ULTRASTRUCTURAL MORPHOLOGY STUDY AND OPTIMIZATION OF GROWTH CONDITIONS FOR *LEPTOSPIRA INTERROGANS*

Kokxin Fang[1], Maizah Mohamed Yatim[2] and Norefrina Shafinaz Md Nor [1,*]

[1]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[2]Electron Microscopy Unit, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

*efrina@ukm.edu.my

Abstract. Leptospirosis is one of the often-neglected fatal zoonotic diseases endemic to most developing countries. The disease transmits mostly through contact of rodent urine contaminated with pathogenic *Leptospira* in the environment. The objectives of this study were to observe and understand the varied *Leptospira interrogans* ultrastructures, and to optimise the growth condition of the bacterium to better prepare for further evaluation and making of treatments against the bacterium. The ultrastructure of the bacterium was investigated using transmission electron microscopy. In this study, we have documented the varied terminal regions (hooked-end, entangled end, and horseshoe structure), Gram-negative-like cell wall, protoplasmic cylinder with or without flagella, detached flagella, cytoplasmic membrane, cytoplasmic formations extruding the cell wall, a variety of bud and outer membrane vesicle formations, and other peculiar features unique to *Leptospira*. These ultrastructures could be influencing the pathogenicity of a *Leptospira* strain but information on them is not well reported, hence the need for more morphological studies on this genus of bacteria. Furthermore, we investigated the growth of the bacterium by comparing the growth curves under different conditions. The necessity of serums and shaking incubation for faster growth and higher *Leptospira* yield were determined. It was observed that shaking incubation was essential for its optimum growth as the bacterium is aerobic. This study focuses on the many basics of *Leptospira interrogans* that were not much mentioned or discussed in other studies and would pave way for more detailed investigations of these characteristics and ultimately help in understanding possible approach for diagnostic and treatment in future.

Keywords: *Leptospira interrogans*, transmission electron microscope, growth condition

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Introduction

Leptospira interrogans is one of the dominant *Leptospira* species causing human leptospirosis in tropical countries like Malaysia [1]. The bacterium is often transmitted from rodents to humans, either through direct contact with the animal's urine, or most of the time, accidental indirect exposure via urine-contaminated environmental water and soil. *L. interrogans* reproduces in the rodent's kidneys and may be secreted out following urination. Despite the pathogenic *Leptospira* does not replicate outside mammalian hosts in the natural environment, it has remarkable survival capacity and can stay viable in sewage sludge, soil or animal waste slurry for months [2]. When in contact, the bacterium is transmitted into humans via skin or mucous membranes and may replicate in the liver and kidneys. The systemic *Leptospira* infection can induce activation of macrophages, act as a B-cell mitogen, and cause severe multi-organ manifestations [3], which could sometime be fatal. Risk for infection is high among workers in sewers, farmers, and those who take part in recreational environmental water activities.

Ultrastructural constituents of the *Leptospira* can affect both its pathogenicity and virulence. For instance, the diversity of antigenic composition among pathogenic *Leptospira* species are believed to be determined by their lipopolysaccharides (LPS) [4], that made up part of the major outer membrane components of *Leptospira*. Due to the varied LPS composition, different *Leptospira* species could have the same antigenic composition (serovar), vice versa [4]. For example, the serovar '*hardjo*' could be found in both *L. interrogans* and *L. borgpetersenii* species, but the *L. interrogans* species alone comprises multiple serovars, including '*icterohaemorrhagiae*' and '*copenhageni*' [5]. Despite these significant differences, most pathogenic *Leptospira* species cause diseases that require similar ministrations. The ultrastructural components of *Leptospira* are also important in vaccine development [6]. Furthermore, detailed descriptions of *Leptospira* ultrastructures observed via electron microscopy were not much published [7], despite their significance in determining the virulence of the bacterium. Our study aims to observe in detail each ultrastructure of *Leptospira interrogans.*

Growth of *Leptospira interrogans* is slow and the bacterial yield is low [8]. The bacterium is obligately aerobic, fastidious and requires unique supplementary factors for growth like vitamins B1 and B12. It is sensitive to pH and temperature, and its source of energy is long-chained fatty acids [9], hence the frequent use of serums in its cultivation [10]. Various optimization and improvements have been made to the media for *Leptospira* growth such as Fletcher, Korthoff, Noguchi, and Stuart media [11], but the Ellinghausen-McCullough-Johnson-Harris (EMJH) are the most used in studies. Nevertheless, in comparison to most other bacteria, *Leptospira* is still considered slow growing which causes the routine isolation of the bacterium from human tissues and fluids not often attempted [9]. Hence, the optimization of its growth is important to speed up future experiments.

