UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

The Effects of *Helicobacter spp.* On Blood Pressure in Rat Models of Hypertension

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, for the degree of Master of Science in Medicine

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Declaration

I, Sarhana Dinat, declare that this dissertation is my own unaided work, except where stated. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this dissertation has not been submitted for any degree or examination in this university or any other university.

Sarhana Dinat, on this ___19th___ day of _____May____ 2021

Professor GP Candy (Supervisor)

Date: May 19th, 2021

Dedication

I would like to dedicate this dissertation to my friends, family, colleagues, and supervisor. A special thank you to my parents, Moosa and Farhana, and sister, Naseera, for supporting me financially and in spirit, and for trying so hard to understand what I do.

A heartfelt thanks goes to my supervisor, Professor Candy, for giving me this opportunity. Thank you for being so understanding, patient and going the extra mile in times of need. To the rest of the Candy Company, thank you for being there every step of the way. Anza, Hannah and Madaliso, thank you for making each day exciting and bringing humour to otherwise drab situations. And lastly, thank you to Sudesh for keeping me laughing throughout this journey.

Abstract

Hypertension affects over one billion people globally and is believed to underlie multiple cardiovascular diseases, however the exact cause remains unknown. *Helicobacter pylori* has been implicated in hypertension. Studies associated high blood pressures with *H. pylori* prevalence and eradication was accompanied by significant decreases in blood pressure. This study assessed the effects of antibiotics targeting *Helicobacter spp.*, and the effect of the human pathogen *H. pylori* on rat models of hypertension. To assess the effects of antibiotics, normotensive Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats were treated with either an antibiotic mix to select for or to eradicate *Helicobacter spp.* To assess the effects of *H. pylori,* SHR and WKY rats with *Helicobacter spp.* eradicated were inoculated with either *H. pylori* cultures or cell-free supernatants of *H. pylori* cultures. Systolic blood pressure was recorded throughout and after these processes. Selecting for *Helicobacter spp.* had no significant effect while eradicating *Helicobacter spp.* accompanied a significant decrease in systolic blood pressures of both SHR and WKY treated rats. A greater decrease in SHR was noted than in WKY, resulting in both strains reaching the same systolic blood pressure. Re-infection with *H. pylori* resulted in increased systolic blood pressure. This was less than the decrease measured following eradication with antibiotics. No significant changes were noted upon treatment with cell-free supernatants. This suggests that *Helicobacter spp*., including *H. pylori,* affects blood pressure, although these bacteria do not influence blood pressure alone. Other microorganisms also targeted by the antibiotics used may also play a role.

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List of Abbreviations

ACE	Angiotensin converting enzyme
ANOVA	Analysis of variance
BHI	Brain heart infusion
BSH	Bile salt hydrolase
CBA	Columbia blood agar
CFU	Colony forming units
CTS	Cardiotonic steroid
FBS	Foetal bovine serum
GIT	Gastrointestinal tract
H_2O_2	Hydrogen peroxide
HSDH	Hydroxysteroid dehydrogenase
K^+	Potassium
MIC	Minimum inhibitory compound
Na^+	Sodium
NO	Nitric oxide
OD	Optical density
PPI	Proton pump inhibitor
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SHR	Spontaneously hypertensive rat
TGF-β	Transforming growth factor β
WKY	Wistar Kyoto

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Chapter 1: Literature Review

1.1 Background

Dubbed the “silent killer”, hypertension affects over a billion of the global population. Hypertension is characterised by persistently high blood pressure in the systemic arteries. It is commonly expressed as the ratio of systolic blood pressure to diastolic pressure. The former is defined as the pressure exerted by blood on the arterial walls when the heart contracts, and the latter occurs when the heart relaxes (Oparil *et al.,* 2018). Blood pressure was noted to increase over time, as lifestyle habits evolved. Blood pressure within individuals also increases with age, as arteries harden and since older individuals have been exposed to environmental factors which raise blood pressure for longer periods of time. These factors include excessive intake of sodium, alcohol consumption, low potassium intake, a lack of physical activity and obesity. Other factors influencing the raised blood pressure of adults includes genetic predisposition and an adverse intrauterine environment (Oparil *et al.,* 2018).

The ideal blood pressure is currently determined to be approximately 120/80 mmHg, with values persistently higher than this classifying as hypertension. A detailed description of the measurements defining hypertension are outlined in Table 1.1, based on the South African Hypertension Practise Guidelines 2014, as determined from randomized clinical trials (Seedat *et al.,* 2014).

Table 1.3: Blood Pressure, as defined by the SA Hypertension Practise Guidelines (Seedat *et al.*, 2014).

Type of Blood Pressure	**Systolic (mmHg)**	**Diastolic (mmHg)**
Normal	≤120	≤ 80
High normal	130-139	85-89
Hypertension	≥ 140	≥ 90

1.1.1 Risks of hypertension

Globally, non-communicable diseases make up 70% of all deaths. The majority of this 70% is a result of cardiovascular events, such as stroke and myocardial infarction, as well as end-stage renal disease, congestive heart failure and peripheral heart disease. A major risk factor for these diseases is hypertension (Steyn *et al.,* 2001; Jongen *et al.,* 2019). Hypertension has repeatedly been shown to have a strong continuous association with the pathogenesis of cardiovascular diseases. These include coronary disease, valvular disease, cardiac arrhythmia and angina, ischaemic and haemorrhagic strokes, abdominal aortic aneurysm and renal disease (Rapsomaniki *et al.,* 2014; Kjeldsen *et al*., 2018). Two out of three adult patients with hypertension had almost a 40% increase in the chance of experiencing a cardiovascular disease event as compared to their normotensive counterparts. Furthermore, hypertensive patients manifest cardiovascular events 5 years earlier than normotensive individuals (Rapsomaniki *et al.,* 2014). Studies have shown raised blood pressure was not necessarily associated with all cardiovascular diseases, and the link between the two differed for systolic and diastolic blood pressures (Rapsomaniki *et al*., 2014). Subsequent studies have demonstrated the relationship between hypertension and cardiovascular disease to be independent of other risks of cardiovascular disease, and assessed solely the impact of hypertension on the onset of cardiovascular disease (Kjeldsen *et al*., 2018).

In an analysis by Lewington *et al.* (2002) the correlation between blood pressure, cardiovascular disease and advancing age was assessed. Increases in blood pressure of 20mmHg and 10mmHg for systolic and diastolic respectively in individuals ranging from 40 to 69 years old more than doubled their risk of ischaemic heart disease mortality or experiencing a stroke. At more advanced ages, such as 80 years old and beyond, the risk of experiencing a cardiovascular event increases almost tenfold (as compared to individuals between 50 and 59 years old) when systolic blood pressure ranging from 120mmHg to 140mmHg was found to increase by 20mmHg (Lewington *et al.,* 2002).

Thus, it is evident that hypertension is a major risk for more detrimental diseases which require more intensive medical care. As a result, early detection, awareness, treatment and lowering the overall burden of hypertension is of paramount importance.

1.1.2 Epidemiology of hypertension

Hypertension affects over a billion people around the world, with the majority of these people living in middle to low-income countries. South Africa has shown a high prevalence of hypertension, ranging from 29.2% to 54.7% prevalence in men and 47.2% to 53.4% in women (Gómez-Olivé *et al.,* 2017).

Despite the high prevalence of hypertension in South Africa, the reported numbers of awareness and control among hypertensive individuals are still low, ranging between 19-56% for awareness and just 14-36% for control (Jongen *et al.,* 2019). High income countries, however, have reported much higher figures, with 82% of awareness and 51% of control amongst hypertensive individuals (Jongen *et al.,* 2019). The amount of awareness and control of hypertension in a country is an important factor in deterring the onset of further cardiovascular diseases, and subsequent mortality thereof. Studies show that the prevalence of hypertension in South Africa is higher than that of other, more developed countries. Within the South African population, the highest prevalence exists in the African urban population, as they have and are adopting a more Western lifestyle (Steyn *et al.,* 2001). Furthermore, it was found that men, in particular those younger than 45, have poor rates of awareness, treatment and control (41%, 39% and 26% respectively) (Steyn *et al.,* 2001).

With the increasing urbanisation and adoption of the Western lifestyle amongst South Africans, a number of risk factors for hypertension have become part of daily life. These include being overweight, excessive salt intake, unhealthy diets, alcohol consumption and a lack of physical activity. A better understanding of the risks and onset of hypertension has been linked to improving the likelihood of hypertensive individuals accepting treatment and bringing the disease under control (Jongen *et al.,* 2019). Despite there being interventions to prevent or control hypertension, both pharmacological and non-pharmacological, these are often not sufficient. Poor treatment and control of hypertension creates a financial and epidemiological burden on South Africa (Steyn *et al.,* 2001).

The cost of hypertension to the South African economy in 1991 was approximately R4 - 5 billion, 7.5% of the healthcare expenditure (Seedat and Rayner, 2012). While the majority of healthcare expenditure is directed towards antiretroviral therapy, death rates from non-

communicable diseases are higher than that of HIV/AIDS, with cardiovascular disease being the leading category (Schutte, 2019).

South Africa has implemented a strategy (National Department of Health Strategic plan for the prevention and control of non-communicable diseases 2013–17) which aims to reduce the death rate due to non-communicable diseases by 25% by the year 2020 (Schutte, 2019). A major aspect of this reduction is effective detection, treatment and control of these diseases. A point of concern is that of patients treated for hypertension, only a third have the disease under control (Schutte, 2019). Those with inadequate control are at a risk for further cardiovascular diseases. The need for cost effective, efficient alternatives for hypertension therapy is vital. In order to develop alternative strategies for treating hypertension, the causes of hypertension need to be elucidated.

1.1.3 Mechanisms of hypertension

Although the exact cause of hypertension is unclear, the mechanisms relating to hypertension are multifactorial and act on a genetic level. Certain genetic predispositions are linked to the increased risk of developing hypertension. This is then exacerbated when coupled with environmental factors, such as increased sodium and/or alcohol intake, poor sleep quality and added stress and anxiety (Hall and Hall, 2018).

Blood pressure is regulated by a number of factors of the cardiovascular system. These include the volume of blood, the amount of blood pumped by the heart per minute and the arterial tone balance. Maintaining physiological blood pressure levels involves an interplay between several key players, including sodium regulation, the renin-angiotensin-aldosterone system (RAAS), natriuretic peptides, the sympathetic nervous system, the immune system and the vascular endothelium (Oparil *et al.*, 2018). A disruption in the function of one or more of any of these factors can lead to an increase in blood pressure.

- Sodium regulation

Regulation of sodium (Na^+) is key in the modulation of blood pressure. Blood pressure is impacted by the ability of the kidneys to excrete salt (Fedorova *et al*., 2010). The Na^+/K^+ -ATPase pump is critical to maintain intracellular sodium and potassium (K^+) ion concentrations and the overall regulation of body sodium concentrations. Three sodium ions are exported in

exchange for the import of two potassium ions with energy derived from the hydrolysis of an ATP molecule. Sodium is also excreted in the urine through natriuresis (Buckalew, 2015).

High levels of sodium in the body promotes water retention, which in turn increases blood volume and as a result, blood pressure. In normotensive individuals, this is regulated by a number of changes, namely a reduction in renal and peripheral vascular resistance as well as an increase in the amount of nitric oxide (NO) produced from the vascular endothelium (Oparil *et al.,* 2018). If NO is not produced sufficiently, it results in an increase in blood pressure. This dysfunction by the vascular endothelium is a major risk for salt sensitivity and hence hypertension. Salt sensitive individuals experience an increase of approximately 10mmHg in systolic blood pressure within a few hours of ingesting 5g or more of sodium. With high intakes of sodium, these individuals will produce excessive amounts of transforming growth factor β (TGF-β), which in turn causes oxidative stress and limits NO bioavailability, as well as increases the risk of fibrosis, which is characterized by the development of fibrous connective in response to injury or damage (Feng *et al.,* 2017). High salt intake was also shown to have effects on the gut microbiome, which in turn resulted in the on the onset of hypertension. Several studies have shown the effect of reducing the survival or completely depleting *Lactobacillus spp.,* in humans and mice respectively, with corresponding influence on blood pressure in each case (Wilck *et al.,* 2017).

Administration of mineralocorticoids, a class of corticosteroids which influence sodium and water balance, causes sodium retention, but if sodium concentrations rise above a threshold, natriuresis occurs. Although an increase in glomerular filtration rate or a decrease in aldosterone, a primary mineralocorticoid, may cause natriuresis, a third factor, believed to be the 'endogenous digitalis-like factors', may be responsible for this observed natriuresis (Buckalew, 2015). Endogenous digitalis-like factors, also known as endogenous cardiotonic steroids, influence sodium levels by inhibiting the Na^+/K^+ - ATPase pump (Fedorova *et al.,* 2010).

- <u>The renin-angiotensin-aldosterone system (RAAS)</u>

The RAAS system is composed of three major compounds, renin, angiotensin II and aldosterone. These components act together to control arterial blood pressure, elevating it when renal blood pressure decreases or when there is a decrease in salt delivery to the distal convoluted tubule. This is done through sodium reabsorption, water reabsorption and vascular tone (Hall and Hall, 2018).

The mechanism of blood pressure regulation via RAAS begins in the arterioles of the kidney, where specialized juxtaglomerular cells become activated, a process which occurs in response to a decrease in blood pressure, beta-activation or indirectly via a decreased sodium load. Once activated, these cells are capable of cleaving the continually secreted prorenin to renin. Once released into the blood, renin acts on its target, angiotensinogen, cleaving it to form angiotensin I. Angiotensin converting enzyme (ACE) then catalyses the conversion of angiotensin I to angiotensin II (Varagic *et al.*, 2014).

By binding to angiotensin II type I and type II receptors, angiotensin II has a number of effects, such as inducing vasodilation through NO generation, as well as acting on the kidney, adrenal cortex, arterioles and brain (Fountain and Lappin, 2018). Angiotensin II increases sodium reabsorption, thereby increasing blood osmolarity and hence arterial blood pressure. This is done by acting directly in the convoluted tubule of the kidney, and by stimulating the release of aldosterone. The latter occurs when angiotensin II acts on the adrenal cortex, stimulating the release of the steroid hormone aldosterone. Aldosterone then binds to nuclear receptors and alters nuclear gene transcription to exert an effect on sodium reabsorption and consequently arterial blood pressure (Hall and Hall, 2018).

Angiotensin II plays a role in vasoconstriction in the systemic arterioles by binding to G-protein-coupled receptors, triggering a signal transduction cascade which ultimately results in vasoconstriction and consequently raises blood pressure (Hall and Hall, 2018).

Angiotensin also has several effects on the brain. It stimulates thirst and water intake by targeting neurons in the hypothalamus and stimulates the release of the antidiuretic hormone which increases water reabsorption in the kidney. Both of these ultimately raise blood pressure (Fountain and Lappin, 2018).

- Natriuretic peptides

When sodium is introduced to the body, atrial natriuretic peptides and brain natriuretic peptides are released, which leads to vasodilation and consequent lowering of blood pressure. This is achieved both directly and indirectly, by affecting sodium uptake and by affecting the release of renin and aldosterone (Woodard and Rosado, 2008). A deficiency of these peptides promotes hypertension. The abovementioned peptides are converted from their precursors to their active forms by a serine protease, corin. A deficiency in corin has been linked to salt-sensitive hypertension and heart failure (Schleuter *et al.*, 2014).

