	50
KCl	-	20	20	10
Aspartatic acid	-	15	15	5
Sodium Pyruvate	-	6	6	6
Glucose	-	100	100	100
$MgCl_2$ anhydrous	1	15	15	5
$CaCl_2.2H_2O$	1	13	13	3
NH_4Cl	25	15	15	7.5
Thiamine HCl	0.5	2	2	1
Sodium acetate	-	35	35	7.5
$NaHCO_3$	-	40	40	40
N-acetyl Glutamine	-	-	15	-
NaCl	100	-	-	-
$ZnSO_4.7\ H_2O$	0.4	-	-	-
$FeSO_4.\ 7H_2O$	5	-	-	-
$CuSO_4.\ 2H_2O$	0.03	-	-	-
BSA	1000	-	-	-
Polysorbate 80	1.25mL	-	-	-
100% Glycerol	0.5mL	-	-	-
Vitamin B12	0.02	-	-	-
MilliQ water	100mL	100mL	100mL	100mL
Final pH at 25^0C	7.2	7.2	7.2	7.2

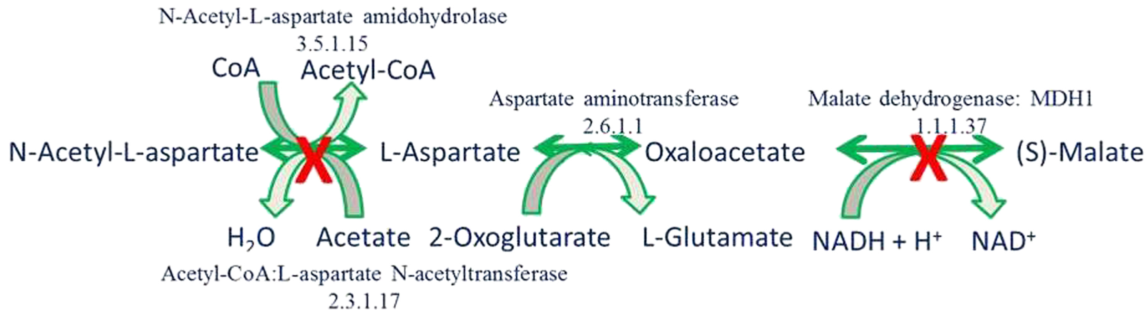


Figure 1

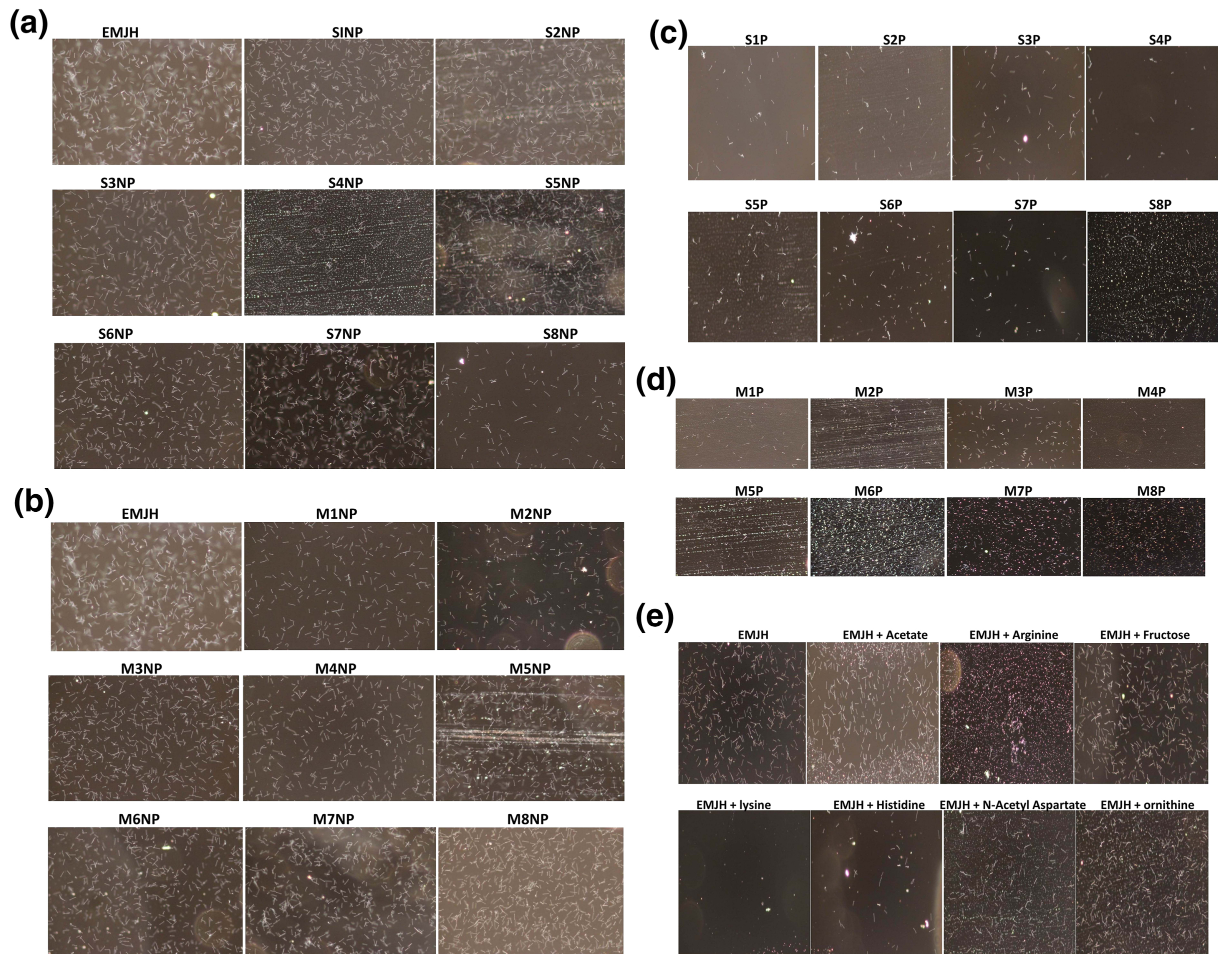
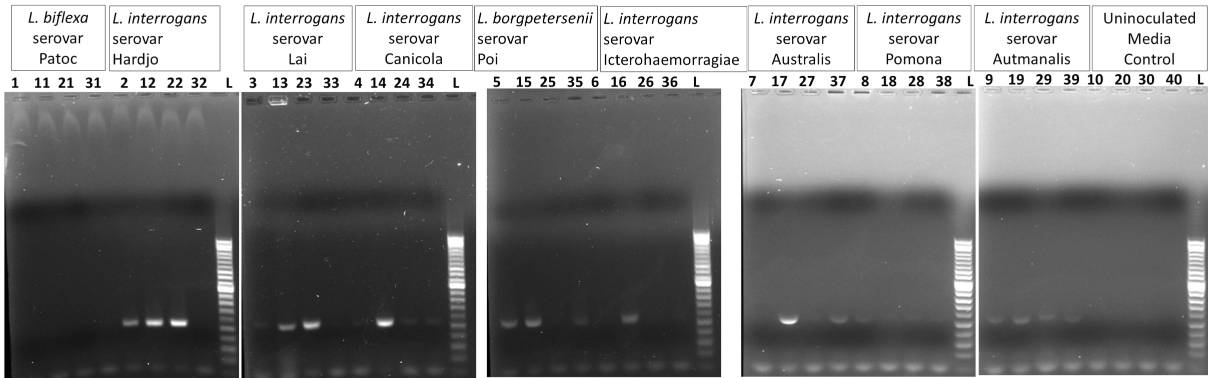


Figure 2

1 M12
1 EMJH
2 M12
2 EMJH
3 M12
3 EMJH
4 M12
4 EMJH
5 M12
5 EMJH

6 M12
6 EMJH
7 M12
7 EMJH
8 M12
8 EMJH
9 M12
9 EMJH
10 M12
10 EMJH

Figure 3



1-10=EMJH Media, 11-20=M12 Media, 21-30=M12Q Media, 31-40=M14 Media

Figure 4