THE ROLE OF *LISTERIA MONOCYTOGENES* AND OTHER BACTERIA IN MENINGITIS AND SPONTANEOUS ABORTION IN SOME TOWNS IN NORTHERN NIGERIA

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DECLARATION

I hereby declare that this work is the product of my own research efforts; undertaken under the supervision of Professor C. I. C. Ogbonna and has not been presented else where for the award of a degree or certificate. All sources have been duly distinguished and appropriately acknowledged.

………………………… ………………

Signature Date

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DEDICATION

To my beloved wife, Celestina and our children, Pirfa, Dinci, Nanchang and Nanbyen.

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ABSTRACT

The involvement of *Listeria monocytogenes* in bacterial meningitis and spontaneous abortion in some towns in northern Nigeria was investigated. Three hundred cerebrospinalfluid (CSF) samples, 300 blood and 300 placenta swab samples were collected from meningitis and spontaneous abortion patients in the study area of Bauchi, Jos and Kano. Three hundred nono (a fermented milk product) samples were also collected from milk hawkers at various locations in the study areas. The samples were inoculated first into *Listeria* enrichment broth. Incubation was at 37° C, followed by cold enrichment at O^O C for samples that did not yield any growth at 37^0 C. Further sub- inoculations were made on *Listeria* selective agar. Parallel inoculation of the samples was also made on blood, chocolate and McConkey agar media using standard bacteriological methods. Listeria-like organisms were subjected to biochemical and serological investigations using type 1a, 4b and polyvalent sera supplied by Difco laboratories (Detroit, Michigan). The bacterial isolates from the CSF samples included *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*. They had the following percentage frequencies of occurrence: 48.8, 16.56, 11.04, 7.36 and 5.58 %, respectively. Furthermore, *Enterococcus faecalis*, *Proteus mirabilis*, *E. coli*, *and L. monocytogenes* were some of the bacteria isolated from the placenta swabs and blood samples. They had the following percentage frequencies of occurrence: 30.43, 15.65, 13.9 and 12.20% respectively. *L. monocytogenes* was not isolated from nono samples. However, a simple *Listeria* selective agar medium, which contained a combination of colistin (1 mg/100 ml) and cefuroxime (2 mg/100ml), enhanced the growth of *L. monocytogenes* and inhibited other gram positive

and gram negative bacteria. *L. monocytogenes* contributed to the burden of bacterial meningitis and spontaneous abortion in the study towns.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY.

Meningitis and spontaneous abortion have been responsible for the death of so many people worldwide. In the United States, 5,755 cases of meningitis were reported in 1995, with 499 deaths (Schuchat et al., 1997). In Africa, 20,000 deaths were reported between 1996 and 1997. Similarly, 3,121 cases of meningitis were reported in Nigeria in 1992 with spontaneous 393 deaths (Greenwood, 1999; Anonymous 1992).

The story is not different for abortion. In Nigeria it was reported that there were 1,000 maternal deaths per 100,000 live births annually (WHO, 1988; Stanley et al., 1998). These reports were not specific for any bacterium. Many cases of death caused by these diseases in Nigeria were not reported. Patients may have died before they could obtain medical help, because of the poor state of some health facilities, and inaccessibility of most of the rural areas (Greenwood, 1999).

The epidemiological data of meningitis in Africa are based on meningitis caused by *N. meningitidis*, otherwise referred to as meningococcal meningitis (Lapeyssonnei, 1963). But it is not the only bacterium reported to cause meningitis. Other bacteria, such as *L. monocytogenes*, *S. pneumoniae*, *S. aureus*, and *Haemophilus influenzae*, have been isolated from CSF of meningitis patients (Hanssler et al*.,* 1990; Zaki et al., 1990). This indicates that focus should also be on these and other associated bacteria, rather than on *N. meningitidis*, as in northern Nigeria (Ejembi et al., 1998).

Immunization recommended by the World Health Organization to be administered before the onset of the meningococcal season failed to stop the transmission of the disease. Cases of meningitis were still reported. Vaccine failure and waning immunity were some reasons given by Blakebrough, Greenwood, Whittle, Bradley, and Gilles (1983) to explain the cases seen. But there are vaccines against *H. influenzae*, and *S. pneumoniae* that are unadministered. It is possible that the cases of meningitis reported after immunization exercise against meningococcal meningitis is caused by the later bacterial agents.

L. monocytogenes is reported to be the principal organism accounting for most cases of spontaneous abortion (Ancona et al., 1980; Lindemann, 1990). Other organisms reported by Khong, Frappel, Steel, Stewart, and Burke (1986) include *Chlamydia, Streptococcus pyogenes*, and *Streptococcus agalactiae.* Reports associating *L. monocytogenes* with cases of spontaneous abortion in northern Nigeria are not documented (Onyemelukwe et al., 1983).

1.2 HYPOTHESIS**.**

Since cases of meningitis due to vaccine failure have been reported in northern Nigeria (Blakebrough et al., 1983), it is possible that other bacteria, such as *L. monocytogenes*, and *S. pyogenes* do not contribute to the burden of meningitis. In addition, there may be no relationship between the incidences of meningitis caused by *L. monocytogenes* with the seasonal epidemics of meningococcal meningitis.

1.3 RESEARCH PROBLEM.

L. monocytogenes is ubiquitous (Cowan & Steel, 1992). Consequently the consumption of unpasteurized cow milk products including nono predisposes the

population to infection by *L. monocytogenes*. Thus, its role in other infections, including spontaneous abortion, cannot be ruled out, given the high maternal mortality rate in Nigeria. Furthermore, the

level of awareness of *L. monocytogenes* amongst health professionals in the study area is low. This could lead to wrong diagnosis of listeriosis.

1.4 JUSTIFICATION FOR THE RESEARCH.

The study areas of Jos, Bauchi, and Kano, lie within the meningitis belt (Lapeyssonnei, 1963; Greenwood, 1999). These are part of the four health zones defined by the Nigerian health authorities (Ejembi et al., 1998; Stanley et al., 1998). Kano was chosen for the purpose of this study to represent the North West; Jos, north central; Bauchi, north east. The population of these zones is largely rural and impoverished. Consequently, some cases of meningitis and spontaneous abortion may not be reported, as patients may have died before reaching any medical help (Stanley et al., 1998; Greenwood, 1999).

Furthermore, the immunizing effects of the conjugate vaccines against meningococcal meningitis administered are not long lasting. This could lead to relapse, and thus an increase in fresh cases of meningitis. However, other cases of meningitis, which may occur in the wake of recent immunization exercises, could be attributed to *L. monocytogenes* and other associated bacteria, since the immunity offered by the vaccines is specific for *N. meningitidis* only. This could add to the burden of mortality and morbidity as well as overstretching the available health infrastructures (Esumeh & Odugbemi, 1992; Birmingham et al., 2003). *L. monocytogenes* is reported to be disseminated through contaminated food, especially unpasteurised milk, vegetable, meat and some other foods (Gray & Killinger, 1966; Harvey & Gilmour, 1993; Jacquet et al., 1993).

Such commodities are produced in the study areas. The consumption of unpasteurised milk as nono infested with *L. monocytogenes* could lead to epidemic or sporadic out-break of infection (Anonymous, 2000). Report by Akpavie and Ikheloa (1992) on the outbreak of listeriosis in a herd of cattle in Nigeria, point to such possibility.

While it seems that climatic conditions in northern Nigeria favour the spread of cerebrospinal meningitis, the general feeding habit of the people may predispose them to infection by *L. monocytogenes*, moreso as the organism is food-borne. Raw milk, meat, water and vegetables have been incriminated as sources of infection by *L. monocytogenes* (Blenden et al., 1987; Harvey & Gilmour, 1993). Food-borne diseases have become major problems worldwide. Such problems are even assuming wider dimensions in countries with good health records (Jose et al., 2001). However, in Nigeria records of food-borne disease outbreaks are rare. Studies on foods consumed in Nigeria have shown that the potential for food-borne disease outbreaks, such as gastroenteritis caused by *E. coli* 0157 and listeriosis exists (Uhiara, 1993). *L. monocytogenes* infections are not common. When patients present cases of meningitis or spontaneous abortion, medical doctors do not directly request for diagnosis of *L. monocytogenes.* Since *L. monocytogenes* may require more than 24 hours growing, laboratory scientists may report negative results, especially in cases of spontaneous abortion (Campbell, 1990; Hof, 1990).