- Sympathetic nervous system

Pressure changes of the circulatory system are detected by baroreceptors and mechanoreceptors. These receptors are located at various points on the arterial tree, at the base of the internal carotid artery. As this artery is stretched with an increase in blood pressure, messages are sent to the brain by the baroreceptors, which signals to decrease the sympathetic outflow of nerve impulses, which in turn decreases blood pressure by changing the tension in the arterial walls (Pijacka *et al.,* 2016). Hypertensive individuals have been found to have a more activated sympathetic nervous system as opposed to their normotensive counterparts. This increased activity was found to be associated with α-21 adrenergic receptor mediated endothelial dysfunction, vasoconstriction, vascular smooth muscle proliferation and an increase in arterial stiffness. All of these aid in developing and sustaining hypertension (Fujita, 2014; Mancia and Grassi, 2014).

- The immune system

Immune responses and inflammation offer a major contribution to the onset of hypertension, through its association with increased vascular permeability and the release of potent mediators, such as reactive oxygen species (ROS), NO, cytokines and metalloproteinases (Harrison and Bernstein, 2018). In hypertensive individuals, both innate and adaptive immune responses have been found to be involved in generating ROS as well as in inflammatory changes in the kidney, blood vessels and brain. Innate responses have been linked to hypertension induced by angiotensin II, aldosterone and a lack of NO (Harrison and Bernstein, 2018).

- Endothelium

The endothelium acts as a major regulator of vascular tone through its interaction with NO. Endothelial cells secrete numerous vasodilators and vasoconstrictors. One of the most important vasodilators is NO which is synthesized in the vascular endothelium (Hermann *et al.,* 2006). Endothelial NO synthase metabolizes L-arginine to NO in the presence of certain cofactors. This endothelial derived NO then stimulates guanylyl cyclase to form 3,5-cyclic guanosine monophosphate. This results in vasodilation of vascular smooth muscle cells, thereby controlling peripheral blood flow and blood pressure (Hermann *et al.,* 2006).

Dysfunction in the endothelium has been linked to the onset of hypertension, as a result of increased oxidative stress, amongst other factors. ROS are generated by several enzymes, such

as NADPH oxidase, xanthine oxidase and cyclooxygenase (Panza *et al.,* 1993). This increase in ROS results in a decrease in NO bioavailability, since excessive superoxide anions bind to NO. A decrease in NO bioavailability is one of the major factors linking endothelial dysfunction and hypertension (Panza *et al.,* 1993; Zalba *et al.,* 2001; Feng *et al.,* 2017). As such, a number of NO therapeutic strategies have been investigated in order to combat hypertension and other cardiovascular diseases such as atherosclerosis and coronary heart disease (Infante *et al.,* 2021).

1.1.4 The role of nitric oxide and arginine

From understanding the mechanisms influencing blood pressure, it is evident that NO plays an important role. Thus, understanding NO and its interactions are paramount to understanding hypertension. Hypertensive patients show a decrease in NO bioavailability, which occurs in several ways. A lack of arginine, or a decrease in the cellular uptake thereof, will result in a reduced amount of NO produced (Moss *et al.,* 2004). Also playing a role is the enzyme NO synthase, as the inhibition or uncoupling of this enzyme as well as a deficiency in the cofactors involved both reduce NO production (Landmesser *et al.,* 2003). In addition, any ROS will reduce NO bioavailability, as discussed previously (Zalba *et al.,* 2001). During hypertension, any combination of the events occurs, decreasing NO bioavailability.

In a study assessing animal models of hypertension, the expression and activity of endothelial NO synthase was found to be reduced in later stages of hypertension (Matsuoka *et al.,* 1996). Taddei *et al.* (1998) assessed patients with primary arterial hypertension and noted a decrease in NO in the early stages, with a breakdown of NO in the late stages. The latter was presumed to be due the production of oxygen radicals (Taddei *et al.,* 1998). Patients of hypertension were also shown to have a reduced metabolization of organic nitrates and other NO donors. The smooth muscular compartment of these patients also showed a decrease in its sensitivity to NO (Kelm *et al.,* 1996). Endothelial dysfunction was detected in the normotensive offspring of hypertensive patients. This dysfunction was reversed solely through the administration of arginine, and not by lowering the blood pressure (Kelm *et al.,* 2003). Thus, it is evident from these studies that NO plays a key role in the development and progression of hypertension through a number of mechanisms.

As NO is produced from arginine by NO synthase, a deficiency in arginine may thus be a contributing factor in hypertension (Vasdev and Gill, 2008). Arginine is involved in a number of important pathways, including protein synthesis, polyamine synthesis and removal of excess nitrogen in the urea cycle (Vasdev and Gill, 2008). Although arginine can be produced intracellularly, arginine is essential in the NO pathway and is predominantly transported into the cells to produce NO. Transport is by y^+ and the y^+L transport systems, with other basic amino acids, such as lysine, competing for transport (Vasdev and Gill, 2008). Deficiencies of arginine, or a change in its metabolism, has potential to increase blood pressure as well as contribute to endothelial dysfunction.

Several studies investigating the effects of arginine levels on hypertension had focused on supplementation studies. Schlaich *et al.* (2004) examined the effect of arginine supplementation in normotensive and hypertensive individuals and found a decrease in the blood pressure of hypertensive patients who failed to control their blood pressure through the use of other pharmaceuticals (Schlaich *et al.*, 2004). Ozçelikay *et al.* (2000) performed a study using rat models, and after feeding arginine orally, rat models of type I diabetes showed a decrease in blood pressure to normal levels after 4 weeks of administering treatment (Ozçelikay *et al.*, 2000). Tay *et al.* (2002) prevented the development of hypertension in fructose-hypertensive rats through oral arginine administration (Tay *et al.*, 2002). The results of these and other studies showed that arginine supplementation is effective in increasing NO production and lowering blood pressure.

1.1.5 *Helicobacter pylori*

One possible factor affecting the availability of arginine is the bacterium *Helicobacter pylori*, a gram negative, microaerophilic bacterium, which utilises arginine to create its own niche and colonise the upper gastrointestinal tract (GIT) (Chaturvedi *et al.*, 2012). Colonization by *H. pylori* causes inflammation in endothelial cells, resulting in diseases such as chronic gastritis, peptic ulcers and gastric cancer (Gobert and Wilson, 2016).

Considered one of the most successful human pathogens, *H. pylori* colonizes more than half of the world's population. The prevalence amongst African South Africans is much higher, reaching almost 90% (Goh *et al.*, 2011). Initially thought to be contaminants from consumed food, it is now understood that *H. pylori* are true gastric colonizers. In 1983, Warren and

Marshall described the successful isolation of a spiral bacterial species from the human stomach, which we now know as *H. pylori* (Warren and Marshall, 1983). The ability of *H. pylori* to colonize the stomach and here cause inflammation and gastritis was confirmed by self-ingestion studies performed by Marshall (1985) and later Morris (1987). The effects of this self-ingestion were reversed by treatment targeting *H. pylori* (Marshall *et al.,* 1985; Morris and Nicholson, 1987). This discovery paved the way for a number of other studies, linking *H. pylori* to a range of upper GIT disorders, such as gastritis, ulcers, and a number of cancers.

Helicobacter species have adapted to colonize the inhospitable conditions of the gastric mucosal surface. It is assumed that all mammals are colonized with some member of the *Helicobacter* genus, be it *pylori* (humans), *felis* (cats) or *mustelae* (ferrets), as well as a number of others (Kusters *et al.,* 2006). All identified *Helicobacter spp.* are urease positive and show high motility due to their flagella. These features allow the *Helicobacter spp.* to colonize the GIT; the urease enzyme allows for survival in the highly acidic gastric lumen while the flagella allow for the bacteria to move towards the more neutral pH of the gastric mucosa (Kusters *et al.,* 2006).

H. pylori successfully colonises the lining of the stomach gastric epithelium, by neutralising the acidic environment by initially converting arginine to urea using an arginase enzyme. A powerful urease enzyme then converts urea to ammonia to raise the pH of the bacterium's environment and create its own niche for colonization. *H. pylori* consumes large amounts of arginine in a short period of time as arginine is essential for its growth (Chaturvedi *et al.,* 2012). Genome analysis of *H. pylori* have revealed the presence of the gene encoding for the enzyme arginine decarboxylase. This indicates that the use of arginase is the main route for arginine degradation by *H. pylori.* Arginase acts as an antagonist to NO synthase, since they compete for the same L-arginine substrate, the former converting it to L-ornithine and urea, and the latter converting it to NO (Chaturvedi *et al.,* 2012). Thus, it can be deduced that colonization by *H. pylori* may result in excess arginine consumption, a decrease in NO production, a decrease in vasodilation and hence an increase in blood pressure.

Infection with *H. pylori* should therefore have a correlation with hypertension, as has been demonstrated in the studies summarised in Table 1.2. Thus, an association between *H. pylori* infection and hypertension is prevalent. As a result, treatment to eradicate *H. pylori* should effectively lower blood pressure, as seen in Table 1.2.

Table 1.2: The correlation between *H. pylori* infection, hypertension and cardiovascular diseases (CVD) associated with hypertension.

Association	Sample Population	Sample Size	Significant Findings	Reference
Blood pressure	Italian	72 hypertensive (34 male and 38 female) and 70 normotensive (35 male and 35 female)	Antibiotic *H. pylori* eradication significantly reduced mean blood pressure	Migneco *et al.* 2003
Blood pressure	Indian subcontinent	40 hypertensive and 40 normotensive	Serology tests detected *H. pylori* in a significant number of patients	Shankar *et al.*, 2012
Blood pressure	Chinese	5246	^{13}C-urea breath tests showed a strong correlation between *H. pylori* and hypertension	Wan *et al.*, 2017
Blood pressure	Chinese	17100	^{13}C-urea breath tests showed a strong correlation between *H. pylori* and hypertension	Xiong *et al.*, 2020
Salt intake and blood pressure	10 nations (in Europe, USA and Japan)	200	Significant correlation in salt secretion and *H. pylori* infection in older patients	Beevers *et al.*, 2004
Atherosclerosis	Congolese	205 (128 *H. pylori* positive and 77 negative)	Association between CVD risk factors, carotid plaque and stroke in *H. pylori* positive patients	Longo-Mbenza *et al.*, 2012
Acute coronary syndrome	Taiwanese	17075 *H. pylori* positive and 68300 negative	Significant correlation between *H. pylori* infection and acute coronary syndrome and its comorbidities	Lai *et al.*, 2015
Coronary artery disease	German	96 with coronary artery disease and 96 without	Serology detected a significant link in *H. pylori* infection in patients with coronary artery disease	Kowalski, 2001
Coronary artery disease	Iranian	62 with coronary artery disease and 58 without	Serology detected *H. pylori* infection was more prevalent in coronary artery disease patients	Vafaeimanesh *et al.*, 2014

1.1.6 *H. pylori* treatment, antibiotics and resistance

Eradication of *H. pylori* as a means of therapy has been of interest for a number of years, due to the role it plays in a variety of diseases. Combinations of bismuth, acid inhibitors and antimicrobials have been used in the past, but these were found to be ineffective in approximately a third of cases (Francesco *et al.,* 2010). An important factor in *H. pylori* eradication is antibiotic resistance. The most common antibiotics described in literature for *H. pylori* eradication is clarithromycin and nitroimidazole, combined with proton pump inhibitors (PPIs) (Mégroud, 2012). Resistance to nitroimidazole, a combination of metronidazole and tinidazole, is highly variable and has increased over time. The prevalence of resistant rates in developing countries is reported to be 80-90% (Francesco *et al.,* 2010). The efficacy of eradication using PPIs and amoxicillin was shown to decrease when used to treat metronidazole-resistant strains (Francesco *et al.,* 2010). The resistance of clarithromycin was found to vary from country to country and is increasing over time. The efficacy of other drug combinations on clarithromycin-resistant strains was found to be highly varied (Francesco *et al.,* 2010). Thus, it is vital that the strains identified be tested for resistance before administering therapy, and a combination of drugs be used. A combination of PPI, amoxicillin and clarithromycin supplemented with levofloxacin has thus far been proven as the most effective means of eradication, provided that the strains being targeted are tested for antibiotic resistance first.

These antibiotics have also been noted to elicit changes on the gut microbiome as a whole, rather than just targeting *H. pylori.* Amoxicillin is a broad-spectrum antibiotic, which has been used to treat a number of infections, from pneumonia to dental abscesses. Treatment with amoxicillin has been shown to result in shifts in the microbiota; Bacteroides was found to decrease while Clostridia and Enterobacteria were shown to increase (Raymond *et al.,* 2015; MacPherson *et al.,* 2018).

Clarithromycin, besides being used to treat *Helicobacter* related ailments, is also used against pneumonia, skin infections and strep throat. Edlund *et al.* (2000) found that treatment with clarithromycin caused shifts in the normal intestinal microbiota, with the populations of *Escherichia coli* decreasing while *Enterococci, Enterobacter, Citrobacter, Klebsiella* and *Pseudomonas* showed significant increase in some volunteers. Slight changes were also observed in *Bacillus* and *Candida.* These changes reverted after 35 days in the volunteers

assessed (Edlund *et al.,* 2000). Similar changes were observed by Matute *et al.* (2002) as well as in several other studies; all noting a decrease in Enterobacteria, with a few studies noting decreases in Bifidobacteria and Bacteroides (Matute *et al.,* 2002; Elvers *et al.,* 2020). All the aforementioned studies found that the respective microbiomes returned to their normal states 20 to 30 days after treatment.

Omeprazole is commonly used in the treatment of gastroesophageal reflux and peptic ulcers, amongst others. Different studies have shown contradicting results with regards to the effects of PPI's such as omeprazole on the gut microbiome. The current consensus is an increase in the amount of Bacteroides and Clostridia, as well as in the *Streptococcus, Holdemania* and *Blautia* genera (Freedberg *et al.,* 2015).

1.1.7 Hypertension and the gut microbiome

Playing a key role in the biological processes of humans and mammals, the gut microbiome has become recognized as the hidden "organ" of the body. The gut microbiome is considered a key player in maintaining the health of an individual, through its role in harvesting nutrients and energy from ingested food, as well as producing numerous metabolites, which in turn regulate the metabolism of its host. Described as "one of the most densely populated and complex ecosystems known", more than 1000 phylotypes have been identified in the human gut microbiota (Ridlon *et al.,* 2015; Shreiner *et al.,* 2016). These can be divided into 6 main phyla, namely Firmicutes, Bacteriodetes, Proteobacteria, Acinetobacteria, Fusobacteria and Verrucomicrobia, with the first two making up more than 90% of the total (Ridlon *et al.,* 2015).

Several studies have shown differences in the gut microbiota between hypertensive and normotensive cases, with hypertensive cases showing a much less diversity (Qi *et al.,* 2017). In a study assessing a Brazilian population, hypertensive individuals showed a reduction in Bacteroidetes with increases in *Lactobacillus* and *Akkermansia* populations. A decrease in the butyrate-producing gut microbiota families was also noted (Silveira-Nunes *et al.,* 2020). Li *et al.,* (2017) noted a decrease in the *Prevotella* group in hypertensive individuals. The gut microbiome profiles of hypertensive individuals were also found to be very similar to that of prehypertensive individuals. This study also demonstrated the ability of faecal transplanted microbiota to cause hypertension in germ-free mice (Li *et al.,* 2017). A study by Adnan *et al.* (2017) assessed the impact of gut dysbiosis on hypertension in rats. This was done by

administering by gavage the microbiota of spontaneously hypertensive rats into normotensive (Wistar-Kyoto) rats and vice versa. Blood pressure was monitored and was noted to increase in normotensive rats with a hypertensive microbiota (Adnan *et al.,* 2017). Sun *et al.* (2019) assessed a wide group of individuals, comprising of a range of racial and geographical backgrounds; a dysbiosis in the gut microbiota of hypertensive individuals was noted, with specific genera associated with hypertension (Sun *et al.,* 2019). Yan *et al.* (2017) assessed 60 hypertensive patients and 60 normotensive individuals. Their results showed a shift in the gut microbiome of hypertensive individuals, with opportunistic pathogenic taxa such as *Klebsiella, Streptococcus,* and *Parabacteroides* present in much higher abundance. These changes and species abundance were also in correlation with severity of disease (Yan *et al.,* 2017).