Materials and Methods

Bacterial Strain and Transmission Electron Microscopy

Leptospira interrogans (pathogenic strain) for screening and optimisation purposes is provided by Dr Vasantha Kumari Neela from University Putra Malaysia. To observe the

ultrastructure, the bacterial samples were negatively stained with uranyl acetate and viewed through electron microscopy in accordance to the published protocol.

Growing the Bacterium

The bacterium was grown in 7 ml BD Difco™ EMJH liquid medium (Ellinghausen–McCullough–Johnson–Harris) with addition of BD Difco™ *Leptospira* enrichment EMJH at 30 °C. The culture was incubated with or without shaking at 180 rpm depending on the objective of the experiment. The growth of the bacterium was tracked by observing the increase in optical density (OD) readings at 405 nm wavelength for 28 days. [12]

Results and Discussion

Ultrastructural Morphology of L. Interrogans

Leptospira terminal regions was observed through TEM. As shown in Figure 1, observing *Leptospira interrogans* through TEM using uranyl acetate negative staining method conferred clear and detailed images of the bacteria, allowing the analysis of their ultrastructures. The bacterium is a thin (~100 nm in diameter), aerobic and motile organism, with helically coiled long body, hence classified in the *Spirochaetes* class. The first structures we observed are the varied terminal regions (Section a), followed by the *Leptospira*'s body/protoplasm cylinder (Section b), and then the extra features (Section c) that occasionally occurs in the immediate vicinity of the bacterium.

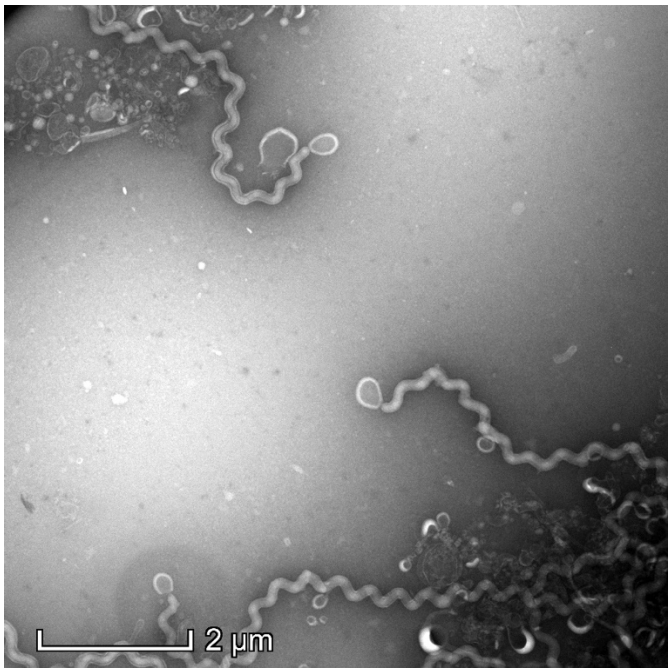

Figure 1: TEM micrographs of *Leptospira* terminal regions.

a) Terminal Regions

There are basically 3 types of terminals observed in *Leptospira interrogans*: the horseshoe structure (Figure 2(a)), the entangled end and the hooked end (Figure 2(b)). The extra features that we observed include buds and cytoplasmic formations.