The cost of procurement of selective laboratory media for the isolation of *L. monocytogenes* is high (Curtis & Lee 1995). These media are imported and may not be readily available to most health facilities in the study areas. This calls for the need to formulate own simple medium that can support the growth of *L.*

monocytogenes. It would also help to increase awareness on listeriosis in the study areas. Furthermore, it would aid in the formulation of appropriate response, classify the burden of disease and evaluate the impact of immunization services (Blakebrough et al., 1983).

1.5 AIMS / OBJECTIVES OF THE RESEARCH.

The present work was designed with the following aims and objectives in mind:

a. To ascertain the relationship between *L. monocytogenes*, other bacteria and cases of meningitis in the study areas.

b. To evaluate the relationship between *L. monocytogenes* and cases of spontaneous abortion in the study areas.

c. To investigate the relationship between nono and listeriosis epidemics caused by *L. monocytogenes*.

d. To categorize the various strains of *L. monocytogenes* present in the study areas.

e. To examine the existing epidemic records of listeriosis.

f To ascertain the level of awareness of the existence of *L. monocytogenes* infections amongst medical doctors and laboratory scientists in the study areas.

g. To develop an appropriate medium for the isolation of *L.monocytogenes.*

CHAPTER TWO

LITERATURE REVIEW

2.1 TAXONOMY.

Listeria monocytogenes is an intracellular Gram-positive non-sporing, non-acid fast anaerobic bacillus measuring 1- 4 µm x 0.5 µm. Though a psychrophile, it grows at 37°C, with tumbling end-to-end motility at 18-22°C using its peritrichous flagellation in nutrient broth (Cossart & Mengaud, 1989; Cowan & Steel, 1992).

It belongs to the genus *Listeria* placed within the *Clostridium* sub-branch (Cowan & Steel, 1992). *L. monocytogenes* remains the only recognied species in this genus. However, six other species, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. innocua* and *L. murrayi* were identified. Opinions differ as to whether they are true *Listeriae*, for they may also represent different but closely related species. These opinions were based on the fact that they lacked haemolytic and virulence properties (Seeliger & Hohne, 1979; Dabiri et al., 1990). Only *L. monocytogenes* is an opportunistic pathogen in humans and various animals (Gerhard et al., 1993).

The natural habitat of Listeriae is decomposing plant matter in which they live as saprophytes (Berche et al., 1987).

2.2 *L. MONOCYTOGENES* IN MENINGITIS AND ABORTION.

2.2.1 Meningitis. Cheesbrough (1984) described meningitis as an inflammation of the meninges of the brain because of infection by microorganisms or trauma. This condition could lead to the presence of microorganisms in the cerebrospinal fluid (CSF) – a normally sterile fluid. Thus, any organism isolated could be responsible for the meningitis (Prats, 1992), as the normal function of the central nervous system would be affected. If not treated, the condition could lead to life

long debility, such as deafness, mental retardation, partial paralysis, or seizure disorders (Macleod, 1980; Jose et al., 2001).

Macleod (1980) explained that the bacteria could gain access to the meninges through the nasal passage or ear. These organisms could be normal flora from these areas, or the result of infection by an "invading" microorganism.

The mortality and morbidity of meningitis and the causative agents have been documented. Works carried out by many investigators (Hanssler et al., 1990; Emele & Anyiwo, 1994; Campagne et al., 1999; Kyaw et al., 2002) showed variations in the organisms from location to location or from one country to another. The major causative bacterium responsible for most cases of meningitis was *Neisseria meningitidis*. Other bacteria were *Streptococcus pneumoniae, Haemophilus influenzae*, *Streptococcus agalactiae*, and *L. monocytogenes* (Moore et al., 1992)

Children, especially neonates or infants, were worse affected than adults (Harvey, 1989; Hanssler et al., 1990). Neonatal meningitis appeared to be more prevalent. Hanssler et al. (1990) reported on cases of late onset of septicaemia in newborn infants in Germany. *L. monocytogenes* was implicated. Zaki et al. (1990) working on bacterial meningitis in the new born in Kuwait, isolated *S. agalactiae* and *E. coli* as well as *L. monocytogenes* that had the highest incidence.

In a report on 280 cases of neonatal meningitis in the Netherlands, *E. coli* topped the list. *S. agalactiae* closely followed it, with *L. monocytogenes* being the third most occurring bacterium (Mulder & Zanen, 1984). Furthermore Bell et al. (1986), in a 14-year period review of meningitis in the newborn in Belfast, did not incriminate *L. monocytogenes*, rather, *E. coli* had the highest frequency of

occurrence, followed by *Staphylococcus aureus*, *Streptococcus viridans*, *Streptococcus pyogenes* and *Candida albicans.* Harvey (1989) reported on a sudden emergence of *L. monocytogenes* in Britain in 1988 in his study of neonatal meningitis.

These reports revealed that children are the most affected group (Christophe et al., 1999). However, in the case of non-pregnant adults, though rare, the disease was associated with epidemics or other underlying diseases as reported by Hernandez et al. (1990), Campbell (1990) and Elcuaz et al. (1996). In addition, the reports revealed that there is a wide variation in the organisms responsible for the meningitis disease, and this suggests the need for each country to mount meningitis surveillance studies. This is because African records on meningitis caused by *L. monocytogenes* are rare (Anonymous, 2000). Heikki (2001) reported that *Streptococcus pneumoniae* is the leading cause of non epidemic meningitis in Africa followed by *Haemophilus influenzae.*

The first reported outbreak of meningitis in Africa was made in 1905 in northern Nigeria (Greenwood, 1999). Most outbreaks were caused by *N. meningitidis*, with few cases involving *Haemophilus influenzae* and *Streptococcus pneumoniae* bacteria.

Emele and Anyiwo (1994) carried out a bacteriological analysis of three successive epidemics of cerebrospinal meningitis infection in Sokoto, a town in northern Nigeria. They isolated *N. meningitidis* as the bacterium responsible for the epidemic. In the study carried out by Ako-Nai et al. (1995) in southern Nigeria, *L. monocytogenes* was not isolated. Unlike northern Nigeria, epidemic meningitis is rare in southern part of Nigeria (Onile et al., 1982).

Seasonal meningitis epidemics in northern Nigeria.

The commonly reported cases of meningitis are those caused by *N. meningitidis* (Lapeyssonnei, 1963; Greenwood, 1999). Person-to-person transmission of this organism is through droplets after coughing or sneezing (Emele & Anyiwo, 1994).The organisms, which are commonly harbored as harmless commensal parasites in the nasopharynx, find their way through cracked skin of the nose caused by dry weather. It is then carried by blood from where the meninges of the brain is infected, resulting in meningitis. The condition that enhances this process is readily available in northern Nigeria (Emele & Anyiwo 1994). Thus, the seasonal epidemic of meningitis occurs as an airborne disease.

But *L. monocytogenes* infection or disease is basically food-borne and zoonotic (Jensen et al., 1996; Schwarzkopf, 1996). Although it exists in diverse environmental sources including the soil, reports of person-to-person transmission through air-borne route is rare, though such a possibility cannot be ruled out (Blenden et al., 1987; Jose et al., 2001). The carriage of *L. monocytogenes* in faeces of healthy carriers as well as in animal droppings is documented (Berg, 1995; Lake, 2002).

In countries where animals are fed with silage (Scott, 1993) there are reports of sporadic and epidemic outbreaks of listeriosis in the population. Siegman-Igra et al. (2002) reported that listerial meningitis occurred throughout the year, with peaks in summer and fall with 70% of cases occurring from May to October in Israel. MacGowan et al. (1994) found that *Listeria* spp were commonest in faeces and soil in July to September. Similar reports for Nigeria are however not available. Most reports were on meningococcal meningitis caused by *N. meningitidis*. Lapeyssonnei (1963) reported of a meningococcal

meningitis belt which stretches across Africa on latitudes 8-16⁰ north of the equator. Nigeria lies within this belt. This zone has a dry season that lasts from October to April, with high temperatures of 30 to 40⁰C and low humidity, thus favouring the spread of meningococcal meningitis. *L. monocytogenes* can survive these conditions as well and cause disease in infected persons.