1.1.8 Hypertension and cardiotonic steroids

A further factor in the aetiology of hypertension is the role played by cardiotonic steroids (CTSs). Nanomolar concentrations of cardiotonic steroids have been detected in the plasma of humans and animal models with hypertension, with concentrations correlating with blood pressure (Pavlovic, 2014). Cardiotonic steroids can be broadly classified into cardenolides, such as ouabain, and bufadienolides, such as marinobufagenin. The former has a 5 membered unsaturated lactone ring attached to the steroid nucleus, and the latter an unsaturated 6 membered ring (Pavlovic, 2014). Ouabain, a cardenolide, has been detected in the blood of patients suffering from hypertension and certain cardiovascular events (Pavlovic, 2014). Evidence has shown that cardiotonic steroids appear to be formed from cholesterol conversions in the adrenal glands, but the exact pathways appear to differ for the two groups of cardiotonic steroids (Pavlovic, 2014).

Certain cardiotonic steroids, such as ouabain, act as Na^+/K^+ pump inhibitors, binding to the pump and inhibiting it (Bagrov *et al.,* 2009) and this reduces the transport of arginine into the cell (unpublished data: Nel and Candy, 2016). As NO production depends on uptake of extracellular arginine into cells, the effect would be to reduce the amount of NO produced and decreased vasodilation, as previously mentioned. Thus, an increase in the amount of cardiotonic steroids present can cause an increase in blood pressure. Investigating the effect of these steroids on hypertension and their reliance on the gut microbiota may reveal a potential target for therapy.

1.1.9 Hypertension in rats

Laboratories around the world have made extensive use of animal models to understand mechanisms causing human disease. Rats and mice can be easily bred and housed, and experimental results can be obtained quickly and are therefore convenient to study disease mechanisms. Studies investigating high blood pressure have used Wistar-Kyoto (WKY) rats that normally have systolic blood pressures of 120 to 130 mmHg (Luo *et al.*, 2008). A strain of in-bred WKY rats were found to develop very high blood pressures and were termed Spontaneously Hypertensive Rats (SHR). These rats develop with systolic blood pressures of between 150 to 164 mmHg at 6 to 8 weeks of age. Systolic blood pressures increase further from 12 weeks old, ranging from 185 to 203 mmHg and plateau after 15 weeks of age (Luo *et al.*, 2008).

The use of antibiotics on rat models has long been employed. In a study assessing the effects of antibiotics on the gut microbiome and its link to hypertension, Galla *et al.* (2018) administered neomycin, minocycline and vancomycin to Dahl-salt-sensitive rats and SHRs. They observed an increase in the systolic blood pressures of salt sensitive rats in response to all three antibiotics, while minocycline and vancomycin was effective in lowering the systolic blood pressures of the SHRs. In both rat strains, the gut microbiomes of the rats were altered by the administered antibiotics (Galla *et al.*, 2018). In another study, Galla *et al.* (2020) administered amoxicillin to two different groups of Dahl-salt-sensitive rats: paediatric rats and dams during gestation and lactation. Systolic blood pressure was lowered in both groups in response to antibiotic administration. Their respective gut microbiomes were also altered, with decreases in the Firmicutes to Bacteroidetes ratio observed, as well as a reduction in the succinate-producing bacteria *Veillonellaceae* (Galla *et al.*, 2020). Khan *et al.* (2016) assessed the effects of antibiotics on the gut microbiome in pregnant Sprague-Dawley rats in an effort to understand how these antibiotics change the gut microbiome and their relevance to pregnancy complications. Amoxicillin, azithromycin and cefaclor was administered by gavage to the rats and was effective in eliciting changes to the rats' gut microbiomes (Khan *et al.*, 2016).

The development of hypertension in the SHR show similarities to that in humans and can be used to study the impact of the microbiome on hypertension (Adnan *et al.* 2017). In addition, the gut microbiome is noted to be fairly conserved in mammals. Rats models used in studies of hypertension have been noted to be colonized with species of *Helicobacter* other than *H. pylori*.

Dahl-salt-sensitive rats have *H. mesocricetorum* and *H. rodentum* with an abundance of 5% whereas SHR have a higher abundance (15%) of *H. mesocricetorum*, *H. rodentum* and *H. ganmi*. Furthermore, and in contrast to humans, these bacteria were not identified in the stomach (unpublished data: Thiba, 2018).

Administering antibiotics to rat models should therefore be effective in selecting for or against bacteria such as *Helicobacter spp.,* as well as eliciting specific changes to the gut microbiome. Thus, employing rat models to study the aforementioned phenomena should provide an insight into that occurring in its human counterparts and the impact of the gut microbiome in hypertension.

1.2 Problem Statement

Stroke and myocardial infarction, congestive heart failure and peripheral vascular disease as well as the consequent end-stage renal disease make up a significant proportion of non-communicable diseases treated worldwide. Elevated blood pressure or hypertension is associated with, and is believed to underlie, these cardiovascular diseases but the cause of hypertension is not known. Hypertension is increasing in sub-Saharan countries, consuming a large slice of scarce healthcare budgets of these countries. Elucidation of the mechanism of hypertension may result in treatments to reduce morbidity and mortality from cardiovascular disease and consequently reduce healthcare costs, particularly in developing countries.

The gut microbiome in both animal models of hypertension as well as in patients with hypertension and cardiovascular disease is altered. Although the identity of a causal bacterium or mechanism is not known, *Helicobacter pylori* has been implicated in the aetiology of human hypertension through many, but not all, epidemiological studies and observations that high blood pressures are associated with the presence of *H. pylori* and successful eradication of the bacterium is accompanied by a significant decrease in blood pressure.

H. pylori is believed to cause inflammation and oxidative stress which are related to blood pressure. However, other possible mechanisms include the reduction of arginine, which is the precursor for NO, from the environment of the bacterium by the bacterium's powerful arginase and urease enzymes; or by having a role in the production of substances which affect blood pressure.

The question is whether *H. pylori* or another bacterium affects blood pressure. Therefore, this study was designed to determine the effects on systolic blood pressure of normotensive and hypertensive rat models

a) by selecting for, and against *Helicobacter spp.* using selective antibiotics,

b) following re-infection with the human pathogen *H. pylori*, in rats where *Helicobacter spp.* were eradicated, and

c) following administration of bacterium-free *H. pylori* culture supernatant, where *Helicobacter spp.* were eradicated to determine the effects of *H. pylori* metabolites alone.

1.3 Hypothesis

The overall hypothesis was that *Helicobacter spp.* and *H. pylori* influences blood pressure, either through a direct interaction, or by producing a metabolite or a precursor to such a metabolite, which affects blood pressure.

1.4 Aims

The aims of this study were to

- determine the effect of antibiotics selecting either for or against *Helicobacter spp.* on hypertension in both normotensive and hypertensive rat models
- determine the effect of *H. pylori* itself, as well as the metabolites it produces on hypertension in both normotensive and hypertensive rat models.

1.5 Objectives

Part 1: Effects of antibiotics on hypertension

- collect faecal samples from the rat models
- isolate *Helicobacter spp.* from faecal samples of rat models using bacterial culture
- assess isolated *Helicobacter spp.* to determine the antibiotic resistance patterns
- use antibiotics to select for *Helicobacter spp.* by administering by gavage to WKY rats (normotensive) and SHR (hypertensive)
- use antibiotics to select against *Helicobacter spp*. by administering by gavage to WKY rats (normotensive) and SHR (hypertensive)
- to record systolic blood pressure using tail cuff measurements

Part 2: Effects of *H. pylori* on hypertension

- prepare cultures of clinical samples of *H. pylori J99*
- determine the number of colony forming units in the *H. pylori* culture using serial dilution
- separate the supernatant from planktonic *H. pylori* culture by centrifuge
- administer by gavage cultures of *H. pylori* to both WKY and SHR
- administer by gavage cell-free supernatants from planktonic cultures of *H. pylori* to WKY and SHR
- assess the efficacy of the above treatments using a faecal antigen testing kit to test for the presence of the *H. pylori* antigen

Chapter 2: Methods and Materials

Overview

This study aimed to assess the effects that antibiotics targeting *Helicobacter spp.* have on rat models of hypertension, as well as the effect of *H. pylori* on hypertension.

This study used Wistar-Kyoto (WKY) and Spontaneously Hypertensive rat models (SHR) to assess the effect of antibiotic eradication of *Helicobacter spp.* and the effect of *H. pylori,* and its metabolites on hypertension.

The antibiotic resistance of *Helicobacter spp.* colonizing the rats' gastrointestinal tract were assessed and antibiotic solutions made up accordingly. Using antibiotics, both rat strains were divided into three groups, selection for *Helicobacter spp.,* selection against (eradicated) *Helicobacter spp.* and a control group. All rat models were housed following usual husbandry, and their systolic blood pressures monitored.

The effects of *H. pylori* were assessed by infecting rat models which had *Helicobacter spp.* eradicated with a clinical strain of *H. pylori.* Both SHR and WKY rats were inoculated with prepared *H. pylori* cultures or the cell-free supernatant of such cultures. A third group of each strain served as the control. Systolic blood pressure was monitored throughout these processes using tail cuff measurements.

2.1: Materials

The materials (reagents and consumables) used in this project are summarised in Appendix A.

2.2: Animal Study

2.2.1 Animals

This study was approved by the University of Witwatersrand Animal Research Ethics Committee (Clearance certificate number 2019/04/31/C) (Appendix B) and carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Animals, 2011). SHR and WKY rats were obtained from Charles River Laboratories – Research Models and Services, Germany and bred at the Central Animal Service at the University of Witwatersrand Faculty of Health Sciences. All rats were approximately 10 weeks old at the beginning of this study and equal numbers of male and female rats used. Their

weights ranged from 250g to 350g, measured weekly. They were housed at the Central Animal Service; individually caged, on a 12-hour light cycle with temperature kept at 30°C. They were fed a standard diet as recommended by the Central Animal Service, with constant access to water. The rats were habituated and checked by a vet weekly. For faecal sample collection, the rats were transferred to metabolic cages overnight. These cages individually house the rats and allow for clean faecal and urine sample collection in receptacles.

Systolic blood pressure was measured using a non-invasive method employing a tail-cuff to occlude blood flow in the tail as it inflates. Upon deflation, sensors measure the systolic blood pressure and pulse (BIOPAC – NIPB250 Tail Cuff System). This non-invasive method has been shown to be reliable and generate dependable results and follows a simple method which does not require calculations post measurement. It is, however, affected by various factors such as heat and restraint. Thus, the use of a large sample size ($n \geq 6$) is vital in generating dependable results. The environment was kept warm (approximately 30°C) and the rats allowed time to acclimate to having blood pressure recorded.

2.2.2 Culture conditions

Supplemented 37g/l Brain Heart Infusion (BHI) served as the liquid media utilised in this project. This was prepared by dissolving the media in distilled water and sterilising by autoclave at 121°C for 15 minutes. After cooling to approximately 50°C, it was supplemented with an antibiotic cocktail (Campylobacter Selective Supplement Skirrows – 5mg vancomycin and 205mg trimethoprim) and 7% foetal bovine serum (FBS). These supplements ensure the effective selection of only *Helicobacter spp.* (Smith *et al.*, 2014). All liquid cultures were incubated under microaerobic conditions using an anaerobic tank and a microaero gas pack, with 100rpm shaking at 37°C. This was cultured continuously, and optical density (OD) readings taken at 600nm to monitor growth. A sample of supplemented BHI served as a blank for all spectrophotometer readings.

Solid media consisted of supplemented 39g/l Columbia Blood Agar (CBA). This was prepared by dissolving media in distilled water and sterilizing by autoclave at 121°C for 15 minutes. Once the media cooled to approximately 50°C, it was supplemented with Campylobacter Selective Supplement (Skirrows) and 7% FBS. This was used to make agar plates for culture.

2.3 Part 1: Effects of Antibiotics on Hypertension

2.3.1 Isolation of *Helicobacter spp* from faeces

Faecal samples were collected from one SHR and one WKY rat using metabolic cages. Faecal pellets weighing 0.2g each were immediately cultured in 10ml supplemented BHI aliquots. After 7 days, the stationary phase of growth was reached, and the cultures were removed from incubation and stored at 4°C. Aliquots (100µl) from this liquid culture were streaked in triplicate onto CBA.

The clinical strain *H. pylori J99* (German Type Culture Collection – Department of Surgery, University of the Witwatersrand) were grown from frozen stock and 100µl streaked onto the prepared plates, in triplicate. These cultures, along with *Bacillus cereus* cultures (Department of Microbiology and Biotechnology, University of the Witwatersrand) served as positive controls when tested against the isolated *Helicobacter spp.*

The streaked plates were cultured for 72 hours. Colonies, which matched the morphology of *Helicobacter spp.* were further subcultured to generate pure cultures for identification and antibiotic resistance assessment.

2.3.2 *Helicobacter spp.* identification

Helicobacter spp. was identified by the Gram stain and oxidase, urease and catalase tests. The faecal isolation was tested along with *H. pylori J99* and *B. cereus* and the results compared.

The Gram Stain

The Gram stain classifies bacteria based on the composition of their cell walls and their ability to retain the crystal violet stain during solvent treatment. Thick cell walls have a high percentage of peptidoglycan and these bacteria thus retain the initial stain, crystal violet, appearing purple in colour and classifying as Gram positive. Thin cell walls have a low percentage of peptidoglycan, and these bacteria therefore retain the final stain, safranin, resulting in their red appearance and their classification as Gram negative (Matheson, 1999; Coico, 2005). The Gram stain was performed by fixing isolated colonies onto slides using heat and then subjecting them to crystal violet, then iodine, alcohol decolourizer and finally

safranin. The slides were viewed under a microscope at 40X magnification. Based on their respective morphologies, *Helicobacter spp.* was expected to stain Gram negative (Van Horn and Dworkin, 1990) and *B. cereus* Gram positive (Bottone, 2010).

The Oxidase Test

The oxidase test assesses whether bacteria possessed cytochrome c oxidase, an enzyme in the electron transport chain. The test was performed by preparing a 1% solution of Kovac's Oxidase Reagent. Filter paper was soaked in this solution and allowed to dry. Colonies were then placed on this filter paper and monitored for colour change. When present, cytochrome c will oxidise the reagent to form indophenols which are purple in colour. *Helicobacter spp.* is known to have a positive oxidase test result (Kusters *et al.*, 2006) while *B. cereus* has a negative oxidase test result (Sharma and Rao, 2015).

The Urease Test

The urease test identifies the presence of the urease enzyme. Urease hydrolyses urea into ammonia and carbon dioxide. The production of ammonia changes the pH of the resulting solution. This shift in pH is shown through the addition of the indicator phenol red (Koumi *et al.*, 2011). Thus, a positive urease result is indicated by a pink colour, and a negative result by a yellow colour. The urease test was performed by dissolving 1g yeast extract, 9.1g potassium phosphate monobasic, 9.5g potassium phosphate dibasic, 20g urea and 0.01g phenol red in 1*l* of distilled water. 3ml aliquots of this solution were inoculated with each of the bacterial colonies and colour change observed after 15 minutes. *Helicobacter spp.* is known to yield a positive urease test result as it hydrolyses urea rapidly (Kusters *et al.*, 2006). *B. cereus* yields both positive and negative urease test results, depending on the particular strain (Mols and Abee, 2008), but for the purpose of this study, a lack of colour change within 15 minutes was recorded as a negative result, as this indicates that the tested colony is not *Helicobacter spp.*

The Catalase Test

This test demonstrates the presence of the catalase enzyme which catalyses the production of oxygen from hydrogen peroxide (H_2O_2). Catalase protects the bacteria from the by-products of oxygen metabolism and is produced by aerobic and facultatively aerobic bacteria. Since *Helicobacter spp.* is considered both a microaerophile and a capnophile (Park *et al.*, 2011), it is expected to test positive for the catalase test. *B. cereus* is a facultative aerobe and therefore should also test positive (Xaplaneteri *et al.*, 2019). The test was performed by preparing a 3%

H_2O_2 solution and placing a single colony in a drop of the solution on a slide. A positive result is indicated by bubble formation as the catalase enzyme decomposes the H_2O_2 into water and oxygen (Iwase *et al.,* 2013).

