4-1-2019

Pathogenic Differences in Listeria monocytogenes 10403s and Cardiotropic Strains Grown under Aerobic and Anaerobic Conditions

Alexander Hayes

Pathogenic Differences in *Listeria monocytogenes* 10403s and Cardiotropic Strains Grown under Aerobic and Anaerobic Conditions

Honors Thesis

Alexander Hayes

Department: Biology

Advisor: Yvonne Sun, Ph.D.

April 2019

Pathogenic Differences in *Listeria monocytogenes* 10403s and Cardiotropic Strains Grown under Aerobic and Anaerobic Conditions

Honors Thesis

Alexander Hayes

Department: Biology

Advisor: Yvonne Sun, Ph.D.

April 2019

Abstract

Listeria monocytogenes is a gram-positive bacillus that is commonly associated with foodborne illness. It is a facultative intracellular pathogen responsible for causing the listeriosis infection in humans which is particularly dangerous to immunocompromised individuals. *L. monocytogenes* pathogenesis occurs through a series of complex interactions between host and bacterial cellular machinery. The wild-type strain as well as a cardiotropic strain with high affinity for infecting cardiac tissue were used to investigate pathogenesis in *L. monocytogenes*. Specifically, the Listeriolysin O protein that is critical for virulence was investigated. Aerobic and anaerobic growth environments as well as short-chain fatty acid supplementation were utilized to understand how growth conditions affect the pathogenic ability of *L. monocytogenes*.

Acknowledgements

I would like to thank my thesis advisor Dr. Yvonne Sun for introducing me to microbiology and the discovery process fundamental to scientific research. I would also like to the thank the University of Dayton Honors Department for supporting my research efforts.

Table of Contents

Introduction

Listeria monocytogenes

Listeria monocytogenes (*L. monocytogenes*) is a gram-positive bacillus that is commonly associated with foodborne illness. Its ability to grow at temperatures lower than standard refrigerators make it a dangerous pathogen. *L. monocytogenes* is a facultative intracellular pathogen that causes the listeriosis infection in humans characterized by "sepsis in the immunocompromised patient, meningoencephalitis in infants and adults, and febrile gastroenteritis"[1]. Treatment of the listeriosis infection usually includes a combination of antibiotics. Ampicillin, a beta-lactam of the penicillin group, acts as an irreversible inhibitor of the bacterial enzyme transpeptidase. This inhibition results in failure to cross-link peptidoglycan leading to bacteriolysis. Gentamicin, a broad-spectrum antibiotic, acts to bind to bacterial ribosomes causing inhibition of protein synthesis and eventual bacterial cell death. These two antibiotics exhibit synergistic effects and are often used together to treat listeriosis[2,3].

Pathogenesis

L. monocytogenes pathogenesis occurs through a series of complex interactions between host and bacterial cellular machinery. *L. monocytogenes* possesses several bacterial proteins that are instrumental in its ability to infect host cells. These proteins, also known as virulence factors, are responsible for promoting adhesion to intestinal epithelium, bacterial internalization into host cells, phagocytic vacuole evasion, and cell-to-cell motility.

Adhesion of *L. monocytogenes* to host cells describes the process by which the bacteria initiate contact to intestinal epithelial cells and remain attached to the cells. *L.*

monocytogenes uses Listeria Adhesion Protein (LAP) to adhere to the intestinal epithelium[4]. Specifically, LAP recognizes and binds to host heat shock proteins as receptors for adhesion to the intestinal epithelial cells[5]. Heat shock proteins are responsible for preventing damage to host cell proteins in response to heat or stressful conditions. Thus, it has been hypothesized that because infection is in itself a stressful event, overexpression of heat shock proteins may be detrimental as they result in increased binding sites for LAP and consequently, increased *L. monocytogenes* adherence[4].

LAP adherence is followed by activity of the internalin proteins. Internalin proteins along with LAP are classified under the term "adhesins" which define bacterial proteins that engage host receptors[6]. Internalin A (InlA) and Internalin B (Inl B) promote invasion and internalization of *L. monocytogenes* by binding to epithelial cellular adhesion molecule E-cadherin as well as the hepatocyte growth factor receptor called Met[6].