The peculiarity of northern Nigeria, a cattle-rearing zone, as an area where the epidemic is rife, suggests that more work should be carried out to ascertain the real incidence of *L. monocytogenes* in cases of meningitis (Onyemelukwe et al., 1983) and spontaneous abortion.

2.2.2 **Spontaneous Abortion.**

Spontaneous abortion and miscarriage are terms that are synonymous. Shiers (2001) defined spontaneous abortion as "the involuntary loss of the products of conception prior to 24 weeks gestation." Bacterial infections, especially with *L. monocytogenes* and *Chlamydia*, were reported as major causes (Khong et al., 1986; Lwin et al., 1991; Shiers, 2001). But Mirdamadi (2005) isolated *S. aureus*, *Streptococcus agalactiae*, *E. coli*, Bacillus spp and *L. monocytogenes* from aborted samples in Iran. Spontaneous abortion was responsible for high mortality and morbidity in both neonates and mothers, as observed by Lindemann (1990). Lwin et al., (1991) suggested that the foetus can be infected through the gastro-intestinal tract during systemic illness to the foetal sac.

Furthermore, person-to-person transmission of *L. monocytogenes* is possible through venereal contact (Onyemelukwe & Lawande 1982; Anonymous, 2000). This was also corroborated by Toaff et al. (1962), in which the bacterium was isolated from human semen. Thus establishing the possibility of a pregnant mother being infected with *L. monocytogenes* through sexual intercourse.

Onyemelukwe et al. (1983) also reported cases of *L. monocytogenes* in males who patronize prostitutes. The data on spontaneous abortion in Africa caused by *L. monocytogenes* are however extremely rare. This may be because little in the way of food-borne surveillance is carried out in Africa, as most infections are food-borne (Anonymous, 2000).

Chiwuzie et al. (1995), working on causes of maternal mortality in a semi-urban Nigerian setting, observed that, globally, 500,000 women die at pregnancy and childbirth yearly, most of whom are from developing countries. Bacterial infection was one of the major causes. They further reported that maternal mortality rate in Nigeria was among the highest in the world, ranging from 800 - 1,500 per 100,000 births. Thus, knowledge of the involvement of other bacteria may be useful in the correlation of instances involving *L. monocytogenes,* in order to obtain an overall picture of the role of this organism in spontaneous abortion, and in nono as vehicle of transmission.

2.3 NONO AND ITS RELATION TO *L. MONOCYTOGENES* INFECTION**.**

The report of an outbreak of listeriosis in 1981, caused by the consumption of coleslaw that contained *L. monocytogenes*, confirmed the first evidence of transmission by food (Schlech et al., 1983; James, 1991; Moura et al., 1993). Afterwards, other outbreaks related to food were reported. These included dairy products such as raw and unpasteurized milk and cheese (James et al., 1985; Moura et al., 1993; RØrvik et al., 1995).

Nono, which is a local form of yogurt, is a fermented product of raw milk from cattle. Meydari and Woel-Kyu (2000) defined yogurt as "a coagulated milk product that results from fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*". The fermented product has low pH.

Gray and Killinger (1966) reported that *Listeria* is acidophobic and can be destroyed at pH below 5.6. Furthermore, Shehu and Lamido (1994) reported an average pH of 3.80 for nono sampled in Zaria, a town in northern Nigeria. But effective fermentation should occur at pH less than 4.4. Consequently, any fermentation at a pH greater than this will be deemed to be ineffective and will favour the survival of *L. monocytogenes* (Barbuddhe et al., 1994; Lake et al., 2002).

With these reported variations in pH values, it would be valuable to ascertain the pH values of nono (yogurt) in the study areas. This is with a view to isolating possible *L. monocytogenes* isolates that could have been shed into the milk from cases of bovine mastitis, or from contaminated hands or utensils used in the preparation (Jacquet et al., 1993; Shehu & Lamido 1994; Jensen et al., 1996). The *L. monocytogenes* isolated from nono, placenta, blood and cerebrospinal fluid may not be of the same strain (Anonymous, 2000).

2.4 STRAINS OF *L. MONOCYTOGENES.*

Sixteen serotypes (serovars) of *L. monocytegenes* have been identified through serological grouping of the H and O antigens (Seeligar & Hohne, 1979). Hof (1984) and Bruce and Clair (1989) reported that serotypes 1/2a, 1/2b and 4b are responsible for more than 90% of Listeriosis cases, while 4b was reported to have caused over 50% of listeriosis cases world wide (Elcuaz et al., 1996; Jose et al., 2001). In Iran, serovars 4b and 1a were responsible for five cases of abortion involving *L. monocytogenes* (Mirdamadi, 2005). Furthermore, serovar 1a was also reported to be responsible for most cases of bovine listeriosis (Harvey and Gilmour 1993; Jacquet et al., 1993; Moura et al., 1993; Jensen et al., 1996). Bortolussi et al. (1984) observed that serovar 4b strains were more

adapted to mammalian tissues than those of serogroup one. But the isolation of serovar 4b in food outbreaks in California and in France suggests that differences in clones and consequently pathogenicity exist (Jose et al., 2001).

Knowledge of these serotypes for any given location would assist in epidemiological studies (Elcuaz et al., 1996). This fact was supported by the observation made by Seeliger and Hohne (1979). These workers reported on the differential regulation of the virulence genes of *L. monocytogenes* between EGD strain and NCTC 7973 strain. They deduced that the differences were influenced by amino acid composition and by environmental parameters. This revealed that a specific serovar isolated from Britain might not act in the same way like a similar serovar in northern Nigeria in terms of virulence. The serovars of *L. monocytogenes* account for the epidemiology of the diseases it causes.

2.5 EPIDEMIOLOGY OF LISTERIOSIS**.**

Knowledge on the epidemiology of human listeriosis is incomplete worldwide (Hanssler et al., 1990; Harvey & Gilmour, 1993). Epidemiological data available still give a distorted picture of the severity of infection. This is because the disease is rare and its incidence in a community may be undetected (Mulder & Zanen 1984). An epidemiological record on listeriosis in the study areas does not exist.

Epidemiological surveys conducted world wide, revealed two patterns of the disease. The first is a predominantly septicaemic state, while the second is meningitis or a meningoencephalitis state. The diseases could be sporadic or epidemic (Elcuaz et al., 1996; McLauchlin, 1987). In the United States for instance, 362 cases occurred between 1970 – year 2000 (Anonymous, 2000).

Absence of simple and affordable medium could have contributed to the poor epidemiological records of listerial diseases especially in Africa.

2.6 METHODS OF DIAGNOSIS OF LISTERIOSIS.

Diagnosis of listeriosis is based on the isolation of *L. monocytogenes* from specimens such as CSF, blood, amniotic fluid and stool. The method of isolation is still a major obstacle in the detection and understanding of its epidemiology (Mavrothalassitis, 1977). Current isolation methods employ enrichment procedures in selective broth and sub-culture on solid medium. This is time consuming, as species identification requires further biochemical tests such as acid production from L- rhamnose, D-xylose, mannose and haemolysin production. Consequently, there is need for new compositions that would enhance the diagnosis of listeriosis.

The design of an appropriate medium has been the subject of several studies (Swaminathan et al., 1988; Cassiday & Brackett, 1989; Lachica, 1990; Kokubo et al., 1990; Kovacs & Ralovich, 1991). Different enrichment broths have been introduced. Lovett (1987) described a broth medium that is used by the Food and Drugs Administration (FDA) in the USA. There are also the primary and secondary enrichment broths described by the University of Vermont (McClain & Lee, 1988). In addition, there is Fraser's broth and the liquid PALCAM (Polymyxin-acriflavine-lithium-chloride-cefazidime-aesculin-mannitol – egg yolk) broth that was designed to be used along with PALCAM solid agar medium.