2.3.3 Antibiotic resistance

Helicobacter spp. isolated from WKY and SHR faecal samples were cultured to assess its antibiotic resistance to amoxicillin, clarithromycin, metronidazole and tetracycline. Assessing the antibiotic susceptibility of the *Helicobacter spp.* colonizing the rats' GIT is necessary to ensure effective eradication thereof. Since *H. pylori* is prone to gaining resistance to antibiotics, calculating an antibiotic dose for effective eradication is vital.

This was done by inoculating 10ml aliquots of supplemented BHI each with a single *Helicobacter spp.* isolate and cultured for 24 hours. This inoculum (500µl) was then used to prepare triplicate spread CBA plates onto which Minimum Inhibitory Compound (MIC) Test Strips of each of the abovementioned antibiotics had been placed. These plates were cultured for 72 hours.

The resistance to the different antibiotics were recorded, and the required antibiotic dose calculated therefrom. The recommended dose was taken as 2-fold higher than the most resistant culture for each antibiotic tested, as read on the concentration gradient on the strips (Mouton *et al.*, 2018).

2.3.4 Antibiotic solutions

The antibiotic solutions (Department of Pharmacy and Pharmacology, University of the Witwatersrand) used in this project are summarised in Table 2.1. A solution to select for *Helicobacter spp.* was prepared to decrease the number of any other bacterial species present which might compete with *Helicobacter spp.* (Werawatganon, 2014). A second solution to select against *Helicobacter spp.* in the GIT of the rat models was prepared based on the antibiotic resistance patterns of *Helicobacter spp.* isolated from the rat models (Jury *et al.,* 2005).

Table 4.1: Antibiotics used to select for or eradicate *Helicobacter spp*.

Antibiotic Mix	**Antibiotics**	**Use**
Mix 1	5mg/ml streptomycin	Selection for *Helicobacter spp.*
Mix 2	0.1µg/ml amoxicillin trihydrate, 12µg/ml clarithromycin, 2.4µg/ml omeprazole and 1.44µg/ml bismuth subcitrate	Eradication of *Helicobacter spp.*

2.3.5 Selection for *Helicobacter spp*.

To select for *Helicobacter spp.,* 6 WKY rats and 6 SHR, both of equal male and female, were used (Figure 2.1). Systolic blood pressure was measured before treatment was administered, serving as a baseline measurement. Antibiotic Mix 1 (Table 2.1) was administered by means of gavage. Each rat in these groups received 0.5ml of Mix 1 (5mg/ml streptomycin) once a day for 5 days.

Systolic blood pressure was recorded for a total of 3 weeks (throughout the process of antibiotic feeding as well for 2 weeks thereafter).

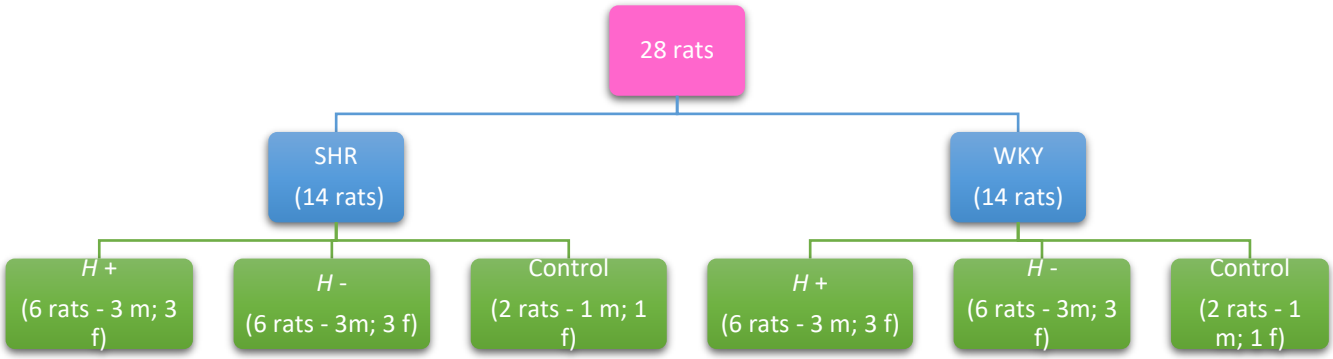


Figure 2.2: Rat groups in Part 1 of this study.
28 rats were used in total and divided as shown. They received treatment to select either for *Helicobacter spp.* (denoted as H+) or against *Helicobacter spp.* (denoted as H-). Controls were fed a standard diet. Each group comprised of equal numbers of males (m) and females (f), all approximately 10 weeks old.

2.3.6 Selection against *Helicobacter spp.*

To eradicate *Helicobacter spp.*, 6 WKY rats and 6 SHR, both of equal male and female were used (Figure 2.1). The rats were rested, habituated and treated with the antibiotics as described above. Antibiotic Mix 2 (0.1µg/ml amoxicillin trihydrate, 12µg/ml clarithromycin, 2.4µg/ml omeprazole and 1.44µg/ml bismuth subcitrate) was used to eradicate *Helicobacter spp.* and systolic blood pressure monitored, as described above.

2.3.7 Control group

A control group for both SHR and WKY consisted of two rats each, one male and one female per strain (Figure 2.1). They received a normal diet and were not administered either of the aforementioned treatments. Their systolic blood pressures were recorded over the same time period as their experimental counterparts.

2.4: Part 2: Effects of *H. pylori* on Hypertension

2.4.1 *H. pylori J99* culture

To assess the effect of *H. pylori* and the metabolites its produces, liquid cultures of clinical samples of the bacteria (*H. pylori J99* from the German Type Culture Collection was provided by the Department of Surgery) were grown using BHI, as outlined previously (Section 2.3.2). The BHI media used in this instance was supplemented with antibiotics only. FBS was eliminated in this culture, to minimize external compounds which could influence the rats' blood pressure, and to treat the rats with the metabolites produced by *H. pylori* only.

To determine the number of cells administered to the rat models, the amount of colony forming units (CFU) was determined. This was done using the serial dilution method. The growth of liquid cultures of *H. pylori J99* made was monitored by recording OD at 600nm. Serial dilutions were then made using each OD sample, ranging from dilutions of $1:10^{-2}$ to $1:10^{-10}$. These dilutions (500µl aliquots) were then used to make spread plates in triplicate on CBA. The CFU were counted and the amount per ml calculated using the dilution factor as per the formula:

$$\text{CFU/ml} = \frac{no.of\ CFU\ counted \times dilution\ factor}{volume\ plated\ in\ ml}$$

A separate aliquot of *H. pylori J99* culture (50ml) was centrifuged to separate the cells from the supernatant of the culture. The bacterial cells were harvested and separated from the supernatant using centrifugation (2000g for 6 minutes).

2.4.2 *H. pylori* culture administration

Six rats each, of both WKY and SHR were used for this section of the study, shown in Figure 2.2. Rats were approximately 24 weeks old. As a preparatory treatment, all rats were administered 0.5ml of the Antibiotic Mix 2 (0.1µg/ml amoxicillin trihydrate, 12µg/ml clarithromycin, 2.4µg/ml omeprazole and 1.44µg/ml bismuth subcitrate) to eradicate *Helicobacter spp.* This was done by gavage once a day for 5 days.

After allowing the rats to rest for a week to clear any circulating antibiotics, 0.5ml of a liquid *H. pylori* suspension consisting of between $5.6x10^8$ and $6x10^8$ CFU/ml was administered once

daily to the rat models by gavage for 5 days. Systolic blood pressure was monitored continuously for a total of 4 weeks (a week before *H. pylori* cell culture was administered, throughout this process and then for 2 weeks thereafter). The rats' systolic blood pressure was again monitored 6 weeks post treatment to assess any possible changes.

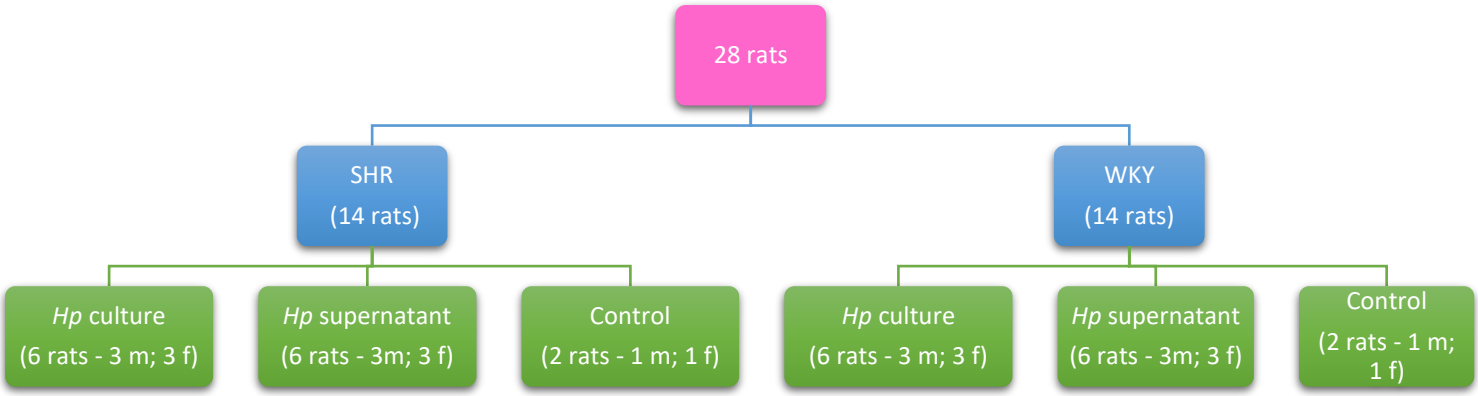


Figure 2.2: Rat groups in part two of this study.
28 rats were used in total and divided as shown. They were administered either live cultures of *H. pylori* (*Hp* culture) or received the cell-free supernatant of *H. pylori* cultures (*Hp* supernatant). Controls were fed a standard diet. Each group comprised of equal numbers of males (m) and females (f), all approximately 24 weeks old.

2.4.3 *H. pylori* culture supernatant administration

Six rats each, of both WKY and SHR were used, as shown in Figure 2.2. Rats were approximately 24 weeks old. As a preparatory treatment, all rats were administered 0.5ml of the Antibiotic Mix 2 to eradicate *Helicobacter spp.*, as outlined previously (Section 2.4.2).

After allowing the rats to rest for a week to clear any circulating antibiotics, the rats were then fed 0.5ml of the cell-free *H. pylori* culture supernatant, administered by gavage once daily for 5 days. Systolic blood pressure was monitored in the same manner as outlined previously (Section 2.4.2).

2.4.4 Control group

A control group consisting of 4 rats, 2 WKY and 2 SHR, each of equal males and females, were used (Figure 2.2). These models were fed a normal diet for the duration of this project, and their systolic blood pressure monitored throughout.

2.4.5 Efficacy of treatment

To assess the efficacy of treatment, faecal samples were collected after treatment with *H. pylori* culture or cell-free supernatant and from controls. The faecal samples were tested for the presence of the *H. pylori* antigen using a faecal antigen detection kit and used according to manufacturer's instructions (CerTest - BIOTEC) (Moon *et al.,* 2013) (Appendix C).

2.5 Statistical Analysis

Data was recorded in EXCEL and reported as mean±std graphically. The blood pressure was compared between the rat models using a non-parametric Kruskal Wallis test. To compare blood pressures within each model over time, a non-parametric Friedman two-way ANOVA was used. A p-value of <0.05 was regarded as significant.

Chapter 3: Results

3.1: Part 1: Effects of Antibiotics Targeting *Helicobacter spp.* on Blood Pressure

3.1.1 Isolation of *Helicobacter spp.* from faeces

Helicobacter spp. was isolated from SHR and WKY rats' faeces using liquid culture. OD_{600} readings were taken to monitor the level of growth. This growth curve is represented in Figure 3.1.

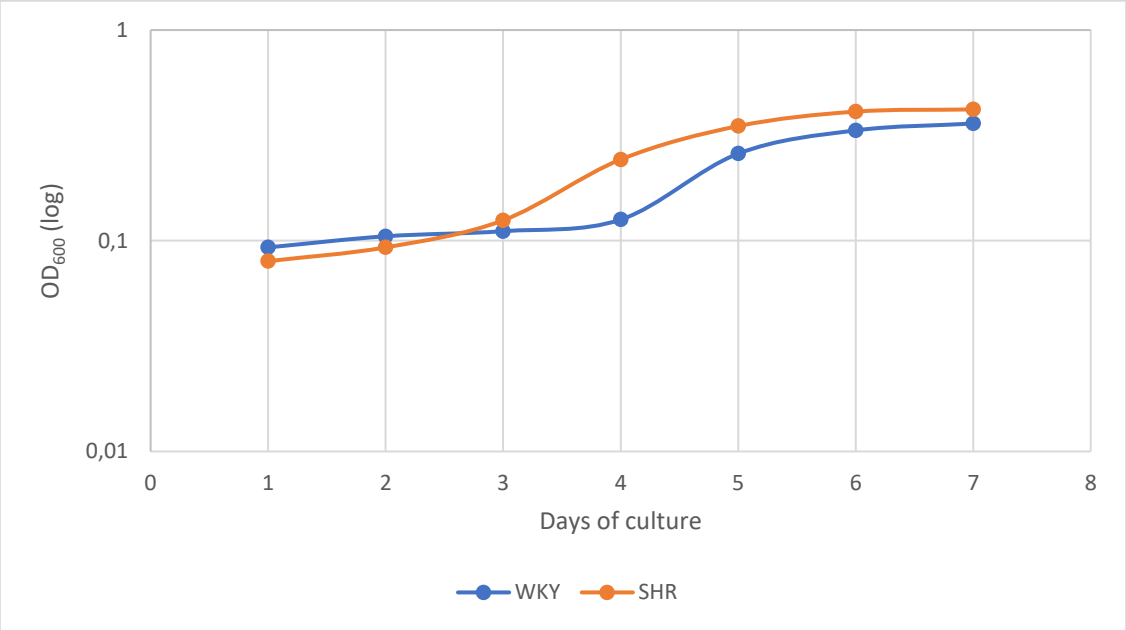


Figure 3.1: Growth curve of *Helicobacter spp*. isolated from faeces.
Growth was stopped after 7 days, as the cells had reached their stationary phase.

3.1.2 *Helicobacter spp.* identification

A series of biochemical tests were carried out comparing the isolated bacteria to the clinical strain *H. pylori J99* and *B. cereus.* These results are summarized in Table 3.1. From the results, the isolated bacteria were identified as *Helicobacter spp*. A purple colour was recorded as Gram positive and a red colour as Gram negative; the presence of purple colour was taken as a positive oxidase test result; a shift in colour to pink was taken as a positive urease test result and the formation of bubbles served as a positive catalase test result.

Table 3.1: Summary of biochemical test results identifying isolated bacteria.

Test	Test Colony: WKY	Test Colony: SHR	*B. cereus* expected	*B. cereus* tested	*H. pylori J99* expected	*H. pylori J99* tested
Gram stain	-	-	+	+	-	-
Oxidase	+	+	-	-	+	+
Urease	+	+	-	-	+	+
Catalase	+	+	+	+	+	+

3.1.3 Antibiotic resistance

The antibiotic resistance of the isolated *Helicobacter spp.* was tested using MIC strips as seen in Figure 3.2. The culture which displayed the most resistance, (the highest MIC) was accepted as the MIC of that particular antibiotic and used subsequently in antibiotic dosage formulations. A summary of the MIC and the dosage calculated therefrom is shown in Table 3.2.