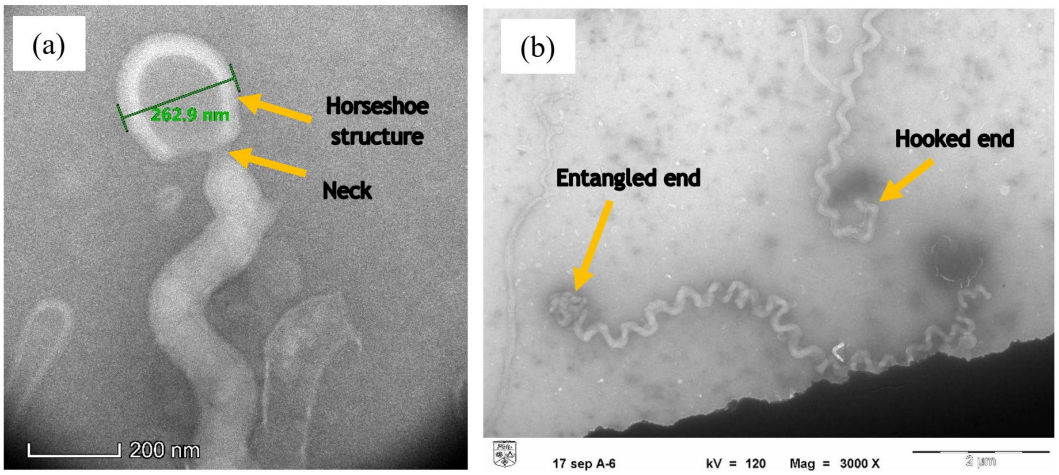

Figure 2: TEM micrographs of *Leptospira* terminal regions (a) horseshoe structure and (b) entangled and hooked ends.

The hooked end of *Leptospira* is what makes the bacterium differ from other spirochetes. The hooked end is formed due to the flagella rotation and is situated at the left side of the bacterium if it is moving towards the right, vice versa [7]. In other words, it serves as the tail of the bacterium. The entangled end is observed occasionally. There are several articles that published images of *Leptospira* with entangled ends, one describing it as 'cyst' [7] but another did not mention about it [13]. To the best of our knowledge, the reason behind the occurrence of these ends and their functions are still unknown. Interestingly, we have frequently observed the horseshoe structures at many *Leptospira* terminal regions. From the figure, we could see that the cytoplasm bridges across from the bacterium to the horseshoe to form a neck. Most studies we came across categorized this structure as bud formations which will bud off and form outer membrane vesicles [14]. However, it is obvious that the horseshoes do not resemble the cell wall or outer membrane, and the cytoplasm is exposed to the environment instead of forming a vesicle. For these reasons, we believe the horseshoe structures differ from bud formations. A study described it as 'terminal appendage/structure' [7]. Nevertheless, further study into this structure is required.

b) Protoplasmic Cylinder

The bacterium has a gram-negative-like cell wall, surrounding the body/protoplasmic cylinder, and filaments that wind around the body. This structure can be observed in Figure 3. The protoplasmic cylinder is about 0.1 µm in diameter but differs in lengths. Generally, one helical wavelength is around 450 nm, and more helix means a longer bacterium (Figure 3(a)). The average length of *Leptospira* is around 6–20 µm [8].