Upon internalization, the bacteria are confined to phagocytic vacuoles which normally act to defend against foreign bodies causing infection. *L. monocytogenes*, as an intracellular pathogen, must be able to escape the vacuole or phagolysosome to continue the process of infection. This is accomplished through the action of Listeriolysin O (LLO). LLO is a pore-forming protein belonging to the family of cholesterol-dependent cytolysins. LLO activity allows *L. monocytogenes* to escape into the cytosol where further growth and replication can occur. It accomplishes this escape by irreversibly binding to cholesterol found in the membrane of the vacuole or phagolysosome[7,8]. The pores formed by LLO may subject the vacuole to osmotic forces which result in its

disintegration; however, the exact mechanism is still unknown[8]. Interestingly, LLO achieves maximal activity at a pH of 5.5[9]. This coincides with the acidic nature of phagolysosomes allowing for the LLO toxin to reach maximal activity in this optimal environmental condition. This acidic requirement for LLO activity also "explains how the pore formation activity is restricted to avoid disruption of the host cell by uncontrolled LLO insertion into the endomembrane system of the host cell"[10]. Schuerch et al. determined the pH-dependent nature of LLO activity is the result of an acidic triad of amino acids consisting of two aspartate residues and one glutamate residue (Asp-208, Glu-247, and Asp-320)[11]. The mechanism of denaturation at an alkaline pH occurs when the carboxylic functional groups of the acidic triad are deprotonated. This deprotonated state on the carboxylic acid side-chain functional group carries a negative charge which creates charge repulsion because of their close proximity in the protein structure. The charge repulsion then contributes to the destabilization of the protein domain and loss of activity[11]. A normal pKa value for a carboxylic acid functional group is estimated to be around 2-4. Accordingly, it would then be expected that these carboxylic acid functional groups would be deprotonated at a pH of 5.5; however, this is not the case. The juxtaposition of these functional groups shifts their pKa values to a higher value ranging from 6-8 resulting in a protonated state at pH values below 6. This then coincides with the maximal activity of LLO seen at pH 5.5 when the acidic triad carries a neutral charge stabilizing the protein domain.

The next step of infection involves the motility of the bacteria which is mediated by the surface protein ActA. ActA mimics the function of host Wiskott Aldrich Syndrome Proteins which *in vivo* work together with Arp 2/3 to polymerize actin. In *L*.

monocytogenes pathogenesis, ActA complexes with Arp 2/3 to polymerize globular actin to filamentous actin. This filament can then propel *L. monocytogenes* throughout the cell which can function to evade phagocytic engulfment as well as propulsion into neighboring cells whereby the process of infection is similarly repeated starting with LLO activity to escape from the following phagocytic vacuole[12].

Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) are classified as fatty acids containing less than six carbons. SCFA production *in vivo* is attributed to the breakdown of nondigestible complex carbohydrates which are "oxidized incompletely in the anaerobic lumen by the intestinal microbiota releasing short-chain fatty acids (SCFA) as fermentation byproducts"[13]. SCFAs play a critical role in both host and bacterial physiology. In humans, SCFAs have been shown to be beneficial through a variety of different mechanisms. In bacteria, the biological activity of SCFAs has been widely investigated. High concentrations of SCFA seem to inhibit the colonization of pathogenic microbes in the intestinal tract. Additionally, diffusion of SCFAs across the bacterial membrane can lead to interference of metabolic reactions and dissipation of the bacterial proton motive force[13].

Materials and Methods

Bacterial Strains

Two strains of *L. monocytogenes* were used which included the 10403s and strain 07PF0776 which is also known as the cardiotropic strain. These two strains belong to two clinically relevant serotypes, 1/2a and 4b, respectively, which account for almost 98% of

documented human listeriosis cases[14]. 07PF0776 describes a strain of *L. monocytogenes* that exhibits "enhanced capacity for establishing cardiac disease *in vivo* resulting from increased bacterial invasion of cardiac cells, a trait potentially linked to amino acid polymorphisms in InlA and/or InlB"[15].