Solid media are also varied. The increasing number of *Listeria* selective agar media is due to the shortcoming inherent in some of them (Lee & McClain, 1986). Most of the media incorporate as many as 8-10 costly and unstable constituents such as lithium chloride, agriflavin, polymyxin, cycloheximide and

bacitracin to mention a few (Leighton, 1979). In addition, some incorporate two indicator systems such as mannitol – phenol red and aesculin – ferric iron complex as in the PALCAM agar. Furthermore, some solid media require special equipment or technique to identify the colonies of *Listeriae,* such as the zoom microscope and oblique lighting technique (Curtis & Lee, 1995).The need for special equipment or an indicator system is due to the tiny colonies of *Listeriae* on these media. The tiny colonies are as a result of non-availability of a suitable growth nutrient, such as glucose that is readily utilized by *Listeria* more than mannitol or aesculin. The mannitol, tryptose and aesculin incorporated in some media are not readily utilized by *Listeria* (Lachica, 1990). Consequently, the cells emerge as small colonies on these media.

The choice of an ideal medium appears to be the responsibility of individual laboratories. Consequently, the development of a simple and affordable medium without unstable reagents (Lachica, 1990; Curtis & Lee, 1995) and selective for *Listeria* is advocated.

Level of Awareness of listeriosis. *L. monocytogenes* infections are not common and, when patients present cases of meningitis or spontaneous abortion, medical doctors do not directly request for a diagnosis of *L. monocytogenes*. Since *L. monocytogenes* may require more than 24 hours to grow, Laboratory scientists may report negative results, especially in cases of spontaneous abortion (Riviera et al., 1993).

There is also a low level of awareness of listeriosis by the general public, food industries and health care providers. Because of the severity of the infection, a zero level tolerance was advocated for *L. monocytogenes* in foods (Mclauchlin, 1987; Jay, 1991; Harvey & Gilmour 1993; Jacquet et al., 1993; Moura et al.,

1993; Anonymous, 2000) as most cases of listeriosis have been linked with the consumption of contaminated foods.

Since infection with *L. monocytogenes* is not as common as malaria, typhoid fever and gastroenteritis, the possibility of awareness of it in Nigeria is doubtful. This raises the need to also create awareness of the disease in the study areas. In addition, control strategies would then be meaningful, thus making further research on the disease relevant.

CHAPTER THREE

MATERIALS AND METHODS

3.1 METHODOLOGY.

Placental swabs, blood, and cerebrospinal fluid samples would be collected from patients in some health facilities in the study area (Cheesbrough, 1984). Nono samples would also be collected from hawkers in the markets. These samples would be processed in the laboratory, in order to isolate *L. monocytogenes* as well as other associated bacteria (Moura et al., 1993). To ascertain the level of awareness amongst health personnel, questionnaires would be distributed to willing Doctors and Medical Laboratory Scientists in the study areas. Statistical analysis would be used to test the significance of the results (Kelly & Onyeka, 1992) at 5 % level of significance.

3.2   THE STUDY'S GEOGRAPHICAL SETTING.

Three northern Nigerian towns: Jos, Bauchi, and Kano were chosen for this study. The towns are state capitals located in the North-central, Northeast, and Northwest geopolitical zones of Nigeria. The study lasted for 22 months (March 1998 to December, 1999). Samples were collected from the following health facilities: Evangel hospital, Vom christian hospital, and Plateau specialist hospital, all in Jos; General Hospital, Toro, and Specialist Hospital in Bauchi; Murtala Mohammed Hospital, Infectious Diseases Hospital, and Nigeria Airforce Medical Centre in Kano. Furthermore, nono was sampled from the following locations: Bukuru, Abattoir, and Central Market in Jos; Wunti and Yelwa Markets and Jos Road Motor Park in Bauchi; Sabon-gari and Kantin-kwori Markets, Mallam Aminu Kano International Airport, Zaria Road Motor Park in Kano.

3.3 PREPARATION OF REAGENTS AND CULTURE MEDIA.

3.3.1 Preparation of Reagents.

Phenol red indicator (Cheesbrough, 1984). A 0.5-g quantity of phenol red powder (BDH chemicals Ltd, England) pH 6.8 - 8.2 was weighed on a mettler analytical NaOH balance (Fisher Scientific, USA) and dissolved in 15 ml of 0.1m. It was made up to 100 ml with distilled water and stored in a brown reagent bottle.

Methyl red (**Cheesbrough**, 1984). A 0.05 g weight of methyl red powder (pH 4.2 – 6.2, SIGMA) was weighed on mettler analytical balance and dissolved in 28 ml of absolute ethanol. A volume of 22 ml of distilled water was added, mixed, and transferred into a clean brown bottle. It was labeled, and stored in a dark place at room temperature.

Bromothymol blue indicator. A 0.1 g weight of Bromothymol Blue pH 6.0-7.6 (May and Baker) powder was weighed on a mettler analytical balance (Fisher Scientific, USA) and dissolved in 2.5 ml of 0.1M NaOH in a beaker. It was then made up to 50 ml with sterile distilled water and stored in a brown reagent bottle in the dark (Cheesbrough, 1984). Phosphate buffer pH7.4 (Cheesbrough, 198). 11.3 g of dried reagent grade disodium hydrogen phosphate (Sigma) and 2.7 g Potassium dihydrogen phosphate (Sigma) were separately weighed on a top loading mettler balance (Fisher scientific, US) and dissolved in one litre of distilled water. The pH was checked with the aid of a pH meter (Gallenkamp, London). No appreciable difference was noted. It was then labelled and stored in a Winchester bottle at 4^0C in the refrigerator.

Sodium hydroxide (0.1M). A 4-g weight of sodium hydroxide (SIGMA) was weighed on a top loading mettler balance and dissolved in 1000ml of distilled water. It was stored in a labeled winchester bottle (Cheesbrough, 1984).

Nalidixic acid (Leighton, 1979)**.** A 500 mg sample (One tablet) of nalidix acid (Glaxo Smith Kline) was crushed in a mortar and dissolved in 5 ml of 1M – NaOH. It was centrifuged at 5 x g for 5 min in a Jouan centrifuge C500 to sediment the tablet base. The clear supernatant was then used as a source of nalidixic acid (100 mg / ml).

10% Potassium hydroxide (KOH). A 10 g sample of KOH (SIGMA) was dissolved in 100 ml of distilled water in a reagent bottle.

3.3.2 Preparation of Culture Media.

Peptone water. A 7.5 g bacteriological peptone powder (Merck, Germany) was dissolved in 300 ml distilled water. It was dispensed in 5.0 ml amounts into bijou bottles and sterilised in the autoclave at 121°C for 15 min at 15 lb pressure.

Peptone water indicator. A 10 g Bacteriological peptone powder (Merck, Germany) was dissolved in 200 ml (w / v) phosphate buffer (pH 7.4) to which 30 ml bromothymol blue was added and made up to 400 ml with the buffer. It was then dispensed in 100 ml amounts and sterilised at 115°C for 30 min in the autoclave (Cheesbrough 1984).

Peptone water sugars. To 100 ml of sterile peptone water indicator solution, 5.0 ml of appropriate sterile ampoule (10g / 100 ml) solution of the sugars: glucose, xylose, mannitol and rhamnose (Oxoid UK) was added. It was dispensed in 4.0 ml amounts into sterile bijou bottles which contained Durham tubes. The solution was steamed in the autoclave to sterilise for 30 min (Cheesbrough 1984).

Listeria **selective agar.** A modification of Curtis et al. (1989) was prepared. A 7.5 g of Columbia agar (Oxoid, England) was dissolved in 200 ml distilled water over a hot-plate. Oxford Selective Supplement (Oxoid U.K.), 1.0g mannitol (Oxoid, U.K.) and 5.0 g phenol red indicator were added and made up to 500 ml

with phosphate buffer (pH 7.4). It was then mixed properly and sterilised in the autoclave at 115°C for 30 min. The medium was then allowed to cool and then dispensed into sterile Petri dishes and allowed to set.