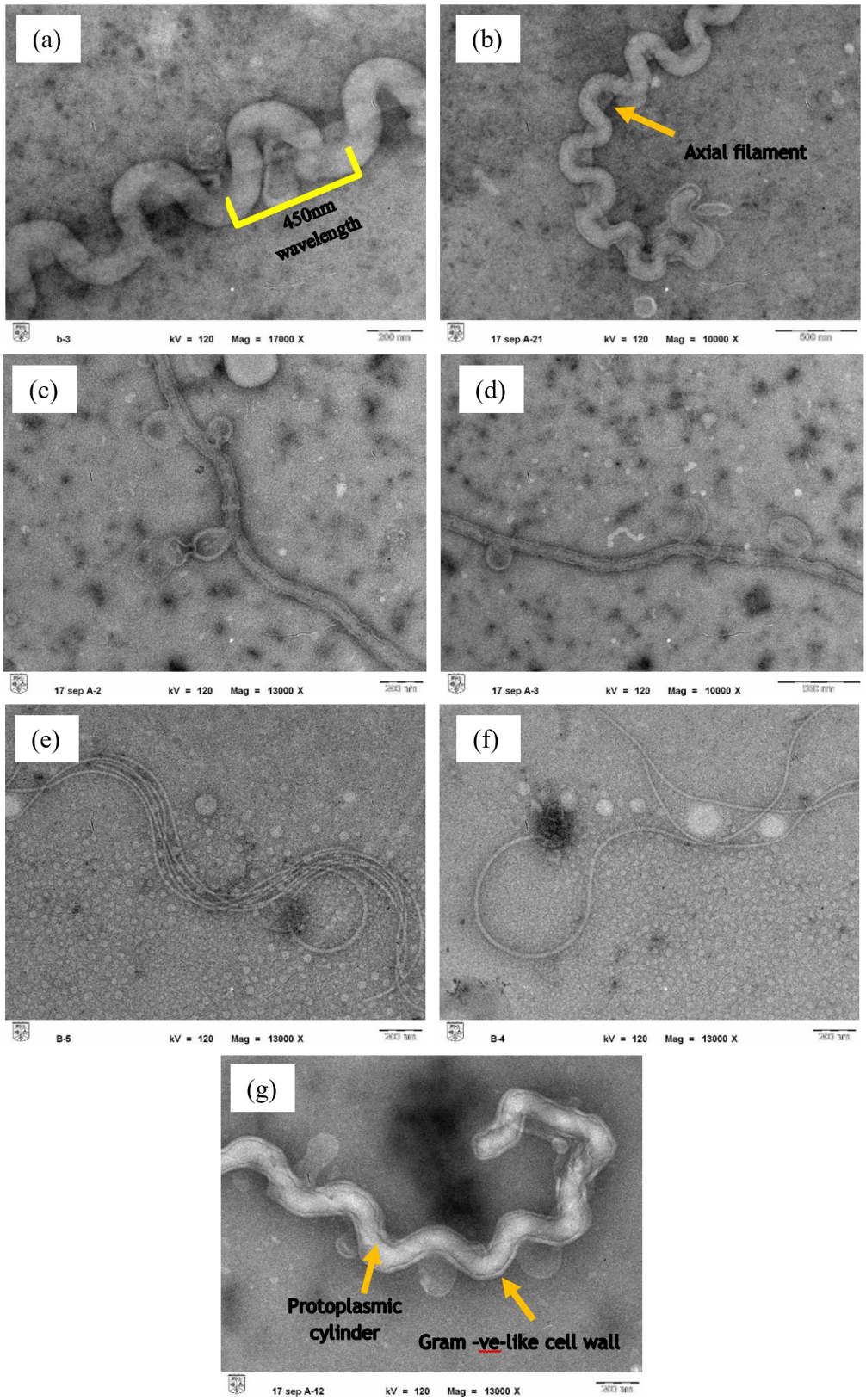

Figure 3: TEM micrographs of *Leptospira* (a,b) clear images of the bacteria, (c,d) *Leptospira* without axial filaments, (e,f) the lost axial filaments and (g) clear image of *Leptospira* protoplasm and cell wall.

The ultrastructures of a particular strain of pathogenic *Leptospira* are key factors in determining its virulence. The axial filaments, or flagella, are present in the periplasmic space of viable *Leptospira* and are responsible for motility (Figure 3(b)). However, in some of the cells this structure was not observed (Figure 3(c) and (d)), while some flagella are found to have detached from the cells (Figure 3(e) and (f)). Although the reason behind this loss is unknown, the effects should include immobility [15] and dissipation of the bacterial helical shape [7]. These flagella separations also prove that the filaments were coiled around the bacteria and did not cross into the middle part of the cell. From this figure we could clearly observe the protoplasm and cell wall of *Leptospira* (Figure 3(g)). The interesting thing about *Leptospira* cell wall is that it falls under the Gram-negative category, but uniquely differs from other Gram-negative bacterial cell walls. This is due to the peptidoglycan layer within the *Leptospira* cell wall is associated with the cytoplasmic membrane rather than the outer membrane like most other bacteria [16].

c) Extra Features - Globular Structures (Buds)

Images of the bud formations of Leptospira was observed and shown in Figure 4. Globular formations (buds) were observed in some cells and originated from either the outer membrane (Figure 4(a)) or the cytoplasmic membrane (Figure 4(b)).