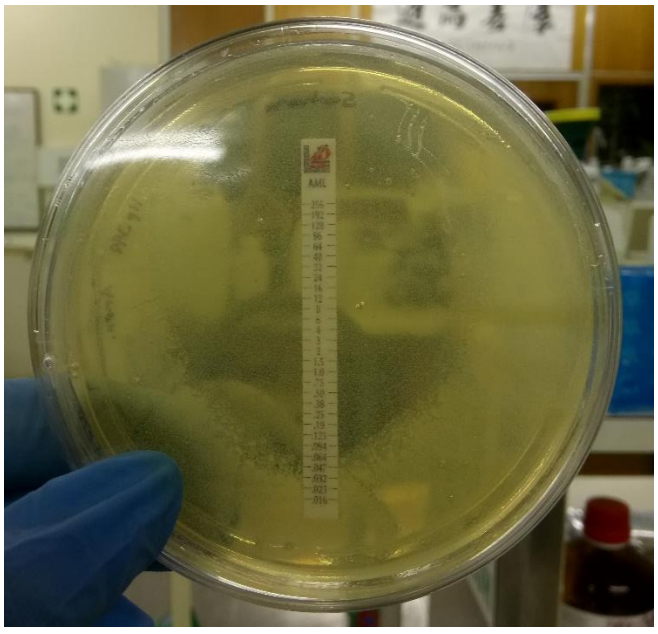


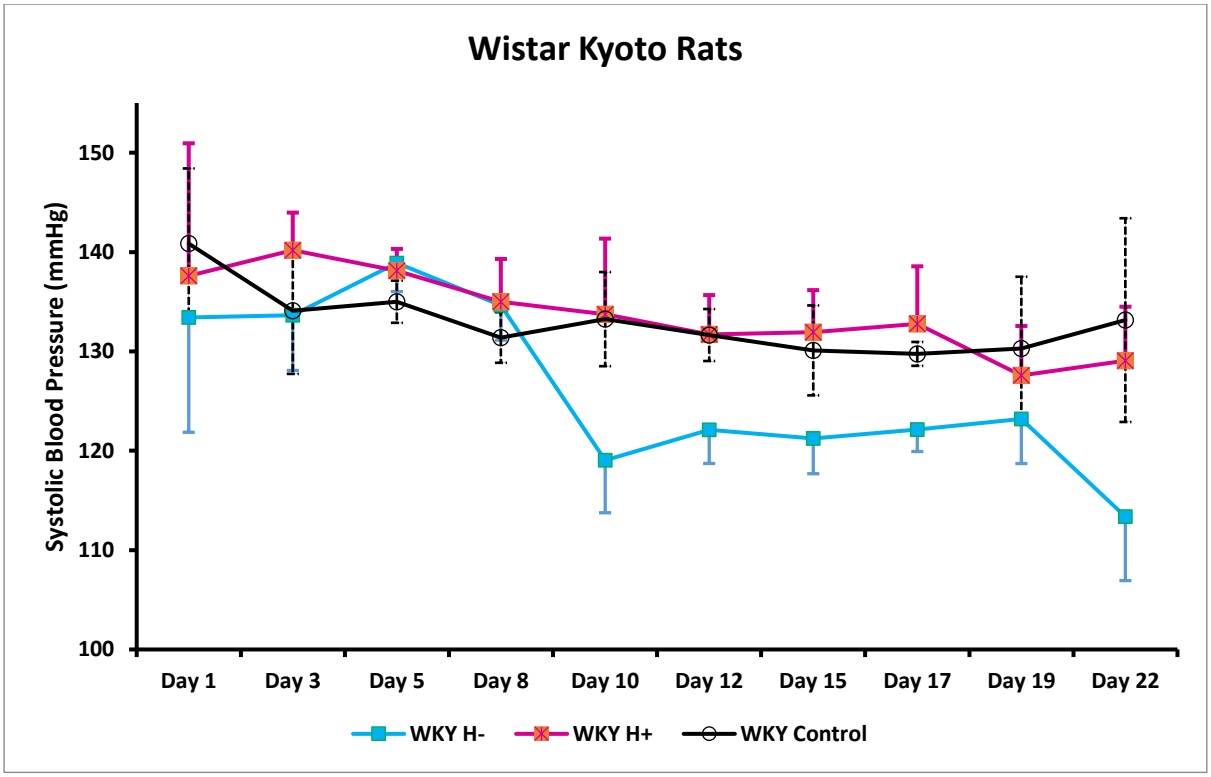
Figure 3.2: A lawn of *Helicobacter spp*. grown in the presence of a MIC antibiotic (amoxicillin) strip.

Table 3.2: Recorded MIC of antibiotics against *Helicobacter spp*. and the antibiotic dosage used.

Antibiotic	MIC Recorded (µg/ml)	Antibiotic Dosage (µg/ml)
Amoxicillin	0.047	0.100
Clarithromycin	5.00	12.00
Metronidazole	Complete resistance	--
Tetracycline	8.00	16.00

3.1.4 Selection for and against *Helicobacter spp.* in WKY rats

Systolic blood pressure was recorded prior to, during the antibiotic treatment, and for 2 weeks thereafter (total of 3 weeks). Systolic blood pressure did not change in the control rats ($p=0.62$) but showed a non-significant decline in the groups treated with antibiotics to select for ($p=0.12$), and against ($p<0.0277$) *Helicobacter spp.* (Figure 3.2). In these respective groups blood pressure showed a non-significant decrease from 138±2mmHg to 129±5mmHg (HP+ vs HP- : $p=0.75$) and from 139±3mm Hg to 113±6mm Hg (HP+ vs HP- : $p=0.0065$).

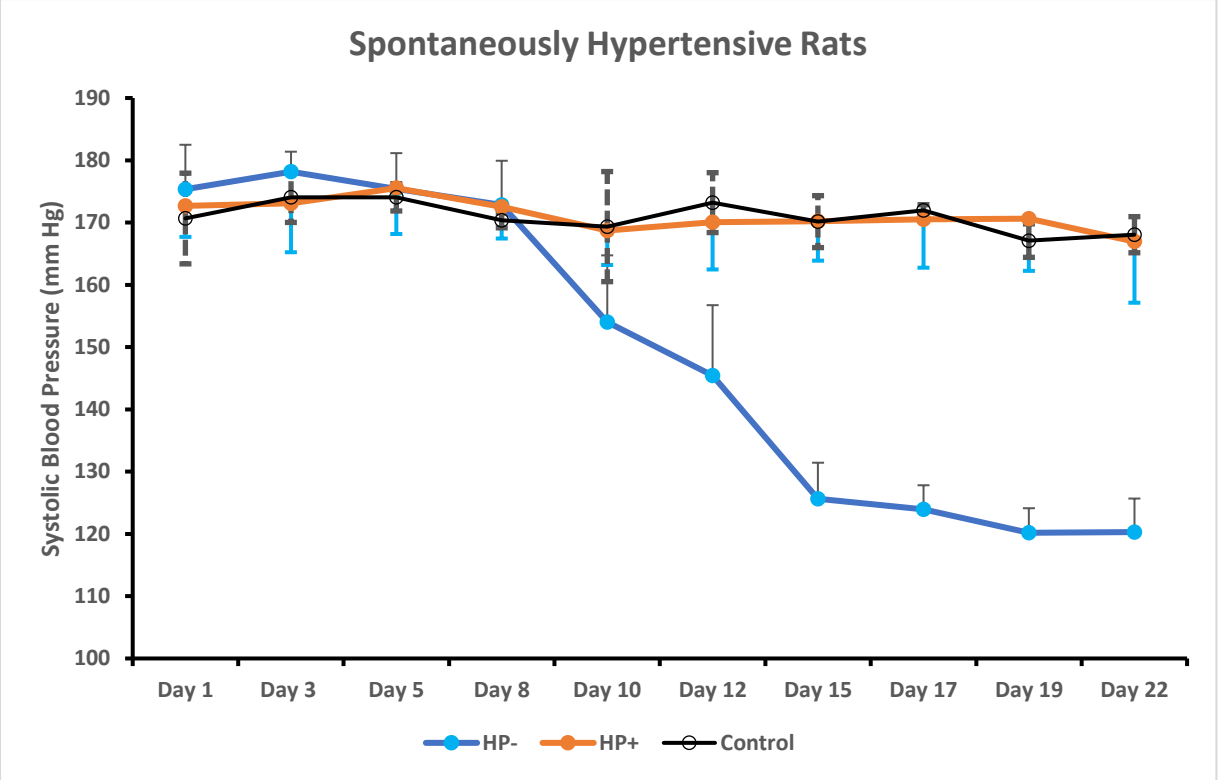


Difference between	Day 1	Day 3	Day 5	Day 8	Day 10	Day 12	Day 15	Day 17	Day 19	Day 22
Group HP- Day 5 vs (p)	-	0.92	0.35	0.92	**0.0464**	0.07	0.12	0.12	0.12	**0.0277**
Group HP+ Day 5 vs (p) (p)	-	0.75	0.75	0.60	0.60	0.17	0.35	0.35	0.12	0.12
Difference Groups HP- vs HP+	0.52	0.054	0.75	0.81	**0.0104**	**0.0039**	**0.0081**	**0.0039**	0.26	**0.0065**

Figure 3.3: The effect of antibiotic treatment to select for (HP+) or against (HP-) *Helicobacter spp.* in WKY rats on mean systolic blood pressure over time.
Antibiotic treatment was administered from day 2 to day 6. All data from each group (n=6 and n=2 controls) is shown as mean±st with changes (p-values) different from the blood pressure on Day 5 indicated in the table below the figure. Significant p-values are in bold.

3.1.5 Selection for and against *Helicobacter spp*. in SHRs

Systolic blood pressure was recorded for a total of 3 weeks, as described previously. Blood pressure did not change in the control rats (p=0.62) but declined in the groups treated with antibiotics to select for (p=0.0464), and against (p=0.0277) *Helicobacter spp.* (Figure 3.4). In these respective groups blood pressure decreased from 176±7mmHg to 167±10mmHg and from 175±6mmHg to 120±5mmHg (HP+ vs HP-: p=0.0039).

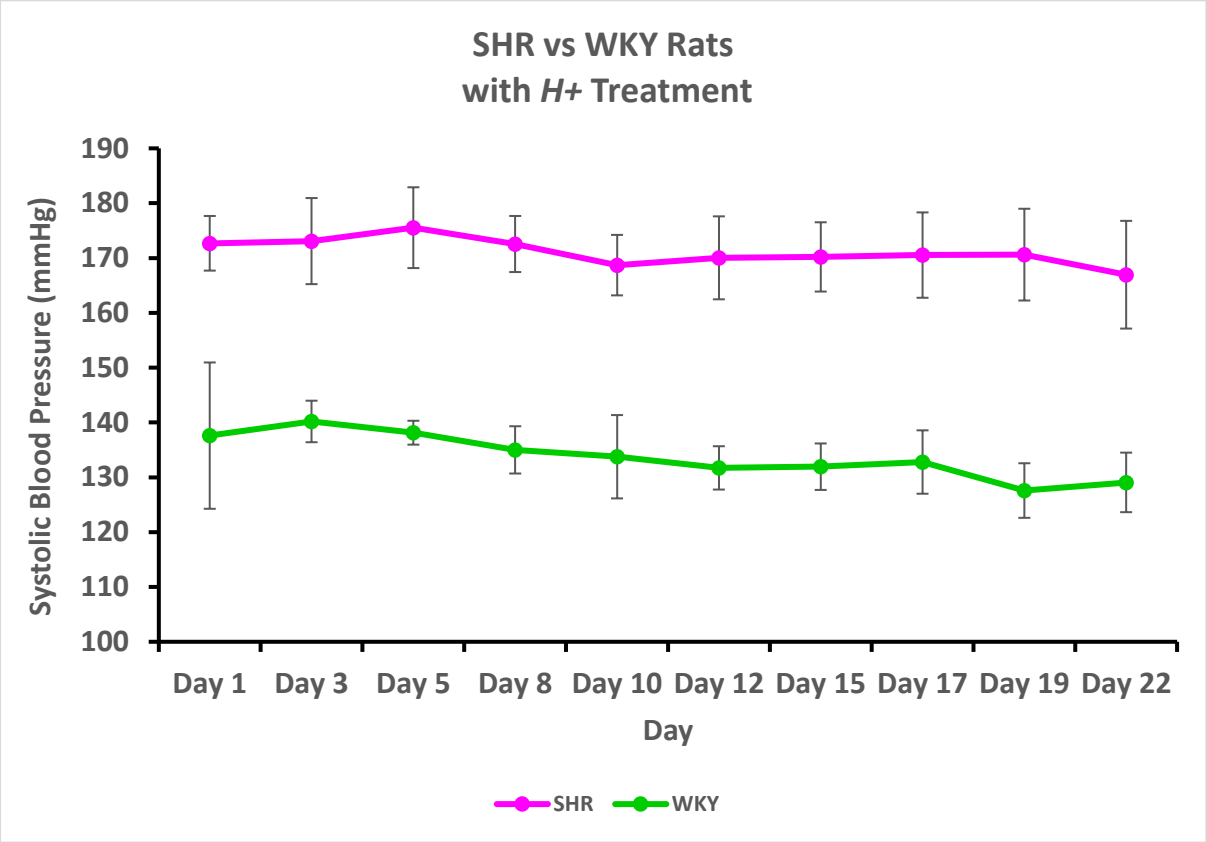


Difference between	Day 1	Day 3	Day 5	Day 8	Day 10	Day 12	Day 15	Day17	Day19	Day 22
Group HP- Day 1 vs (p)	-	0.17	0.92	0.60	**0.0464**	**0.0277**	**0.0277**	**0.0277**	**0.0277**	**0.0277**
Group HP+ Day 1 vs (p) (p)	-	0.75	0.60	0.75	**0.0431**	0.07	0.46	0.75	0.92	**0.0464**
Difference HP- vs HP+ Groups	0.63	0.15	0.81	0.94	**0.0250**	**0.0104**	**0.0039**	**0.0039**	**0.0039**	**0.0039**

Figure 3.4: The effect of antibiotic treatment to select for (H+) or against (H-) *Helicobacter spp.* in SHR rats on mean systolic blood pressure.
Antibiotic treatment was administered from day 2 to day 6. All data from each group (n=6 and n=2 controls) is shown as mean±std, with changes (p-values) different from the blood pressure on Day 5 indicated in the table below the figure. Significant p-values are in bold.

3.1.6 Changes in blood pressure between SHR and WKY rats following antibiotic treatment

As indicated above (Figure 3.3 and Figure 3.4) two weeks after antibiotic treatment to select for *Helicobacter spp.*, blood pressure decreased in the SHRs (p=0.0464) but not in WKY rats (p=0.12). The SHR blood pressures were consistently higher than measured in WKY rats (p=0.0051; Figure 3.5) over the duration of the study. The median change in blood pressure (Figure 3.7) was -6.4mmHg [range -24.1 - -0.2mmHg] in the WKYs compared to -8.1mmHg [range -20.2 - -1.4mmHg] in SHRs (difference between change in WKY vs. SHR p=0.69).