Brain heart infusion broth (3.7 g / 100 ml). A 7.4 g BHI broth powder (Difco, Michigan) was dissolved in 200 ml distilled water in a 250 ml

conical flask on a hot-plate. It was dispensed in 5.0 ml amounts into bijou bottles and then sterilised at 115°C for 30 min at 15 lb pressure.

10% Blood agar (Baker and Breach, 1980). A 1.8g sample of nutrient agar (Biotec, U.K.) was dissolved in 450 ml of distilled water under a Bunsen flame in a 1.0 L conical flask. It was then autoclaved at 121°C under 15 lb pressures in the autoclave for 15 min. After cooling to about 45°C, 50 ml of sheep blood was added and thoroughly mixed. The medium was immediately dispensed into sterile Petri dishes and allowed to set on a bench.

Chocolate agar (Baker and Breach, 1980). A 1.8 g of nutrient agar (Biotec,U.K.) was dissolved in 450 ml of distilled water with the aid of a bunsen flame in a 1.0 L conical flask. It was autoclaved at 121°C under 15 lb pressures in the autoclave for 15 minutes. Immediately, 50 ml sheep blood was added to give a chocolate colour. The medium was allowed to cool to about 50°C and dispensed into Petri dishes, to set on a bench.

MacConkey agar. 24.30 g of MacConkey agar (Biotec, U.K.) was weighed and dissolved in 500 ml distilled water in a 1.0 L conical flask placed on hot-plate maintained at 100°C. It was then covered with non-absorbent cotton wool and autoclaved at 121°C at 15 lb pressure for 15 min. After all cooling, the medium was dispensed into Petri dishes to set.

***Listeria* enrichment broth.** A 37 g powder of *Listeria* enrichment broth (Difco, Michigan) was dissolved in 500 ml phosphate buffer (Ph 7.4) over a hot-plate. This was mixed, and made up to 1000 ml with phosphate buffer (pH 7.4). It was dispensed in 5.0 ml amounts into bijou bottles, and sterilised at 115°C for 30 min.

3.4 SAMPLE COLLECTION.

3.4.1 Cerebrospinal Fluid.

Cerebrospinal fluid (CSF) samples were collected monthly (March 1998 to December 1999) from patients diagnosed as suffering from meningitis. One hundred samples were collected from each of Jos, Bauchi and Kano. A total of 300 samples were collected. A 0.5 ml of CSF was drawn from each patient with the aid of a syringe, and emptied into a bijou bottle. Samples within Jos were transported to the laboratory within 10 minutes and refrigerated at 4ºC. Samples from Bauchi and Kano were also refrigerated in the respective hospitals. They were subsequently transported to Jos in a food flask containing ice. The samples were categorized into nine age groups (years) of patients viz: - <1mo, 1 - 11mo, 1- 4, 5- 9, 10 – 14, 15 – 19, 20 – 24, 25 – 29, ≥30 years. This range was chosen in order to reflect the ages affected. Equal samples were not collected for each age group due to the attendant difficulty posed by the small number and ages of patients who reported to the hospitals, as well as the time limit for this study. Patients voluntarily did not want to fill any consent forms.

3.4.2 Placental Swabs and Blood Samples.

Three hundred placental swabs and 300 blood samples were obtained from patients. One hundred placenta swabs and 100 of the blood samples (0.5 ml) in 5.0 ml of 3.7 g / 100 ml brain heart infusion broth (BHI, Difco, Michigan), were collected from each experimental town. The samples were preserved in a

refrigerator at 4°C. The samples from patients were categorised into six age groups (years) that consisted of 15-19, 20 – 24, 25 – 29, 30 – 34, 35 – 39, and $\geq$ 40. All the experimental samples were voluntarily taken from the patients after they had completed and signed consent form (Appendix A1).

3.4.3 Nono Samples.

Equal number (100) of Nono samples were randomly collected from unsuspected hawkers in Jos, Bauchi, and Kano. These gave a total of 300 samples. A 5.0 ml sample was transferred into each of two pre-labelled sterile universal bottles bearing the date of collection, location and sample number,and transported promptly to the laboratory, where they were stored in the refrigerator at 4°C. Furthermore, samples collected outside Jos were transported in food flasks containing blocs of ice. One pair was for pH determination while the second pair was inoculated onto culture media.

3.5 TREATMENT OF SAMPLES.

3.5.1 Inoculation of Samples.

 Cerebrospinal fluid.

Portions of each of the CSF sample was inoculated in blood, chocolate, MacConkey (Biotec, U.K.) and *Listeria* enrichment broth (Difco, Michigan) media contained in Petri dishes and bijou bottles. The blood and MacConkey agar and *Listeria* enrichment broth cultures were incubated aerobically at 37°C for 18 - 48 hours. The Chocolate agar cultures were incubated anaerobically at 37°C in a candle jar for the same period. All cultures were examined daily for growth.

Placental swab / blood.

Each swab sample was inoculated asceptically into 3.7g /100 ml BHI broth (Difco, Michigan) and 3.2g / 100 ml *Listeria* enrichment broth (Difco, Michigan). All the

broth cultures were then incubated at 37ºC for 24 hours and examined for growth. A blackening of the *Listeria* enrichment broth medium was noted as evidence of growth as described by Curtis and Lee (1995). The blood samples were subjected to the same treatments given to the placental samples. All the media were examined for growth.

Nono samples

A 2.0 ml of each sample was dispensed into sterile test tubes and centrifuged at 3000 rpm (ALCO 4222, Italy) for 15 minutes to concentrate any bacteria that may be present. The bulk of the supernatant was removed and the deposit resuspended in the remaining portion of liquid. This was used to inoculate 3.2 g /100ml *Listeria* enrichment broth medium incubated at 37ºC for 24 hours. The medium was examined for the presence of *Listeria* -like organisms.

3.5.2 Examination of Cultures.

The culture plates were examined daily for characteristic growth. Colony appearance and reaction of the indicator medium were noted. Growth on blood agar was noted for haemolysis around the colonies and these reactions were reported either as alpha (α) or beta (β) haemolysis. Growth on chocolate agar medium was noted for tiny, translucent, whitish colonies or golden coloration. On MacConkey agar medium, colonies were also noted for whitish, pinkish or swarming appearance with fishy odour. A darkening of the *Listeria* enrichment broth medium was noted as evidence of growth.

Sub-inoculation.

Broth culture of each test isolate was inoculated onto *Listeria* selective (Difco, Michigan) and MacConkey agar plate media. The plates were incubated at 37ºC for 18 – 48 hours, and examined daily for the appearance of black halos around

colonies on the *Listeria* selective agar plate and of tiny magenta-colored colonies on the MacConkey agar plate medium.

Cold-enrichment.

Broth cultures that did not show any evidence of growth were returned to the refrigerator at 4ºC to enable listerial cells present to grow. Periodic Sub-inoculation onto 7.5 g / 100 ml *Listeria* selective agar was carried out fortnightly over a period of two months after which they were discarded as negative if no *Listeria* species was isolated.

Control culture.

A *L. monocytogenes* isolate (SLCC 2379 Wurzburg) from a *Listeria* study group at the National Veterinary Research Institute, Vom, was similarly inoculated on the test media. This served as a control culture.

3.5.3 pH Values of Nono Samples.

The pH of each Nono sample was determined with the aid of a Gallenkamp pH meter (Gallenkamp, London) using a calomel reference electrode.

3.5.4 Characterization of Presumptive Isolates. This was carried out in order to identify the isolates according to their biochemical characteristics (Cowan and Steel, 1992).

Gram staining.

The CSF samples and the presumptive isolates were subjected to gram staining using the procedure described by Baker and Breach (1980). Their gram reactions were recorded and compared with the descriptions of Seeliger and Jones (1986) and Cowan and Steel (1992).

Catalase test.

The *Listeria*-like isolates and other presumptive isolates were subjected to catalase test as described by Baker and Breach (1980).

Coagulase test.

Coagulase test to confirm the pathogenicity of *S. aureus* isolates was carried out according to the method of Baker and Breach (1980.)

Motility test.