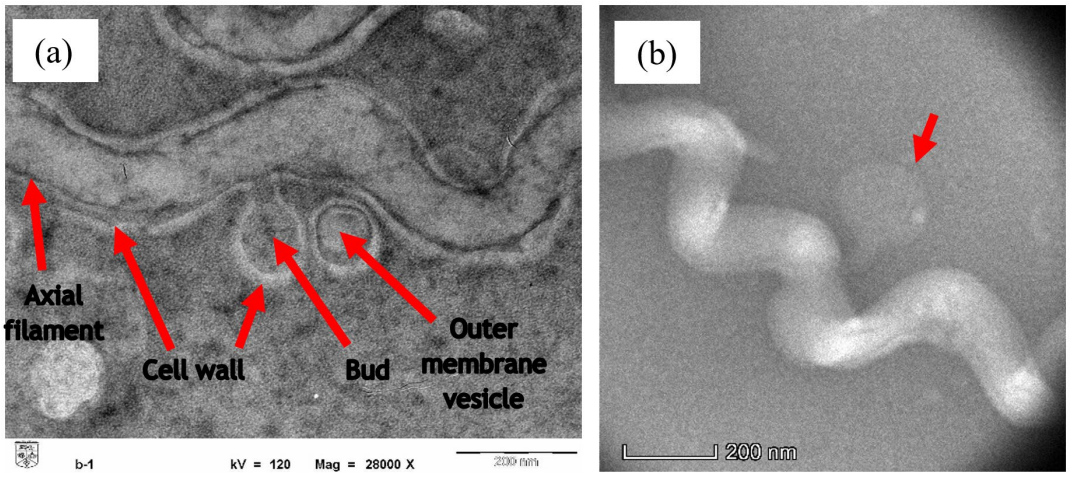

Figure 4: TEM micrographs showing the bud formations of *Leptospira*

These reoccurring structures that look like buds are commonly present in most Gram-negative bacteria [17]. They originate from the cytoplasm throughout the cell and might detach from the bacteria to form outer membrane vesicles. The production of these vesicles allows bacteria to interact with their environment, including promoting pathogenesis, enabling bacterial survival during stress and regulating microbial interactions within bacterial communities [14]. We also observed many cytoplasmic formations that most of the time look like huge bacteriophages. The function of these formations has yet to be elucidated and are presumed to form due to internal pressures. The shape of these cytoplasmic formations is not constant, as shown in Figure 5(a) to (c). It was described as 'globose formations' in a study [7]; similar structures were also captured under the TEM from another study [18], albeit not much being mentioned.

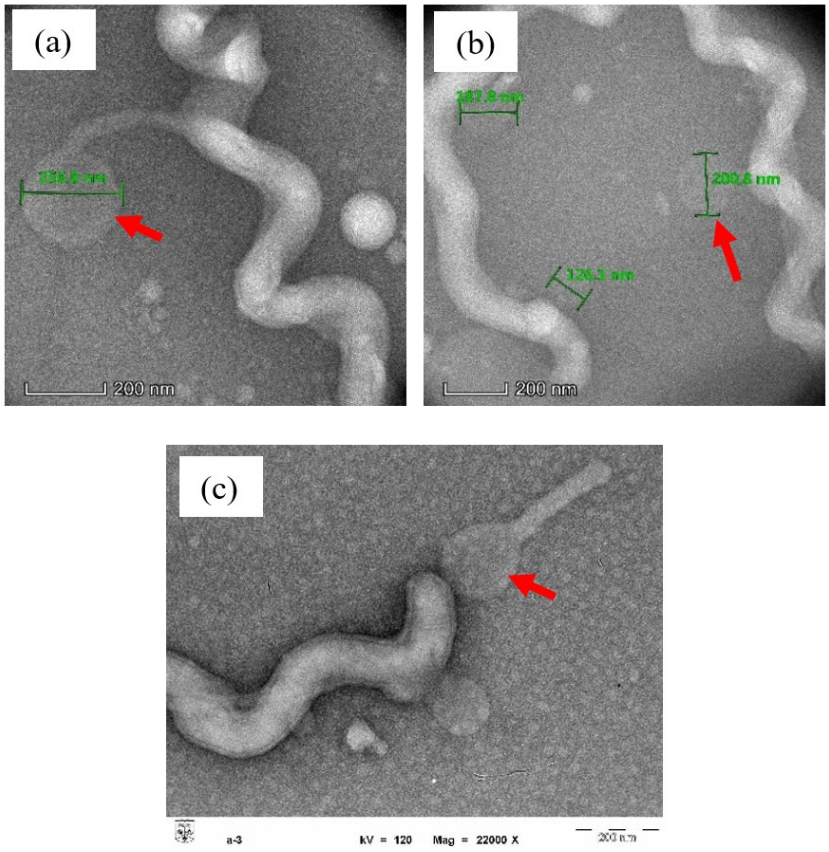

Figure 5: Different TEM micrographs of the bacteriophage-like bud formations of *Leptospira.*

These formations with diameters much greater than the width of the *Leptospira* and originate from within the cytoplasm are located indistinctly throughout the cell. Inconsistent bud sizes were observed, hence the assumption that they were formed due to internal pressures.