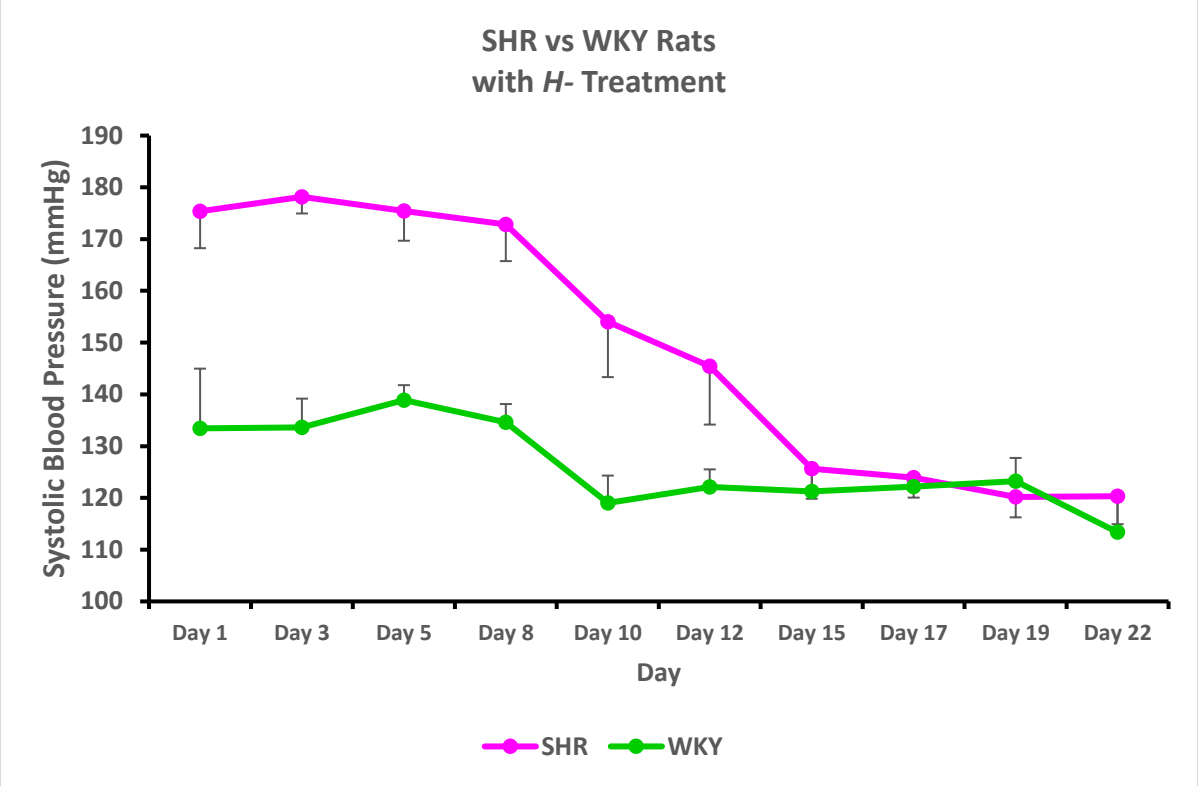


Difference p=	**0.0051**	**0.0050**	**0.0051**	**0.0051**	**0.0051**	**0.0051**	**0.0051**	**0.0051**	**0.0051**	**0.0051**

Figure 3.5: Differences between systolic blood pressures of SHR and WKY rats after treatment for *Helicobacter spp.* (*H+*).
Treatment with antibiotics was issued from day 2 to day 6 and systolic blood pressure in WKY rats is compared with that of SHRs over time. All data from each group shown as mean±std, with changes (p-values) indicated in the table below the figure.

In contrast, antibiotic treatment to eradicate *Helicobacter spp*. resulted in blood pressure of SHRs decreasing within 10 days to that recorded in the WKY rats ($p>0.05$; Figure 3.6). Indeed, median change in blood pressure (Figure 3.7) was -24.7mmHg [range -33.7 - -16.4 mmHg] in the WKY rats compared to -56.1mmHg [range -65.1 - -41.8mmHg] in SHRs (p=0,0051 vs WKY).

The change in blood pressure (Figure 3.7) between WKY control rats (median -1.8mm Hg [range -7.6 - +3.9mmHg]) was not different to the change recorded for the SHR controls (median -6.0mmHg [range -6.5 - -5.5mm Hg])



p=	0.0051	0.0050	0.0051	0.0050	0.0051	0.0051	0.17	0.63	0.30	0.0656

Figure 3.6: Differences between systolic blood pressures of WKY and SHR rats after treatment to eradicate *Helicobacter spp.* (*H*-).
Treatment with antibiotics was issued from day 2 to day 6 and systolic blood pressure in WKY rats is compared with that of SHRs over time. All data from each group shown as mean±std, with changes (p-values) indicated in the table below the figure.

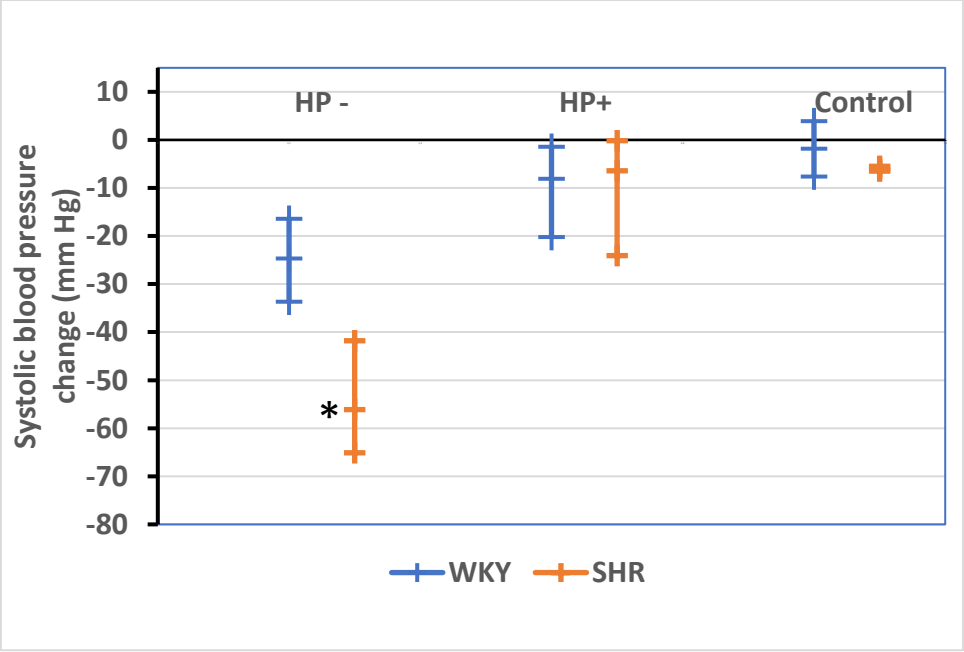


Figure 3.7: Median and range of systolic blood pressure changes recorded in WKY and SHR rats following antibiotic treatment to select for, and against *Helicobacter spp*. * p=0.0051.

3.2 Part 2: Effects of *H. pylori* on Hypertension

3.2.1 *H. pylori J99* culture

The clinical strain *H. pylori J99* was cultured from frozen stock and enumerated for feeding, as shown in Figure 3.8.

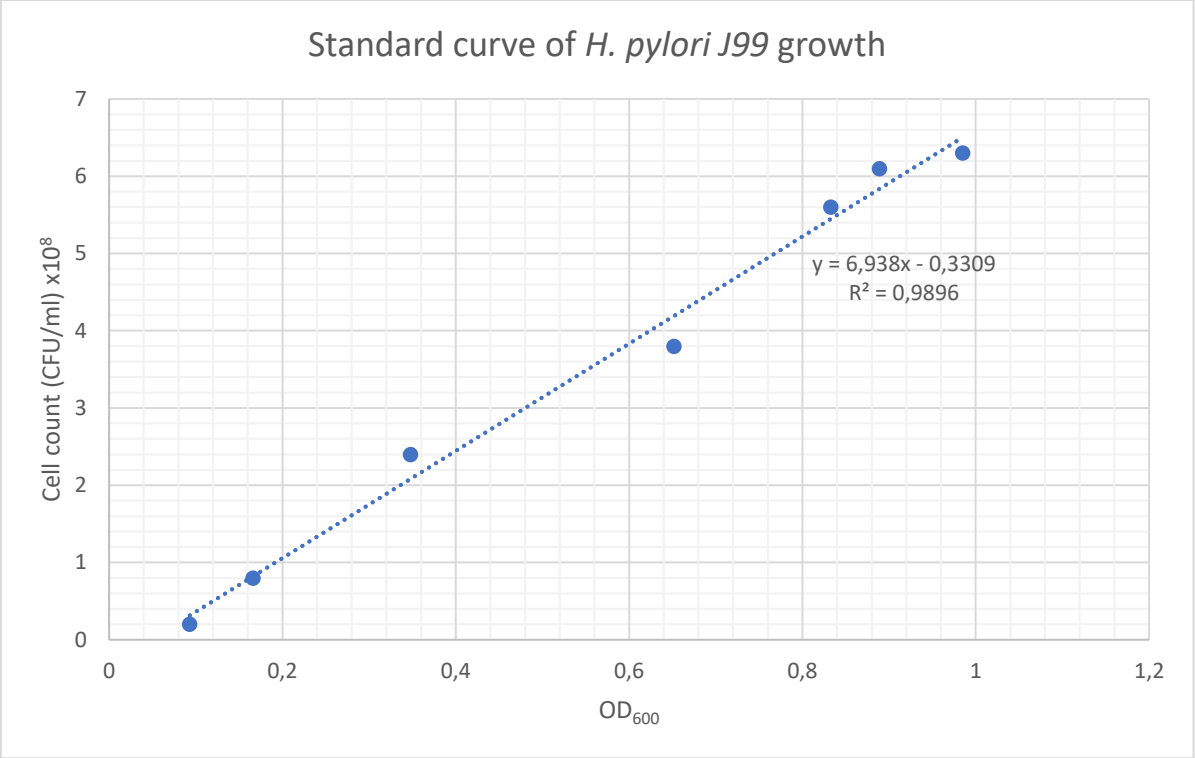


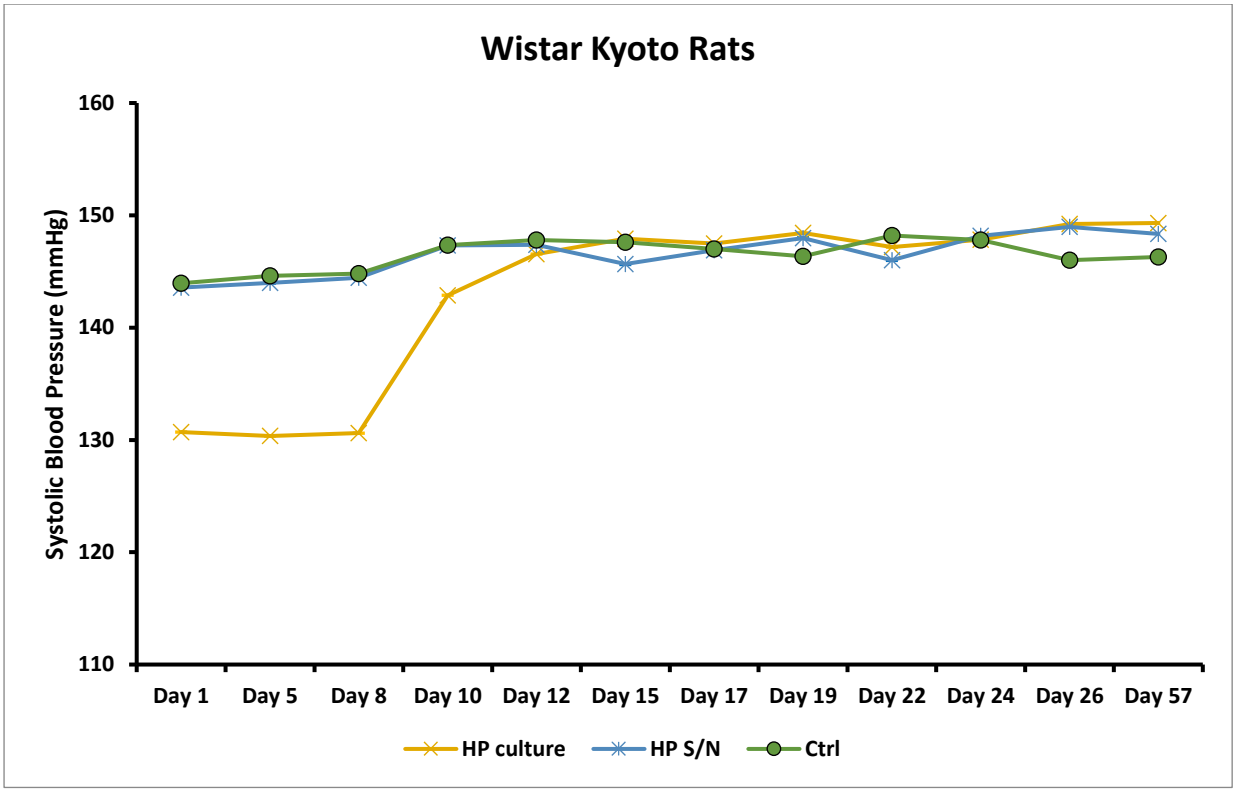
Figure 3.8: CFU calculated from serial dilution culture plates of *H. pylori J99*.
Serial dilutions were prepared for each corresponding OD reading taken at 600nm over a period of 24 hours. Cells were harvested once logarithmic growth had begun to plateau. Thus, cultures containing between $5.6x10^8$ and $6x10^8$ CFU/ml were administered to the rat models. From the same phase of growth, a portion of these cultures (50ml) were centrifuged to separate the supernatant, which was administered to a different cohort of rat models.

3.2.2 Effects of *H. pylori* re-infection and *H. pylori* supernatant on blood pressure

WKY and SHR rats (n=6 in each group) were given antibiotics to eradicate *Helicobacter spp.* as described in the methods section, and then re-infected with *H. pylori*, the species commonly infecting humans.

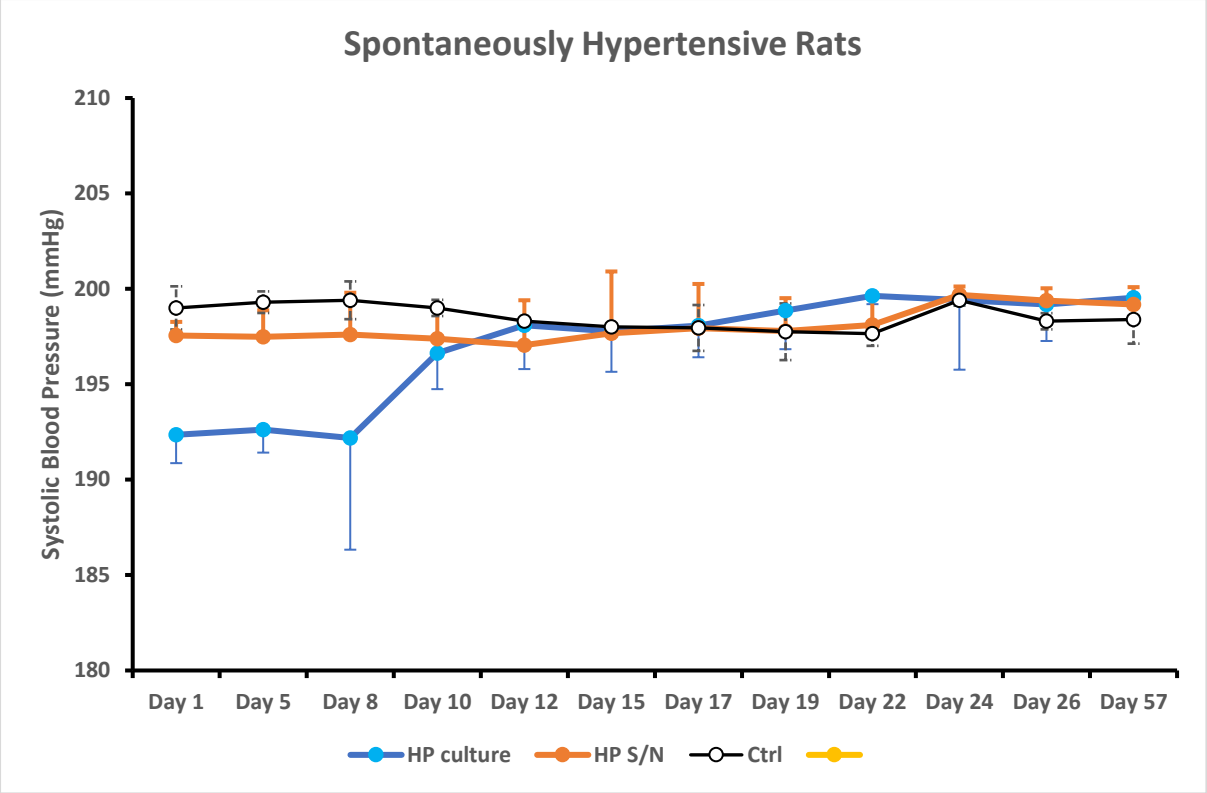
Systolic blood pressures were then recorded each second day prior to, during the administration of live *H. pylori* culture or the cell-free supernatants and for the following 2 weeks (total of 4 weeks) as well measured 6 weeks post treatment (Figure 3.9). Control animals of similar age were not treated.