The *Listeria*-like isolates and presumptive isolates from MacConkey agar medium were subjected to motility test according to the procedure of Baker and Breach (1980.)

Oxidase test.

The ability of the isolates to produce oxidase was tested according to the method of Cowan and Steel (1992.) This confirmed the presence of *N. meningitidis* and other microbes.

Carbohydrate fermentation test.

The *Listeria*-like isolates were examined for their abilities to ferment glucose, xylose, mannitol and rhamnose (Oxoid Ltd, U.K.). The method adopted was that described by Cheesbrough (1984). In this method, a broth culture of the *Listeria*-like isolate was made by inoculation in 2.5 g / 100 ml peptone water (Biotec, U.K.) and incubated at 37ºC for 30 min. A loopfull of the broth culture was then added to peptone water (2.5 g /100 ml) sugar (10%) indicator (bromothymol blue) medium contained in a bijou bottle, with the aid of a sterile wireloop. The medium contained also an inverted Durham tube to enable the detection of any gas production. A change of colour from blue to yellow after a period of 24 - 48 hr incubation at 37°C was indicative of sugar fermentative ability of the organism,

while the displacement of the medium in the Durham tube also confirmed gas production by the same organism.

Methyl red / Voges – Proskauer (VP) tests.

The ability of the suspected *Listeria* species to produce acid and or acetoin after the utilization of glucose was tested. The experimental peptone water glucose broth utilized in the carbohydrate fermentation test was employed for the experiment according to the method of Baker and Breach (1980).

CAMP test.

In this particular test, 10% sheep blood agar plate was prepared and a standard *Staphylococcus* species culture was then streaked across the medium, while the *Listeria* species was streaked at right angle to it but without touching the *Staphylococcus* species culture. *Enterococcus faecalis* was used as a negative control. It was also streaked at right angle to the *Staphylococcus* species culture.

The cultures were then incubated at 37ºC for 24 hr after which they were observed for any arrow-head shaped area of haemolysis as described by Seeliger and Jones (1986) and Cheesbrough (1984.) Arrowhead shaped area was indicative of interaction between the diffusible extra cellular protein of *Listeria* species and the haemolytic factor of the *Staphylococcus* species.

3.5.5 Serology

The *Listeria* genus was identified to a particular serotype by serotyping it with Bacto-*Listeria* – O antisera supplied by Difco Laboratories Ltd., Detroit, Michigan. A rapid slide test procedure was carried out using types 4 and I and polyvalent sera. In this procedure, a suspension of the isolate was made in 1.0 ml of phosphate buffer (pH 7.2). The organism suspension was then heated at 80ºC for 60 minutes in a water bath. The suspension was centrifuged and the bulk of the

supernatant fluid removed. The organism was re-suspended in the remaining portion of liquid. A drop of the heated organism was added to a drop of the antiserum diluted to 1: 20 in saline solution on a glass plate and rocked back and forth for 1 – 2 minutes. Similarly, a drop of the organism suspension was added to a drop of negative control buffer solution and rocked for 1 – 2 minutes. They were observed for agglutination.

Confirmation of isolates. All presumptive isolates were further confirmed by the Diagnostic Division and the *Listeria* Study Group at the National Veterinary Research Institute Vom, Nigeria.

3.5.6 CSF White Blood Cell Count.

The methodology adopted for this experiment was that described by Cheesbrough (1984). Three drops of the CSF were run into a dry precipitin tube with a Pasteur pipette. A pipette was used to transfer three drops of toluidine blue White Blood Cell (WBC) fluid to the CSF contained in the precipitin tube. The contents were mixed. A Fuchs-Rosenthal haemocytometer was charged with the diluted CSF and then allowed to settle for 2 - 3 minutes. The White blood cells were then counted using a x10 objective of a binocular microscope. The white cells were counted in five squares and the total count was multiplied by 2 (dilution factor) to give the number per ml of CSF.

Awareness survey on listeriosis. Questionnaires (Appendices A2 and A3) were distributed to 100 medical doctors and 100 laboratory scientists in the study towns. The survey was conducted to ascertain the level of awareness of listeriosis amongst health personnel. Emphasis was not placed on equal participation from the three study towns due to the dearth of manpower.

3.5.7 Statistical Analysis.

Data obtained were subjected to analysis of proportion and standard deviation tests as described by Kelly and Onyeka (1992) to ascertain the significance of the occurrence of bacterial agents and pH values of nono in Jos, Bauchi and Kano, respectively.

3.5.8 Case Definition.

A case of bacterial meningitis was confirmed when at least one of the following criteria was met (Lwin et a*l*., 1991):

 a. A white blood cell count $>$ 5 cells per ml.

 b. A direct Gram stain smear of the CSF that show the presence of gram-positive cocci or gram-negative rods or large gram-positive spherical cells (yeast cells).

 c. Isolation from CSF of *N. meningitidis* or other pathogenic bacteria.

Similarly, a case of spontaneous abortion occurred when:

 a*. Listeria monocytogenes* was isolated from placenta and blood sample.

 b. Isolation of other bacterial pathogens from placenta and blood samples of test   patients.

3. 5. 9 Meteorological Data for Jos, Bauchi and Kano.

Data on mean monthly temperatures and relative humidity were obtained from National Root Crops Research Institute, Potato Programme, Vom and the Nigerian Air port Authority Kano.

3.6 GROWTH OF *L. MONOCYTOGENES* ON FORMULATED MEDIA.

Four media were prepared: Colistin (Glaxosmithcline) selective agar (CSA) 1.0 mg / 100 ml; cefuroxime (Glaxosmithcline) selective agar (CeSA) 2.0 mg / 100

ml; colistin - cefuroxime selective agar (CceSA) and Brain heart infusion (BHI) agar as a control medium. *Listeria* species and other organisms were grown on these media in order to ascertain their selectivity and also to define the appropriate medium for the isolation of *Listeria* species.

3.6.1 Preparation of Stock Antibiotics.

Colistin (1. 0 g / 100ml).

A 1.0 g vial of colistin was dissolved in 100 ml sterile distilled water. The solution was dispensed into sterile universal bottles, each bottle containing 20 ml. The bottles were then stored in a refrigerator maintained at 0^0C.

Cefuroxime (750 mg / 100 ml).

A 750 mg vial of cefuroxime was dissolved in 100 ml of sterile distilled water and dispensed in 10 ml amounts in universal bottles and then stored in a refrigerator maintained at 0^0C.

3.6.2 Preparation of Working Antibiotic Solution.

Colistin (1.0 mg / 100 ml.) pH 7.4. A 0.1 ml of stock colistin was made up to 100 ml phosphate buffer pH 7.4 to give a working concentration of 1.0 mg / 100 ml (v / v).

Cefuroxime (2.0 mg / 100 ml.) pH 7.4. A 0.27 ml of stock cefuroxime was made up to 100 ml sterile phosphate buffer (pH 7.4) to give a working concentration of 2.0 mg / 100 ml (v / v).

3.6.3 Preparation of Selective Media.

Colistin selective agar pH7.4 (1.0 mg / 100 ml).

A 3.7 g sample of BHI agar (Difco, Michigan) was dissolved in 100 ml working colistin antibiotic solution (v / v) in a 250 ml conical flask placed over a Bunsen flame. A 0.01 ml sample of nyastatin (Pharmamed, Malta) was added. The flask

was shaken and then sterilized in the autoclave at 121^0C and 15 lb pressure for 30 minutes. This was then allowed to cool to about 44^0C. The medium was then dispensed into Petri dishes and allowed to set. The resultant culture plates were labeled appropriately.

Cefuroxime selective agar (2.0 mg / 100 ml), pH 7.4.

Similarly, 3.7 g / 100ml BHI agar was dissolved in 100 ml of working cefuroxime (pH 7.4) contained in a 250 ml conical flask and 0.01 ml of nyastatin (12.5 units) was then added. The flask was plugged with non-absorbent cotton wool and sterilized in an autoclave at 121^0C at 15 lb pressure for 30 minutes. The medium was allowed to cool and it was then dispensed into sterile culture plates, each plate containing 15 ml of the medium.

Colistin-Cefuroxime selective agar (pH 7.4).