Growth Conditions

There were 12 unique growth conditions. The characteristics of each unique sample resulted from differences in the strain used, presence of oxygen, and type of supplementation. As mentioned above, either the 10403s or 07PF0776 strains of *L. monocytogenes* were used. Next, bacteria were grown under both aerobic or anaerobic conditions. The final difference was whether the bacteria were supplemented with a mixture of SCFAs (M1 or M2) or not supplemented at all (NS). The SCFA mixtures consisted of acetate, propionate, and butyrate. M1 contained 76. 5 microliters (μL) of acetate, 6.75 μL of propionate, and 6.75 μL of butyrate. M2 contained 330 μL of acetate, 210 μL of propionate, and 60 μL of butyrate. The preparation of bacterial cultures consisted of an addition of 3 milliliters (mL) of brain heart infusion (BHI) broth to a standard culture tube. This was followed by addition of the M1 or M2 mixtures of short-chain fatty acids if they were to be supplemented. Finally, a single colony from a growing petri dish was transferred with a wooden applicator stick to the broth mixture. The colonies were then incubated at 37 degrees Celsius in an aerobic or anaerobic incubator to allow growth for 12-16 hours.

	WT + NS	WT + M1	WT + M2	WT - NS	WT - M1	WT - M2
STRAIN	10403s	10403s	10403s	10403s	10403s	10403s
O_2 PRESENCE	Aerobic	Aerobic	Aerobic	Anaerobic	Anaerobic	Anaerobic
SUPPLEMENT	None	Mix 1	Mix 2	None	Mix 1	Mix 2

	H + NS	H + M1	H + M2	H - NS	H - M1	H - M2
STRAIN	07PF0776	07PF0776	07PF0776	07PF0776	07PF0776	07PF0776
O_2 PRESENCE	Aerobic	Aerobic	Aerobic	Anaerobic	Anaerobic	Anaerobic
SUPPLEMENT	None	Mix 1	Mix 2	None	Mix 1	Mix 2

Table 1. Depiction of the 12 unique growth conditions used

Protein Extraction, Precipitation, Buffer Loading, and Heating

After the allotted growth time, the optical density of each culture was measured at 600 nanometers and then normalized to ensure the relative concentration of *L. monocytogenes* remained the same across each sample. A normalized volume of each culture was added to 1 mL centrifuge tubes along with the addition of BHI to bring the total volume of the tube to 1 mL. Next, the samples were centrifuged at 10,000 revolutions per minute (RPM) for 3 minutes. Following this spin cycle, 900 µL of the normalized culture supernatant was extracted and transferred to a new centrifuge tube. 100 µL of trichloroacetic acid (TCA) was then added to each sample to promote the precipitation of proteins out of solution. The samples were then incubated at 4 degrees Celsius for 1 hour. Following this incubation period, samples were centrifuged at 15,000 RPM for 15 minutes at 4 degrees Celsius. The supernatant was then discarded and 500 µL of acetone was added to each protein pellet. The samples were then centrifuged again at 15,000 RPM for 15 minutes at 4 degrees Celsius. The supernatant was then discarded again, and the protein pellet was left to dry for at least 5 minutes. After, 12 µL of a 2X sodium dodecyl sulfate (SDS) sample buffer was added to each pellet. The SDS is an anionic surfactant that contributes to the denaturing of protein samples by disrupting non-covalent interactions. It distributes a negative charge to the protein samples giving each

protein a similar net negative charge. The samples were then heated at 95 degrees Celsius for 5 minutes to further promote denaturation.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Following heating, 10 μL of each sample were loaded into the wells of a sodium dodecyl sulfate-polyacrylamide gel. The porous gel is designed to allow for the proteins to migrate through based on size where smaller proteins are able to migrate at a faster rate. As mentioned above, the SDS distributes a uniform negative charge on each protein sample so the migration rate is mainly based on size of the protein rather than the strength of charge. The SDS-polyacrylamide gel consists two distinct layers called the stacking gel and separating gel. The stacking gel functions to concentrate the proteins present in each sample into a thin protein band before reaching the separating gel[16]. The voltage of the power supply was set to 70 volts for approximately 20 minutes in which the proteins would pass through the stacking gel. This was followed by 120 volts for the remainder of gel electrophoresis as the proteins migrated through the stacking gel.