Some embedded buds protruding from the periplasmic cylinder were also observed in the *Leptospira* (Figure 6(a) and (b)). These formations resemble granules and are called 'blebs' [19].On the other hand, outer membrane vesicles detached from the bacteria like the ones shown in Figure 6(c) and (d) had cell walls surrounding them and are presumed to have bud off from the organism. The buds as seen in Figure 6(e) to (h) could be similar to the bacteriophage-like formations in Figure 6(a) and (b) but with more elongated structures.

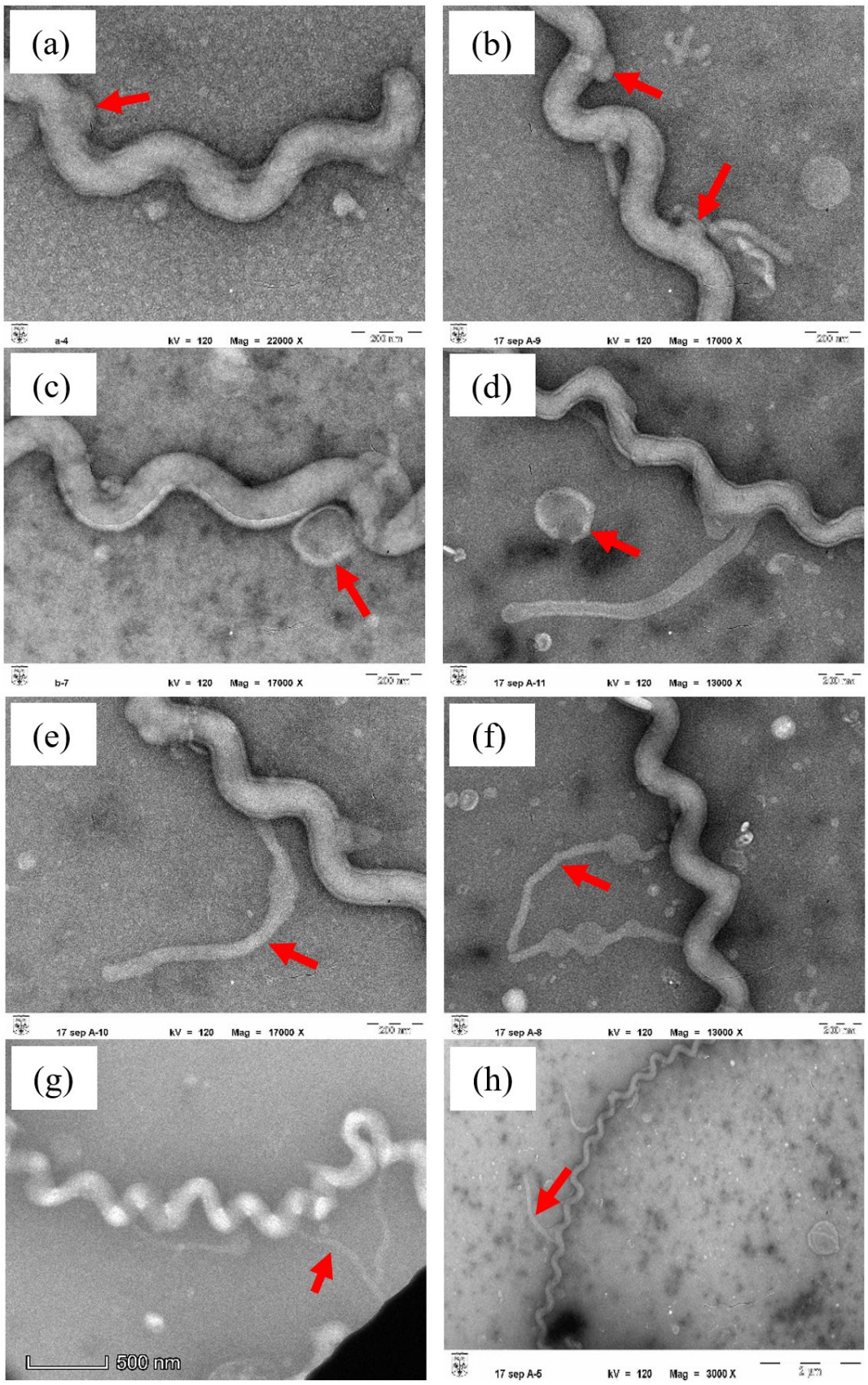

Figure 6: TEM micrographs of (a,b) the embedded bud formations, (c,d) the detached buds (outer membrane vesicles), and (e,f,g,h) elongated bud formations of *Leptospira interrogans*

Growth Conditions of L. Interrogans

Unlike other common bacteria, the growth of *Leptospira* is slow and requires extensive care and supplementary mediums. The necessity of animal serum for bacterial growth was determined by comparing the growth of *Leptospira* inoculated with or without the addition of rabbit serum. The difference in growth rate of *Leptospira* incubated under static and shaking (180 rpm) conditions was also compared. The hypotheses are: additions of serums could increase bacterial yield due to higher nutrient contents, while shaking incubation could heighten reproduction rate as it helps incorporate oxygen into the culture and the bacterium is aerobic.

The growth was observed through changes in optical density of the cultures. As shown in Figure 7, incubating under shaking condition had improved the growth of the bacterium with or without addition of the serums. The increment in OD of the culture was lowest when not shaker incubated, even with the addition of the serums. Therefore, aeration is more important, compared to the addition of serums in promoting the speed of bacterial growth. It is also obvious that *Leptospira interrogans* took about 2 weeks to reach its exponential growth phase (as indicated by the green lines), even in a shaking condition, and the change in OD from inoculation to stagnant phase is only 0.05.