Brain heart infusion agar was weighed (3.7 g) and then dissolved in 100 ml of equal volume of working colistin and cefuroxime antibiotic solution in a 250 ml conical flask placed over a Bunsen flame. A volume of 0.02 ml nyastatin (12.5 units) was added. The flask was plugged with non-absorbent cotton wool and sterilized in the autoclave as described. The dispensation of the medium was aseptically carried out as described previously.

Brain heart infusion agar (pH 7.4).

A 3.7 g of Brain heart infusion agar (Difco, Michigan) was dissolved in 100 ml phosphate buffer (pH 7.4) in a 250 ml conical flask. A 0.01 ml nyastatin (12.5 units) was then added and sterilized as previously described. The medium was stored at 4^oC.

3.6.4 Testing of the Media.

The selectivity of the prepared media was tested by using strains of *Enterococcus faecalis, Escherichia coli, Proteus mirabilis, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus pneumoniae, Klebsiella spp , Candida albicans* and SLCC (2379 Wurzburg*) L. monocytogenes* type 4b. Except for *L. monocytogenes* which was streaked on BHI control agar medium, other organisms were streaked on the antibiotic media. Each streaking was done with the aid of a sterile wire loop. Plate cultures were incubated at 37^{0}C in an incubator for 24 - 48 hours. The media were examined for their ability to inhibit or support microbial growth. The ability of the BHI agar to give rise to larger colonies of *Listeria* compared with *Listeria* selective agar was studied. Profuse, moderate, scanty, and no growth were scored as +++, ++, +, and -, respectively.

3.6.5 Comparative Cost Analysis.

The costs of Oxford *Listeria* agar and PALCAM agar and *Listeria* enrichment broth were compared with brain heart infusion agar, colistin and cefuroxime antibiotics (Appendix A4). A local retailer of laboratory reagents was consulted.

CHAPTER FOUR

RESULTS

4.1 SAMPLE COLLECTION.

Out of 300 samples of cerebrospinal fluid collected, 69 were positive for meningitis bacteria in the months of March to May of 1998 and 79 in 1999 respectively. The details are in Figure 1.

4.1.1 Examination and Characterisation of Isolates.

The motility and other biochemical tests revealed that 41 isolates from the CSF and 48 from the placenta / blood were motile. Twelve isolates from the CSF and 26 from the placenta / blood were also CAMP test positive. Only isolates from CSF were positive for oxidase reaction. The details are in Table 1. Similarly, 12 isolates from the CSF and 14 from the placenta / blood utilised rhamnose and glucose (Table 2).

Gram stain reaction.

Twelve isolates from the CSF and 14 from the placenta / blood were Gram positive bacilli (Table 3).

Isolates from Cerebrospinal fluid. A total of 215 CSF samples were culture positive for nine species of confirmed bacterial pathogens with *Neisseria meningitidis* having the highest percentage occurrence of 48.84. Other bacteria were *Streptococcus pneumoniae* (16.74 %), *Escherichia coli* (11.16 %), *Staphylococcus aureus* (7.44 %) and *Listeria monocytogenes* (5.58 %). The details are in Table 4. Further more, a break down of the bacterial pathogens in the study towns showed that Kano had the highest occurrence of *N. meningitidis* (45), *S. pneumoniae* (10) and *E. coli* (10), while *L. monocytogenes* (6) had the highest occurrence from CSF samples from Jos. Furthermore, *N. meningitidis*
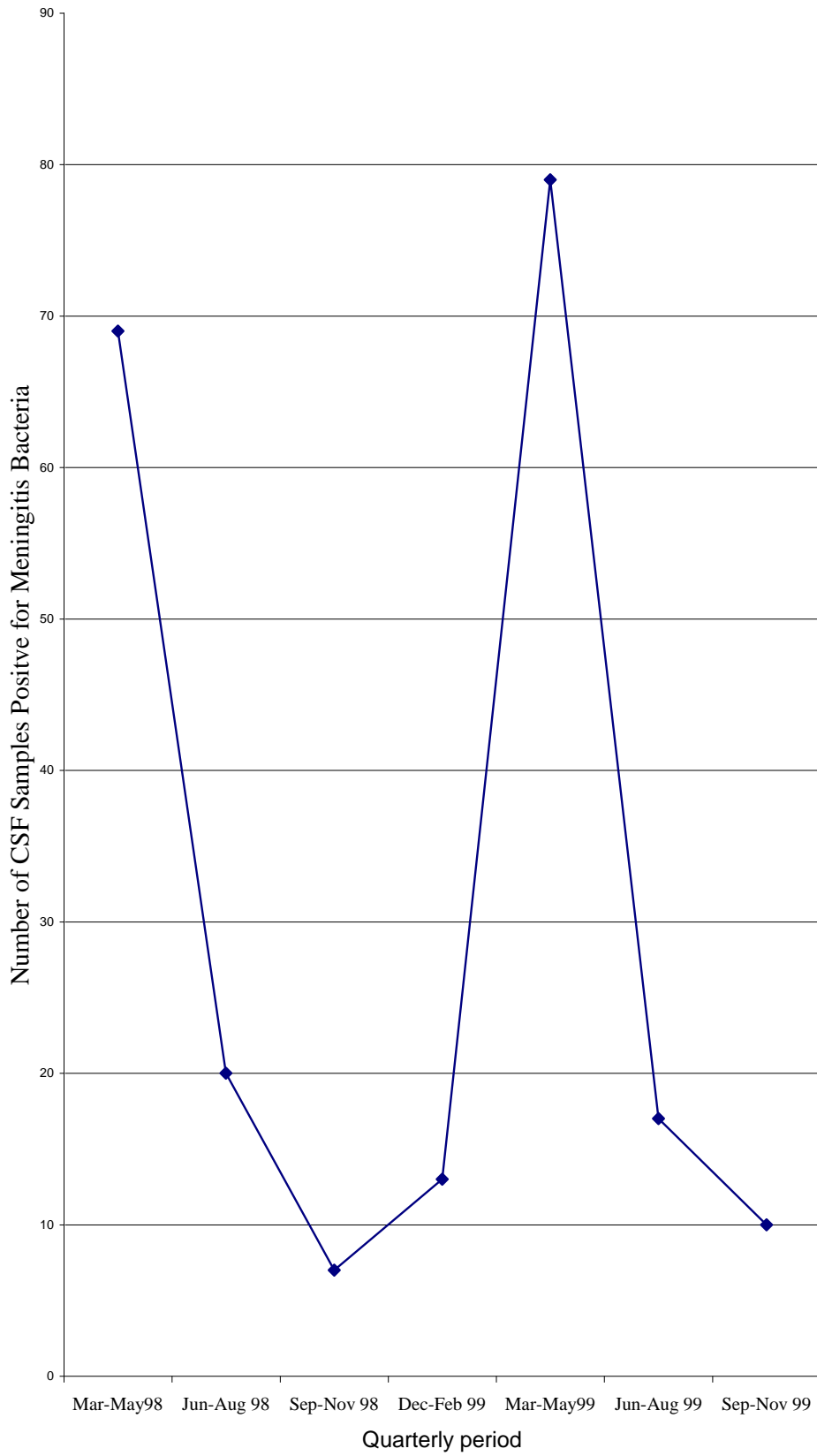
Figure 1. Number of Positive CSF Samples from Mar 98 to Dec 99.

Table 1. The Number of Isolates showing Motility and Biochemical Reactions.

Name of test	Sample CSF	Placenta	Nono
Positive motility	41	48	-
Negative motility	6	-	-
Catalase reaction	28	26	-
Coagulase reaction	16	13	-
Methyl red reaction	41	48	-
V P reaction	-	-	-
CAMP- test	12	26	22
Oxidase reaction	105	-	-

Key:
- represents no motility or reaction.

Table 2. Carbohydrate Fermentation Reaction of Presumptive *L. monocytogenes* Isolates from CSF, Placenta and Blood.

Carbohydrate	Number of isolates	
	CSF	Placenta and blood
D- xylose	-	-
D – mannitol	-	-
Rhamnose	12	14
D –Glucose	12	14

Key:

‒ stands for no reaction.