Western Blot

After the proteins had sufficiently separated in the gel after gel electrophoresis, preparation began to transfer the proteins to a polyvinylidene difluoride (PVDF) membrane. First, the PVDF membrane, because of its highly hydrophobic nature, was soaked in methanol to increase protein binding when the gel is applied. Next, a "sandwich" was prepared in the semi-dry transfer apparatus by placing the PVDF membrane on a thick piece of filter paper. Next, the gel was gently laid on top of the PVDF membrane followed by another piece of filter paper and dry paper towel. The power supply was then set to 20 volts (V) and the transfer was allowed to run for 30

minutes. After the transfer, the membrane was placed in a box with deionized water. The membrane is then placed in a blocking solution consisting of Tris-buffered saline, 0.05% Tween 20, and 3% milk. 10 mL of the blocking solution was used for each membrane. The purpose of the blocking procedure is to minimize any non-specific protein binding interaction with the PVDF membrane. After 20 minutes allotted for the blocking procedure had lapsed, the blocking solution was replaced with a new solution consisting of the Tris-buffered saline, 0.05% Tween 20, and 3% milk along with an anti-LLO antibody. This solution was allowed to sit overnight to promote antibody binding to the target protein LLO. The next morning, a secondary antibody conjugated with an enzyme called horseradish peroxidase (HRP). The secondary antibody functions to bind to the fragment crystallizable region of the primary antibody. Following this binding and a series of washes, a chemiluminescent substrate is added to the membrane. HRP catalyzes a reaction with chemiluminescent substrate which yields the emission of light which was then visualized using a gel documentation system specific for chemiluminescence[17].

Results

Figures 1 through 4 show the results of the western blot performed to assess relative LLO abundance between different strains and growth conditions. Figures 2 and 3 show two separate membranes from the cardiotropic and 10403s strains, respectively. M1 shows a reduced abundance of LLO more under aerobic than anaerobic conditions for both strains. M2 shows a reduced abundance of LLO under aerobic and anaerobic conditions for both strains.