Blood pressures were initially lower following eradication and increased in both WKY rats (Figure 3.9) and SHR (Figure 3.10) within two days after re-infection. Blood pressures increased from 131±6.1mmHg at day 8 to 149±0.4mmHg at day 26 ($p=0.0027$) in WKY rats and a non-significant increase from 192±6mmHg to 199±2mmHg at day 26 ($p=0.07$) in SHRs (200±1mmHg at day 57; $p=0.0277$). Blood pressure was unchanged in rats treated with *H. pylori* cell free supernatant and control animals (Figure 3.11). The change in blood pressure was significantly greater in WKY rats (median: +16.6mmHg and range: +10.2- +27.3 mmHg) treated with *H. pylori* than the SHR group (median +7.9 mmHg and range: -4.1 - +18.0mm Hg) (Figure 3.12).



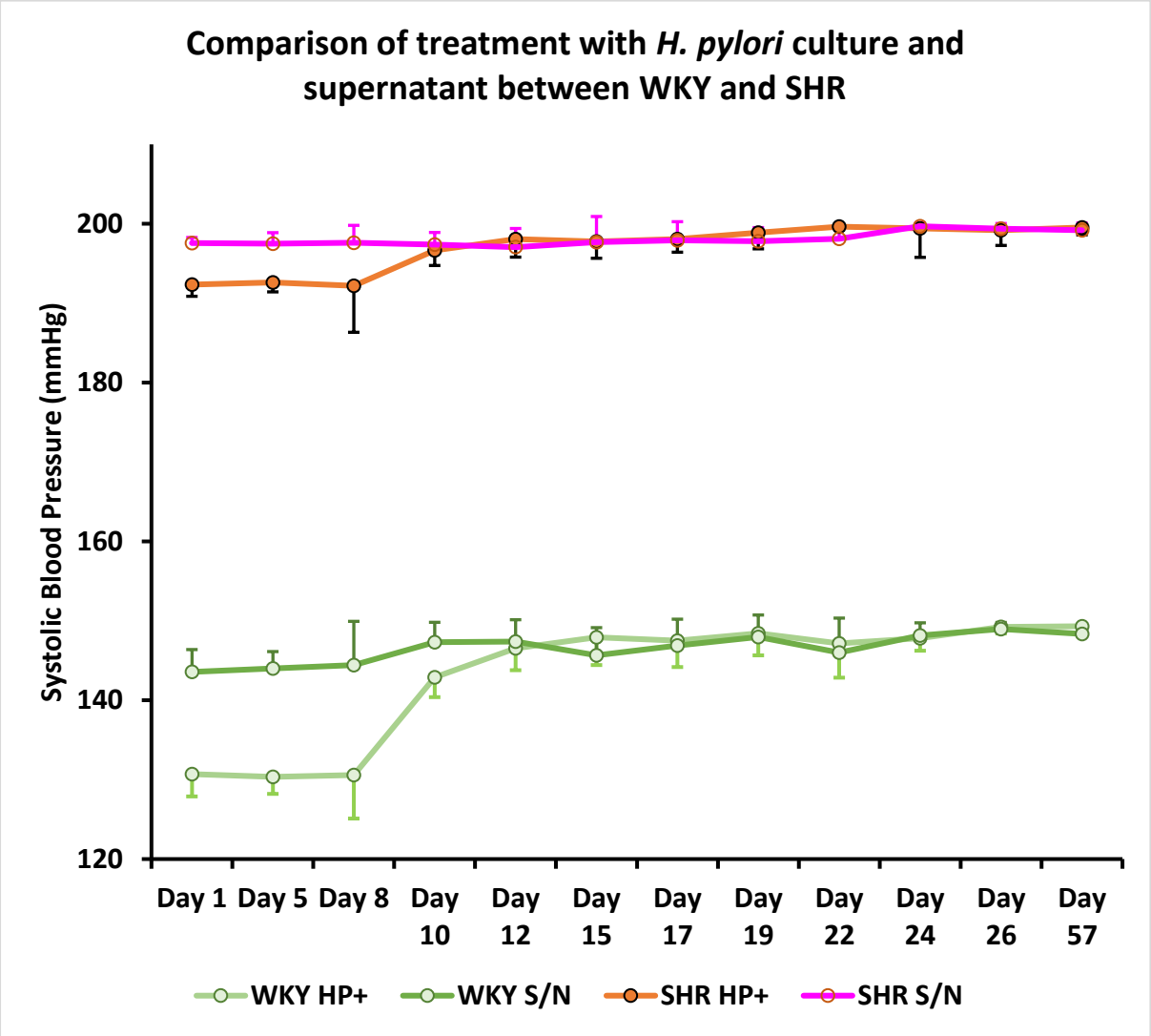
Difference between	Day 1	Day 5	Day 8	Day 10	Day 12	Day 15	Day 17	Day 19	Day 22	Day 26	Day 57
Group +HP Day 1 vs (p)	0.92	0.92	1	**0.0277**	**0.0277**	**0.0277**	**0.0277**	**0.0277**	**0.0277**	**0.0277**	**0.0277**
Group +SN Day 1 vs (p)	0.35	0.46	1	0.60	0.25	0.92	0.35	0.22	0.60	0.07	**0.0277**
Control	0.18	0.65	1	0.18	0.18	0.18	0.18	0.65	0.18	0.18	0.65
Difference Groups + HP vs S/N	**0.0039**	**0.0005**	**0.0209**	**0.0209**	0.08	0.56	1	0.25	1	1	0.25

Figure 3.9: Changes in mean systolic blood pressure following gavage with either live *H. pylori* (HP) or cell-free *H. pylori* culture supernatant (HP S/N) in WKY rats.
Treatment was issued from day 8 to day 12. All data from each group (n=6 and n=2 controls) is shown as mean±std, with changes (p-values) indicated in the table below the figure. Significant p-values are in bold.



Difference	Day 1	Day 5	Day 8	Day 10	Day 12	Day 15	Day 17	Day 19	Day 22	Day 24	Day 26	Day 57
+HP Day 8 vs (p)	0.75	0.75	1	0.12	**0.0464**	0.12	0.06	0.07	**0.0464**	0.07	0.07	**0.0277**
+SN Day 8 vs (p)	0.75	0.92	1	0.89	0.60	0.75	0.60	0.92	0.46	0.08	0.12	0.12
Control	0.65	0.65	1	0.65	0.18	0.18	0.65	0.18	0.18	1.00	0.18	0.65
Difference Groups HP- vs HP+	**0.0039**	**0.0039**	0.15	0.63	0.34	0.87	0.94	0.69	**0.0235**	0.81	0.94	0.52

Figure 3.10: Changes in mean systolic blood pressure following gavage with either live *H. pylori* (HP) or cell-free *H. pylori* culture supernatant (HP S/N) in SHRs.
Treatment was issued from day 8 to day 12. All data from each group (n=6 and n=2 controls) is shown as mean±std, with changes (p-values) indicated in the table below the figure. Significant p-values are in bold.



Difference between WKY vs SHR	**Day 1**	**Day 5**	**Day 8**	**Day 10**	**Day 12**	**Day 15**	**Day 17**	**Day 19**	**Day 22**	**Day 24**	**Day 26**	**Day 57**
HP culture gavage	**0.0051**	**0.0051**	**0.0051**	**0.0049**	**0.0049**	**0.0049**	**0.0047**	**0.0050**	**0.0049**	**0.0050**	**0.0051**	**0.0050**
S/N gavage	**0.0051**	**0.0051**	**0.0050**	**0.0051**	**0.0051**	**0.0050**	**0.0050**	**0.0049**	**0.0049**	**0.0048**	**0.0050**	**0.0051**

Figure 3.11: Changes in mean systolic blood pressure following gavage with *H. pylori* culture (HP+) and cell-free supernatant (S/N) in SHR and WKY rats.
Treatment was administered from day 8 to day 12. All data is shown as mean±std, with changes (p-values) indicated in the table below the figure.

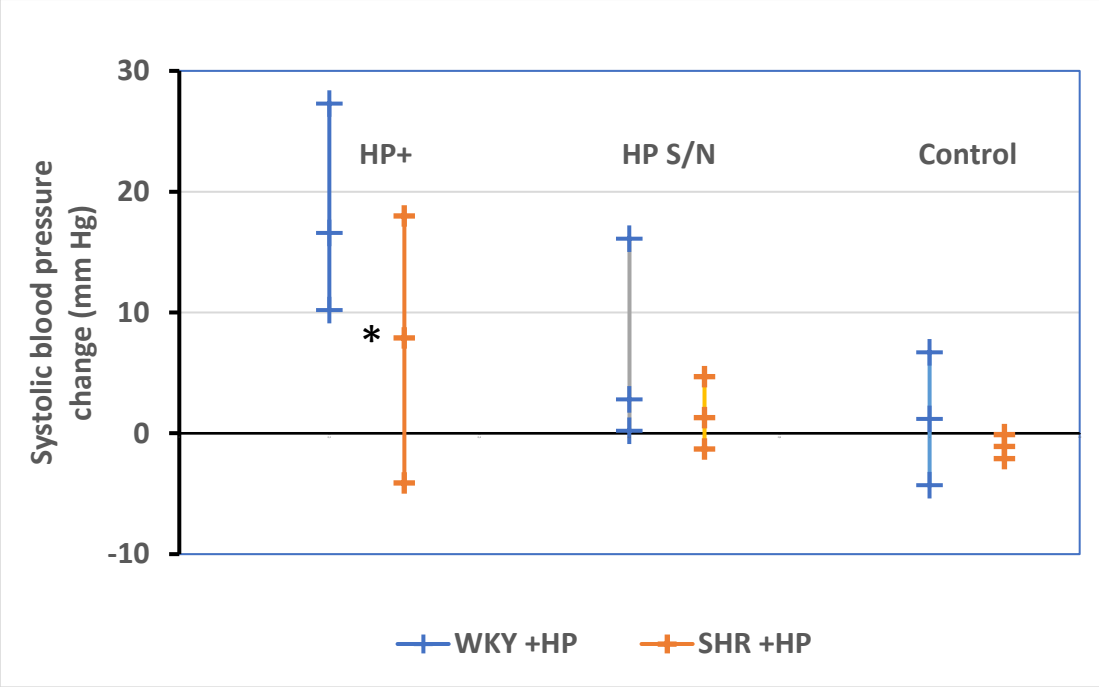


Figure 3.12. Median and range of systolic blood pressure in WKY rats compared to SHRs treated with *H. pylori* culture (HP+) or *H. pylori* cell free supernatant (HP S/N) and control rats.

HP+: p=0.0306 (*); HP S/N p=0.57; Control p=1.

3.2.3 Efficacy of treatment

The efficacy of treatment was assessed using a faecal antigen testing kit, an example of which is shown in Figure 3.13. The results of the antigen tests are summarised in Table 3.3. From the results, it can be concluded that both hypertensive and normotensive rat models of *H. pylori* infection were established.

Figure 3.13: Faecal antigen test strips used to detect *H. pylori* infection.
Top to bottom: The first strip (2) shows a positive result from the group of SHRs treated with *H. pylori* culture. The second strip (17) shows a negative result when the faeces of a WKY rat treated with cell-free culture supernatant was assessed. The third strip (20) shows the negative outcome of testing the faeces of a SHR control. The fourth strip is a positive control, as provided in the test kit.

Table 3.3: A summary of the faecal antigen test outcomes for each rat group.

Rat Group		**Test Outcome**
WKY	*Hp* culture	all positive
	Hp metabolites	all negative
	control	all negative
SHR	*Hp* culture	5/6 positive
	Hp metabolites	all negative
	control	all negative

Chapter 4: Discussion

Overview

There are a number of studies reporting the association of the presence of *H. pylori* and blood pressure as well as cardiovascular complications of elevated blood pressure (Table 1.2). The aim of this study was to determine whether *Helicobacter spp*. and in particular *H. pylori* infection could affect blood pressure. Rat models of hypertension are known to be infected with *Helicobacter spp*. and the microorganism needed to be eradicated prior to testing the effect of infection with the human pathogen *H pylori*.

The main findings of the study showed a significant decrease in systolic blood pressure following multiple antibiotic treatment for *Helicobacter spp*., which was sustained for more than two weeks after treatment. The greater decrease in systolic blood pressure in SHRs compared to the WKY rats resulted in the SHR systolic blood pressures being no different to that of the WKY rats. Re-infection of eradicated rats with *H. pylori* resulted in a systolic blood pressure increase less than the decrease measured following eradication. The increase in blood pressure was greater in the WKY rats, which had lower initial pressures than the SHRs. Taken together these data suggests *Helicobacter spp*. including *H. pylori* affect blood pressure, although these bacteria alone do not fully account for the observed changes. Multiple antibiotics would have eradicated not only *Helicobacter spp*. but also affected other microorganisms of the gut microbiome. The greater systolic blood pressure decrease following eradication and subsequent increase following re-infection respectively, and variation (Figure 3.7 and Figure 3.12) between animals of these responses, suggests other microorganisms also contribute to blood pressure and their contribution might be greater than that of *Helicobacter spp*., alone.

4.1: Part 1: Effects of Antibiotics Targeting *Helicobacter spp.* on Hypertension

This study tested changes in blood pressure following antibiotic treatment in SHR and WKY rats to select for, and against *Helicobacter spp*. The antibiotic treatment was based on the resistance patterns of *Helicobacter spp.* isolated from faeces of the rats. The isolated bacterial colonies were assessed in a series of biochemical tests which yielded results expected for *Helicobacter spp.*, thus identifying the presence of these bacteria.

The resistance to MIC antibiotic test strips for clarithromycin and tetracycline was high, yet not complete, and thus the former was used in the formulation fed to the rat models. This

resistance is in accordance with *Helicobacter spp.* resistance patterns, as observed in literature (Francesco *et al.,* 2010). The cultures displayed complete resistance to metronidazole. As a result, metronidazole as well as nitroimidazole (of which metronidazole is a component) was disregarded as possible treatment methods. This complete resistance is likely due to the rats being exposed to doses of metronidazole or nitroimidazole or both, as they are common drugs used to treat a range of ailments (Mabeku *et al.,* 2019). Interestingly, the *Helicobacter spp.* isolates were highly susceptible to amoxicillin. As such, amoxicillin was selected as part of the antibiotic formulation, along with bismuth. This selection was found to be most effective in eradicating *Helicobacter spp.* (Francesco *et al.,* 2010).