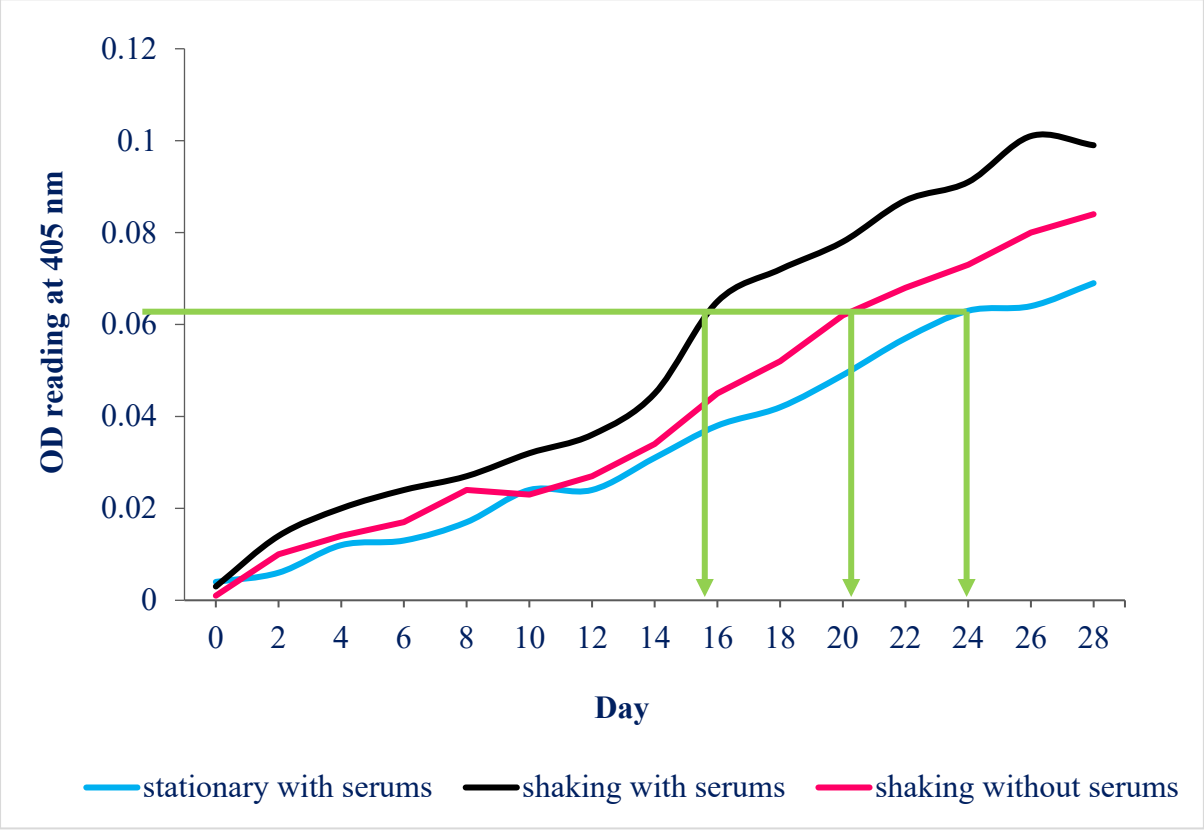

Figure 7: Graph showing the growth curve of *Leptospira interrogans* with or without serums and under different shaking conditions.

From Figure 8 we can see that when all the cultures were left stationary after the experiment, *Leptospira* sank to the bottom, partly due to its length that is around 15 micrometers, which is quite long compared to other bacteria. The bacterial sediment, which indicates bacterial yield, is low in cultures that are incubated under stationary conditions, but

not a lot of difference between cultures with and without serums. These highlights the importance of aeration for *Leptospira* growth, because the bacterium is aerobic.

Addition of animal serums did not alleviate the bacterial yield enough, so we concluded that the usage of serums could be excluded if need to. Besides being very costly for daily use in research, the technical disadvantages to using serum include the unclear nature of serums, batch-to-batch changes in composition, and the high risk of contamination. The bacterial culture can be easily contaminated due to its slow growth, as contaminants tend to grow at a higher rate; nevertheless, any foreign microbe could be spotted very easily. However, if necessary and the lab was cash-rich, the use or maybe testing of more varied serums could be considered due to the highly lagging growth of the bacterium. The doubling time of *Leptospira* under optimal conditions is 6-8h [20]. This could be partly due to the fact that pathogenic *Leptospira* do not multiply outside the host [2].

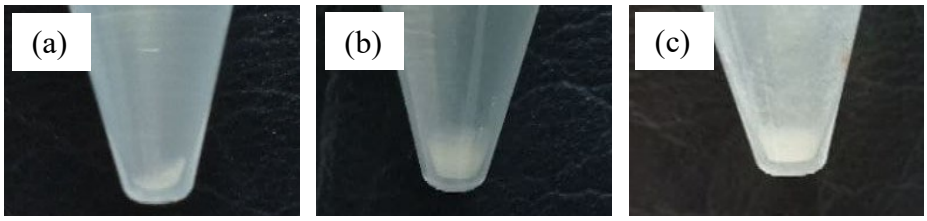

Figure 8: The sediments in the tubes after culturing under different conditions (a) stationary with serums, (b) shaking with serums and (c) shaking without serums.

Conclusions