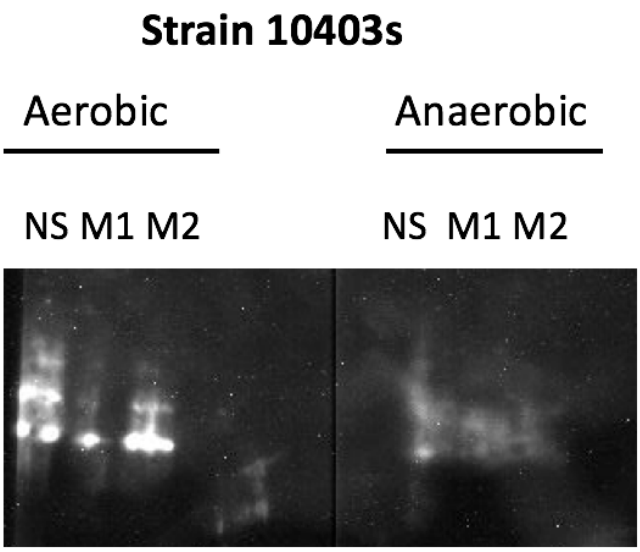

Figure 1

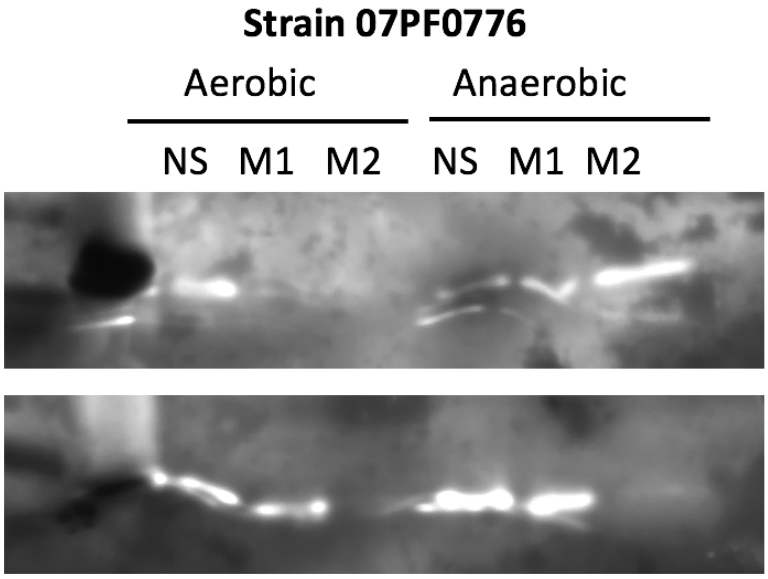

Figure 2

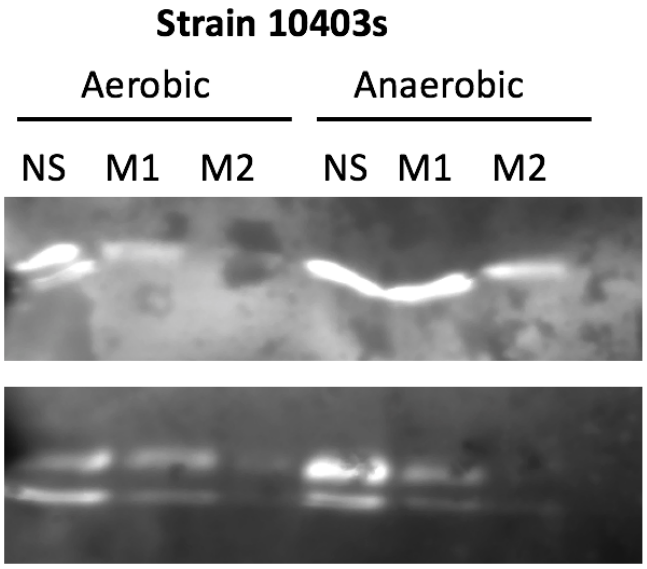

Figure 3

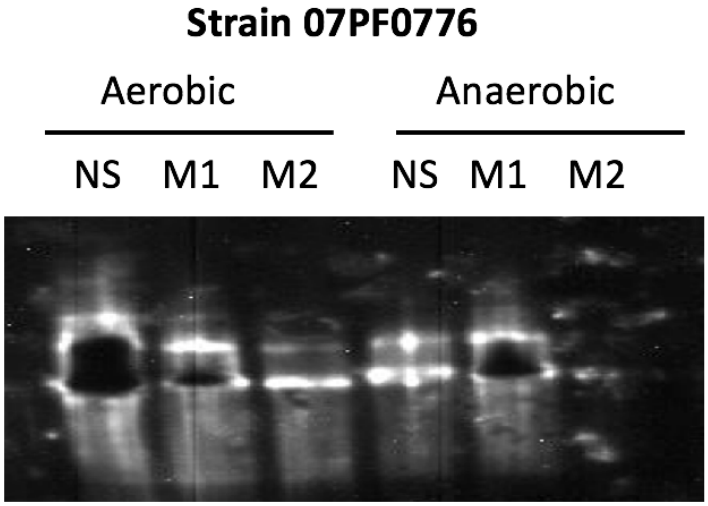

Figure 4

Discussion

The results from the western blot point to a few interesting conclusions. *L. monocytogenes* is a facultative anaerobe and thus prefers aerobic growth conditions but is capable of growing under anaerobic conditions. This preference may explain why LLO abundance appears to be reduced under anaerobic conditions (Figure 1). Also, short-chain fatty acid supplementation is known to cause growth inhibition of *L. monocytogenes*[13]. Nevertheless, the results show *L. monocytogenes* is able to produce more of its key virulence factor LLO under anaerobic conditions when supplemented with M1 SCFAs (Figures 2 and 3). However, the abundance of a protein does not equate to its activity meaning increased LLO abundance in a certain growth condition may not necessarily result in greater virulence. One possible explanation for the greater abundance of LLO comes from the preferred acidic environment of *L. monocytogenes* LLO. As mentioned before, the LLO protein domain is stable at a slightly acidic pH when certain key residues remain neutrally charged. Because SCFAs are organic acids, they function to slightly lower the pH of a solution. Regarding M2 supplementation, inhibition was seen in both strains under both aerobic and anaerobic conditions. This widespread inhibition compared to M1 may be explained by the higher concentrations of SCFAs present in M2 versus M1. Future investigations may look into the interplay between overall inhibition of *L. monocytogenes* LLO production through SCFA supplementation while also examining the influence of pH on LLO activity. The aforementioned research topic is important as are all basic science research topics that seek to develop a deeper understanding of the natural world and the diverse organisms in it. *L. monocytogenes* is a

clinically relevant bacterial pathogen in our world that through research we can illustrate a clear picture of its characteristics.