The prepared formulation was administered to the rat models, and blood pressure recorded during this time. The systolic blood pressure in both WKY and SHR rats treated to eradicate *Helicobacter spp.* showed a significant decrease from day 8, and continued to decrease for a week, before reaching a plateau (Figure 3.6). When treated to select for *Helicobacter spp.,* the systolic blood pressures of both the WKY and SHR groups showed no significant change (Figure 3.5). When comparing the changes in blood pressure of WKY with that of SHR, for both eradication and selection of *Helicobacter spp.,* there was no significant difference in the overall distribution. This means that each treatment had the same effect on both the rat strains.

As seen in Figure 3.6, when comparing the systolic blood pressures of SHR and WKY rats when treated with Antibiotic Mix 2 (amoxicillin, clarithromycin, omeprazole and bismuth), SHR systolic blood pressure decreased to the point of equalising with that of the WKY rats. This could be due to certain bacterial groups present in higher abundance in the hypertensive rats being decreased or eradicated, ultimately resulting in a gut microbiome resembling a normotensive rat. Similar findings were seen in a study by Adnan *et al.,* (2017); when the gut microbiome of SHRs were administered to WKY rats, the normotensive WKY rats experienced an increase in blood pressure. Increases in the ratio of Firmicutes to Bacteroidetes in the gut microbiomes of these rats were also observed (Adnan *et al.,* 2017).

The results of this study showed that administering an antibiotic cocktail of amoxicillin, clarithromycin, omeprazole and bismuth subcitrate decreased systolic blood pressure in both normotensive and hypertensive rats, with a greater decrease in hypertensive rats. As these antibiotics were formulated to act on the gut microbiome, it can be deduced that they reduced or eliminated a group or groups of bacteria, which play some role in the onset of hypertension, and are thus more prevalent in the gut microbiomes of SHRs. As this formulation was intended

to target *Helicobacter spp.*, it is likely that this group was eliminated or reduced. The possibility of other bacteria being targeted by these antibiotics cannot be ruled out.

Amoxicillin is a broad-spectrum antibiotic which has been shown to result in shifts in the microbiota; Bacteroides was found to decrease while Clostridia and Enterobacteria were shown to increase (Raymond *et al.,* 2015; MacPherson *et al.,* 2018). Clarithromycin was found to cause shifts in the normal intestinal microbiota, slight changes observed in *Bacillus* and *Candida*, as well as decreases in Bifidobacteria and Bacteroides (Edlund *et al.,* 2000; Elvers *et al.,* 2020). Omeprazole was noted to increase the amount of Bacteroides and Clostridia, as well as in the *Streptococcus*, *Holdemania* and *Blautia* genera (Freedberg *et al.,* 2015). As observed in several studies, when administering antibiotics to rat models, their gut microbiomes were altered, with one study noting changes in the Firmicutes and Bacteroides groups (Khan *et al.,* 2016; Galla *et al.,* 2018; Galla *et al.,* 2020). Thus, while this formulation was carefully designed to target *Helicobacter spp.,* the possibility of other bacterial groups being targeted cannot be overlooked. From the aforementioned studies, it can be noted that certain bacterial groups are affected by all components of this formulation, namely changes in Clostridia and Bacteroides. Studies are required to determine their role in the observed changes in systolic blood pressure.

4.2: Part 2: Effects of *H. pylori* on Hypertension

Re-infection with the human pathogen *H. pylori* resulted in significant systolic blood pressure increases in both the SHR and WKY rats. This increase was less than the decrease observed following *Helicobacter spp.* eradication. However, gavage with cell-free *H. pylori* supernatant including possible generated metabolites, had little effect on blood pressure. Results from the faecal antigen tests indicated that colonisation of *H. pylori* infection in both SHR and WKY, was successful (Table 3.3).

Following eradication of *Helicobacter spp*. in part 1 of this study, blood pressures of both strains were found to return to their respective normal pre-treatment values. These rats were then used in the second part of this study. SHR systolic blood pressure was found to increase steadily once the rats reach 12 weeks old, as was described in literature (Luo *et al.,* 2008). Hence, despite using the same rats for both part 1 and part 2 of this study, the baseline values for SHR for each part differ.

Interestingly, one group each of SHR and of WKY rats was found to have an average baseline systolic blood pressure lower than the rest of the rats in their strain (Figure 3.5 and 3.6). These groups (one SHR and one WKY) were treated to select against *Helicobacter spp.* in part 1 of this study, and then served as the groups reinfected with *H. pylori* cultures in part 2. As a result, these groups were found to have a slightly lower systolic blood pressure when compared to the rest of the SHR and WKY. Despite the systolic blood pressures reverting after receiving antibiotic treatment to eradicate *Helicobacter spp.*, these rats' systolic blood pressure was still found to be lower than the rest of the rats in their particular strain.

Several studies have shown positive correlation between *H. pylori* and hypertension, as mentioned previously (Table 1.2). These studies demonstrated this correlation by detecting the presence of *H. pylori* using serological tests or ^{13}C-urea breath tests. Eradication studies used antibiotics, most commonly clarithromycin and amoxicillin, and found a significant decrease in blood pressure, as corroborated in this study. Thus, it can be concluded that *H. pylori* plays a role in the aetiology of hypertension, but that eradication using antibiotics targeting *Helicobacter spp.* is simultaneously eliciting an effect on other groups of bacteria which might also be involved in blood pressure. It is likely that the other bacterial groups in the gut microbiome affected are Clostridia and Bacteroides, as these are the most common groups affected by every antibiotic used to eradicate *Helicobacter spp.* in this study, as previously mentioned. Furthermore, this study showed re-infection with *H. pylori* resulted in an increase in blood pressure. Since the increase in systolic blood pressure recorded upon re-infection with *H. pylori* was less than the decrease noted in response to eradication with antibiotics, it is likely that *H. pylori* does not influence blood pressure alone. *H. pylori* or *Helicobacter spp.* might create an environment for other microorganisms causal in hypertension to establish themselves and create a dysbiosis of the gut microbiome which exists in hypertensive individuals (Qi *et al.,* 2017).

4.3 Limitations and Future Prospects

Selected antibiotics were employed in two specific formulations and further studies could assess the effects these antibiotics have on the microbiome using techniques such as 16S rRNA gene sequencing to identify the bacteria present before and after treatment. Administering different antibiotics, in different combinations as well as individually, should also provide further insight into the mechanisms behind the observations on blood pressure. Furthermore, longer term blood pressures needed to be measured after antibiotic treatment to determine the durability of these effects. The increasing blood pressures with age in the SHRs was a confounding factor. This was taken into account by taking several measurements prior to treatments. The blood pressure measurement taken at the last intervention was then used as the baseline pressure measurement.

4.4 Conclusion

This study described the antibiotic resistance patterns of *Helicobacter spp.* naturally colonizing SHR and WKY rat models and successfully established both hypertensive and normotensive rat models of *H. pylori* infection. It showed that the use of antibiotics successfully lowered systolic blood pressure in hypertensive models, allowing these models to maintain a systolic blood pressure characteristic of a normotensive rat. Re-infection with *H. pylori* increased systolic blood pressure in both hypertensive and normotensive rats, albeit a small increase. Thus, this study suggests that hypertension is not influenced by *H. pylori* alone, but it is rather the result of interplay between other bacterial groups also impacted by the antibiotics used. These findings will contribute to identifying the mechanism of hypertension and contribute to finding new ways to eradicate hypertension. Furthermore, this study provides support for the significant role of the microbiome in blood pressure regulation. These results have implications for studies using animal models of hypertension where the use of antibiotics employed prophylactically for interventional studies may affect results. Additionally, the results might have implications for the treatment of *H. pylori* in the event of treatment failure which might select for this bacterium.

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Chapter 6: Appendices

Appendix A: List of Materials

Table A1: Microbiological culture and inoculation supplies.

Product	Manufacturer
Brain Heart Infusion	Oxoid™
Columbia Blood Agar	Oxoid™
Foetal Bovine Serum	Thermo Scientific™
Campylobacter Selective Supplement Skirrows	Oxoid™
MicroAero gas pack	Thermo Scientific™
Disposable inoculating loops	Thermo Scientific™
Petri dishes	Thermo Scientific™
Anaerobic jars	Oxoid™
Falcon tubes	Thermo Scientific™

Table A2: Biochemical tests supplies.

Product	Manufacturer
Gram stain kit	Sigma-Aldrich
Kovac's Oxidase Reagent	Sigma-Aldrich
Yeast extract	Oxoid™
Potassium phosphate monobasic	Merck
Urea	Merck
phenol red	Sigma-Aldrich
Hydrogen peroxide	Merck

Table A3: Antibiotic resistance tests supplies.

Product	Manufacturer
Amoxicillin MIC Test Strips	Liofilchem®
Clarithromycin MIC Test Strips	Liofilchem®
Metronidazole MIC Test Strips	Liofilchem®
Tetracycline MIC Test Strips	Liofilchem®

Appendix B: Ethical Clearance Certificate

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2019/04/31/C

APPLICANT: Ms S Dinat
School: School of Clinical Medicine; Department: Surgery;
Location: CAS

PROJECT TITLE: The effects of Helicobacter spp. on blood pressures in rat models of hepertension

Category: C; Species and Numbers Involved: *54X +/- 200g male WKY and SHR Rats*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 30 Apr 2019. This approval remains valid until 30 Jan 2021.

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ Date: 03/02/2020
(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

Signed: _____ Date: 03/02/2020
(Registered Veterinarian)

CC: Student supervisor: Prof G Candy
Director Central Animals Service: Dr Kim Jardine

CERTEST *H. pylori*

One Step test to detect Helicobacter pylori in card format/
Prueba de un solo paso para detección de Helicobacter pylori en formato cassette

SPECIMEN COLLECTION AND PREPARATION

Stool samples should be collected in clean containers. The samples can be stored in the refrigerator (2-8ºC) for 1-2 days prior to testing. For longer storage, maximum 1 year, the specimen must be kept frozen at -20ºC. In this case, the sample will be totally thawed and brought to room temperature before testing. Homogenise stool samples as thoroughly as possible prior to preparation.

Specimen preparation (see illustration):

1. Take out the cap of the collection tube (1) and use the stick to pick up sufficient sample quantity. Then, introduce the stick once into 4 different parts of the stool sample (2) making sure that, at each insertion, only the stick's screw is covered with the sample to take the appropriate amount of faecal sample (approx. 50mg) and add it to the collection tube. Any additional sample that exceeds the stick's screw could cause wrong results. For liquid samples, add approx. 125µL in the collection tube using a micropipette.
2. Close the tube with the diluent and stool sample. Shake the tube in order to assure good sample dispersion (3).

MATERIALS

MATERIALS PROVIDED

- CerTest *H. pylori* card tests
- Instructions for use
- Stool collection tubes with diluent

MATERIALS REQUIRED BUT NO PROVIDED

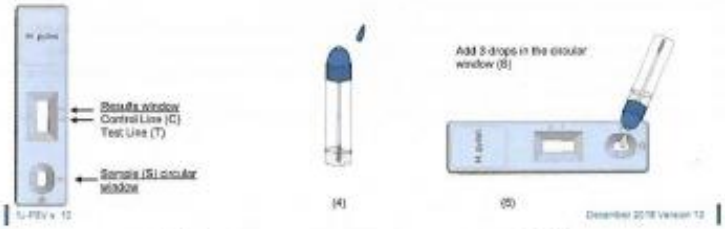
- Specimen collection container
- Disposable gloves
- Timer

TEST PROCEDURE

Allow tests, stool samples and controls to reach room temperature (15-30ºC) prior to testing. Do not open pouches until the performance of the assay.

1. Proceed to shake the stool collection tube in order to assure good sample dispersion.
2. Remove the CerTest *H. pylori* card test from its sealed bag just before using it.
3. Take the stool collection tube, cut the end of the cap (4) and dispense 3 drops in the circular window marked with the letter S (5). Avoid adding solid particles with the liquid.
4. **Read the results at 10 minutes**. Do not read the test result later than 10 minutes.

If the test does not run due to solid particles, stir the sample added in the sample window (S) with the stick. If it doesn't work, dispense a drop of diluent until seeing the liquid running through the reaction zone.

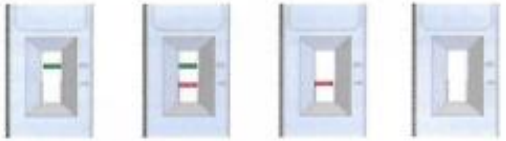


CerTest BIOTEC S.L. Pol. Industrial Río Gállego II, Calle J, Nº 1, 50840, San Mateo de Gállego, Zaragoza (SPAIN) www.certest.es

CERTEST *H. pylori*

One Step test to detect Helicobacter pylori in card format/
Prueba de un solo paso para detección de Helicobacter pylori en formato cassette

INTERPRETATION OF RESULTS (please refer to the illustration below)



	H. pylori	Interpretation of the results
1.	- GREEN	There is no *Helicobacter pylori* presence. No infection caused by *Helicobacter pylori*.
2.	+ GREEN-RED	There is *Helicobacter pylori* presence. *Helicobacter pylori* infection, which might mean gastrointestinal diseases (stomach and duodenum) like gastritis, peptic ulcer disease or gastric carcinoma.
3.	Any other result	Invalid result, we recommend repeating the assay using the same sample with another test.

NEGATIVE: Only the GREEN line appears across the results window in the site marked with the letter C (control line).

POSITIVE: In addition to the GREEN control line, a RED line also appears in the site marked with the letter T (test line) in the results window.

INVALID: Total absence of the control coloured line (GREEN) regardless the appearance or not of the test line (RED). Insufficient specimen volume, incorrect procedural techniques or deterioration of the reagents are mostly the main reasons for control line failure. Review the procedure and repeat the assay with a new test. If the symptoms or situation still persist, discontinue using the test kit and contact your local distributor.

NOTES ON THE INTERPRETATION OF RESULTS

The intensity of the red coloured band in the test line (T) in the results window will vary depending on the concentration of antigens present in the specimen. However, neither the quantitative value nor the rate of increase in antigens can be determined by this qualitative test.

QUALITY CONTROL

Internal procedural control is included in the test. A green line appearing in the control line (C) in the results window is an internal control, which confirms sufficient specimen volume and correct procedural technique.

LIMITATIONS

1. The test must be carried out within 2 hours after opening the sealed bag.
2. An excess of sample could cause wrong results (brown bands appear). Dilute the sample with the diluent and repeat the test.
3. The intensity of test line may vary from very strong at high antigens concentration to faint when the antigens concentration is close to the detection limit value of the test.
4. CerTest *H. pylori* should be used only with samples from human faeces. The use of other samples has not been established. The quality of the test depends on the quality of the sample; proper faecal specimens must be obtained.
5. Positive results determine the presence of *Helicobacter pylori* in faecal samples; nevertheless, a positive result should be followed up with additional invasive techniques (endoscopy) to confirm the results. A confirmed infection should only be made by a physician after all clinical and laboratory findings have been evaluated and must be based in the correlation of the results with further clinical observations.
6. A negative result is not meaningful because of it is possible the antigens concentration in the stool sample is lower than the detection limit value. If the symptoms or situation still persist, a *Helicobacter pylori* determination should be carried out, on a sample from an enrichment culture or using an invasive technique.
7. Bloody stool samples can contain components that may cause non-specific reactions in the test. Every bloody stool sample whose result is positive should be followed up with other techniques of diagnosis to confirm the result.

CerTest BIOTEC S.L. Pol. Industrial Río Gállego II, Calle J, Nº 1, 50840, San Mateo de Gállego, Zaragoza (SPAIN) www.certest.es

Appendix D: Plagiarism Report

Gmail — Sarhana Dinat <sarhana.dinat@gmail.com>

Plagiarism Report

Ekene Nweke <Ekene.Nweke@wits.ac.za> Fri, Feb 12, 2021 at 10:37 AM
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