The observation of the *Leptospira interrogans* with a transmission electron microscope by the negative staining method, was rapid and showed the ultrastructures and characteristic morphology of the bacterium with great clarity. The method gave good contrast and high resolution to the images of the specimens. We have observed varied terminal regions and cytoplasmic formations that are not well studied in previous projects, hence their unknown reasons of presence & functions. From here on, we concluded that another study is needed for screening of the surface proteins, which would help in understanding the cause of virulence, and developing drugs, vaccines and phage therapy against the pathogen. The functionality of these ultrastructures should also be researched for a enhanced understanding of the *Leptospira* physical biomaterials. The necessity of shaking incubation for a better *Leptospira* growth was clearly seen, while the use of serums is optional based on the results of this study. This discovery would be useful for future research as the old way of incubating *Leptospira* in static conditions could be improved for better growth. Nevertheless, the growth conditions of *Leptospira* are inconsistent among strains, so further study on the requirements of the targeted strain is necessary.

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Author Contributions

Conceptualization, F, K., Y, M. M. and N, N. S. M.; methodology, data analysis and interpretation, F, K. and N, N. S. M.; investigation, F, K.; original draft preparation F, K. and critical revision of the article, F, K., N, N. S. M.; Funding, N, N. S. M. All authors have read and agreed to the published version of the manuscript.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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