Personal Reflection

The Honors Thesis Project was a great introduction to biological research and scientific writing. Thanks to my thesis advisor Dr. Yvonne Sun, over the course of the project, I had the chance to learn laboratory protocols as well as the scientific principals behind each step. Several challenges arose through the course of the project; however, every challenge or unsuccessful experiment always resulted in a learning opportunity. These missteps I experienced contributed to a resilience that I have developed along with a greater respect for the meticulous and concise nature of scientific research. As I seek to become a physician-scientist, I hope to be able to continue to develop the skills I have learned in my thesis journey to benefit the scientific and medical community in the future.

References

1. Acheson, D. & Schlech, W. F., III. Foodborne Listeriosis. *Clinical Infectious Diseases* **31**, 770–775 (2000).
2. Lindsey, K. D., Koranyi, K. & Azimi, P. H. Listeria monocytogenes: Synergistic Effects of Ampicillin and Gentamicin. *American Journal of Clinical Pathology* **72**, 974–977 (1979).
3. Moellering, R. C., Jr, Medoff, G., Leech, I., Wennersten, C. & Kunz, L. J. Antibiotic synergism against Listeria monocytogenes. *Antimicrobial agents and chemotherapy* **1**, 30–34 (1972).
4. Burkholder, K. M. & Bhunia, A. K. Listeria monocytogenes Uses Listeria Adhesion Protein (LAP) To Promote Bacterial Transepithelial Translocation and Induces Expression of LAP Receptor Hsp60. *Infect Immun* **78**, 5062–5073 (2010).
5. Wampler, J. L., Kim, K.-P., Jaradat, Z. & Bhunia, A. K. Heat Shock Protein 60 Acts as a Receptor for the Listeria Adhesion Protein in Caco-2 Cells. *Infect Immun* **72**, 931–936 (2004).
6. Bonazzi, M., Lecuit, M. & Cossart, P. Listeria monocytogenes internalin and E-cadherin: from bench to bedside. *Cold Spring Harbor perspectives in biology* **1**, a003087–a003087 (2009).
7. Cossart, P. *et al.* Listeriolysin O is essential for virulence of Listeria monocytogenes: direct evidence obtained by gene complementation. *Infect. Immun.* **57**, 3629 (1989).
8. Tweten, R. K. Cholesterol-Dependent Cytolysins, a Family of Versatile Pore-Forming Toxins. *Infect. Immun.* **73**, 6199 (2005).

9. Geoffroy, C., Gaillard, J. L., Alouf, J. E. & Berche, P. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from Listeria monocytogenes. *Infection and immunity* **55**, 1641–1646 (1987).

10. Osborne, S. E. & Brumell, J. H. Listeriolysin O: from bazooka to Swiss army knife. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **372**, 20160222 (2017).

11. Schuerch, D. W., Wilson-Kubalek, E. M. & Tweten, R. K. Molecular basis of listeriolysin O pH dependence. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 12537–12542 (2005).

12. Yoshikawa, Y. *et al.* Listeria monocytogenes ActA-mediated escape from autophagic recognition. *Nature Cell Biology* **11**, 1233 (2009).

13. Sun, Y. & O'Riordan, M. X. D. Regulation of bacterial pathogenesis by intestinal short-chain Fatty acids. *Advances in applied microbiology* **85**, 93–118 (2013).

14. Kyoui, D. *et al.* Genetic distance in the whole-genome perspective on Listeria monocytogenes strains F2-382 and NIHS-28 that show similar subtyping results. *BMC Microbiology* **14**, 309 (2014).

15. Alonzo, F., Bobo, L. D., Skiest, D. J. & Freitag, N. E. Evidence for subpopulations of Listeria monocytogenes with enhanced invasion of cardiac cells. *Journal of Medical Microbiology* **60**, 423–434 (2011).

16. Schägger, H. & von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* **166**, 368–379 (1987).

17. Mahmood, T. & Yang, P.-C. Western blot: technique, theory, and trouble shooting. *North American journal of medical sciences* **4**, 429–434 (2012).