Table 3. Number of Gram Stain Reactions of Isolates from CSF, Placenta, Blood and Nono.

Gram reaction	Number of isolates from:		
	CSF	Placenta / blood	nono
Gram positive cocci	63	63	74
Gram positive bacilli	12	14	-
Gram negative diplococci	105	-	-
Gram negative bacilli	35	34	-
Budding large Gram positive cells	-	4	-
Total	215	115	74

Key:

‾ represents no isolate.

Table 4. Number (and percentages) of Pathogens Isolated from CSF Samples from Jos, Bauchi and Kano.

| | Study Towns | | | |
Pathogens	Jos	Bauchi	Kano	Total (%)
Neisseria meningitidis	22	38	45	105(48.84)
Streptococcus pneumoniae	8	12	10	36 (16.74)
Escherichia coli	2	12	10	24 (11.16)
Staphylococcus aureus	4	5	7	16 (7.44)
Listeria monocytogenes	6	3	3	12 (5.58)
Streptococcus pyogenes	4	2	2	8 (3.72)
Haemophilus influenzae	4	1	1	6 (2.79)
Proteus mirabilis	-	2	3	5 (2.33)
Enterococcus faecalis	1	1	1	3 (1.40)
Total	51	76	88	215(100)

S.E= 5.66 1±.71 (Jos); 8.44± 3.23 for Bauchi and 9.78± 3.78 for Kano.

Key: - represents no isolates.

alone accounted for 48.84% of the bacterial pathogens isolated. Other bacteria (excluding *Listeria*) accounted for 45.58 %, while *L. monocytogenes* was 5.58 % (Table 4).

A break down of the ages of patients sampled showed that *N. meningitidis* occurred more in the age range 1 – 4, 5 – 9, 10 – 14 and 15 – 19 years. Patients in the age range < 1and 1 – 11 months as well as ≥ 30 years had *L. monocytogenes* isolated from their CSF. The age group 5 – 9 years had the highest number of bacterial isolates (Table 5).

Isolates from placenta swabs and blood samples.

One hundred and two (102) placenta swab and 13 blood samples were culture positive for eight confirmed species of bacterial pathogens isolated from placenta swabs and 2 from blood samples (Table 6). *Enterococcus faecalis* was the most isolated pathogen, followed by *Proteus mirabilis, E. coli* and *L. monocytogenes*. *L. monocytogenes* and *Streptococcus agalactiae* were isolated from blood samples. In addition, the distribution of the pathogens in the experimental towns showed that Kano had the highest occurrence of the total number of bacterial pathogen (46) with Bauchi (41) and Jos (28) following this (Table 7). Infection in the age range of the patients showed that the number of bacterial pathogens isolated was more in the older patients. *L. monocytogenes* was not isolated from patients in this age range (Table 8).

Isolates from nono. *E. faecalis* and *S. agalactiae* were isolated from 52 and 22 samples respectively.

Control culture. The control culture of *L. monocytogenes* (SLCC 2379) grew on *Listeria* selective agar medium

Table 5. Occurrence of Prevalent Meningitis Pathogens Amongst Various Age Groups.

| | | | | | Age Range (years) | | | | | |
Pathogens	<1	1-11	1-4	5-9	10-14	15-19	20-24	25-29	≥30	Total
N. meningitis	-	2	12	42	29	10	6	2	2	105
S .pneumoniae	6	8	3	3	-	-	-	-	16	36
E. coli	2	8	8	2	3	-	-	-	1	24
S. aureus	2	-	2	2	1	-	2	3	4	16
L. monocyto-genes	2	4	-	-	-	-	-	-	6	12
S. pyogenes	1	2	1	-	-	-	-	-	4	8
H. influenzae	1	4	1	-	-	-	-	-	-	6
P. mirabilis	-	2	1	1	-	-	-	1	-	5
E. faecalis	1	2	-	-	-	-	-	-	-	3
Total	15	32	28	50	33	10	8	6	33	215

Key:

- represents no occurrence.

Table 6. The Number and Percentage of Species of Micro-organisms Isolated from Placenta and Blood Samples

Species of Microorganisms	Number of Positive Samples from:		Total (%)
	Placenta	Blood	
Enterococcus faecalis	35	-	35 (30.43)
Proteus mirabilis	18	-	18 (15.65)
Escherichia coli	16	-	16 (13.9)
Listeria monocytogenes	7	7	14 (12.20)
Staphylococcus aureus	13	-	13 (11.30)
Streptococcus agalactiae	6	6	12 (10.43)
Candida albicans	4	-	4 (3.48)
Streptococcus pyogenes	3	-	3 (2.61)
Total	102	13	115 (100)

Key:

‑ represents no isolates.

Table 7. The occurrence of Microorganisms Isolated from Placenta Swabs and

Blood Samples of Patients in the Study Towns (Jos, Bauchi and Kano).

| | Towns | | | |
Microorganisms	Jos	Bauchi	Kano	Total
Enterococcus faecalis	6	8	21	35
Proteus mirabilis	6	8	4	18
Escherichia coli	4	7	5	16
Listeria monocytogenes	3	4	7	14
Staphylococcus aureus	3	4	6	13
Streptococcus agalactiae	4	8	-	12
Candida albicans	1	1	2	4
Streptococcus pyogenes	1	1	1	3
Total	28	41	46	115

Key:

⁻ represents no isolates.

Table 8. Occurrence of Microorganisms (Isolated from Placenta Swab and Blood) in the Age ranges of the Patients.

	Age Range (Years)						
Microorganisms	15-19	20-24	25-29	30-34	35-39	≥40	Total
Candida albicans	3	1	-	-	-	-	4
Escherichia coli	10	-	-	-	6	-	16
Listeria mono-cytogenes	-	4	3	7	-	-	14
Proteus mirabilis	-	-	-	-	-	18	18
Staphylococcus aureus	13	-	-	-	-	-	13
Streptococcus agalactiae	-	-	-	4	-	8	12
Enterococcus faecalis	-	-	-	14	-	21	35
Streptococcus pyogenes	3	-	-	-	-	-	3
Total	29	5	3	25	6	47	115

Key: - represents no isolates.

Serological investigation. Out of a total of 26 confirmed isolates of *L. monocytogenes*, (12 from CSF and 14 from placenta swab and blood samples), 18 were of serovar 4b, while 8 were 1a (Table 9).

White Blood Cell Count (CSF) Samples.

Two hundred and forty seven (247) CSF samples had WBC count greater than 5 cells / ml, while 53 had less than 5 cells / ml.

4.1.2 Statistical Analysis.

There was a significant difference in the means of the bacterial isolates from the CSF samples in Jos, Bauchi and Kano at $p > 0.05$ ($\chi^2 = 14.92$; $df = 2$).

pH of nono samples. The mean pH of nono sampled from Jos was 4.2727 ± 0.078, 4.395 ± 0.087 for Bauchi and 4.47 ± 0.078 for Kano.

4.1.3 Meteorological Data.

Mean temperature and relative humidity distribution.

A plot of the mean temperature showed that Jos experienced a mean temperature of 28.05^0C and 32.59^0C in the month of April 1998 and 1999 respectively while Bauchi and Kano recorded 42^0C each. The details are in Figure 2. Similarly, the mean relative humidity was low for the study towns in the months of January to March but experienced a steady rise from April to October and decline from November to December (Figure 3).

4.1.4 Outcome of Awareness Survey. All of the 100 medical doctors who participated in the awareness survey in the study towns had handled cases of meningitis, while 71 had handled cases of spontaneous abortion. Details of the data are in Table 10. Similarly, only 34 Laboratory scientists out of 100 knew about *Listeria monocytogenes*. None had isolated the organism routinely.

Table 9. Number and Percentage of Occurrence of Serovars of *Listeria monocytogenes* Associated with Meningitis and Spontaneous Abortion ases in the Study Towns (Jos, Bauchi and Kano).

	Towns			
Serovars	Jos	Bauchi	Kano	Total / Percentage
1a	1	2	5	(30.77)
4b	4	8	6	1(69.23)
Total	5	10	11	26 (100)
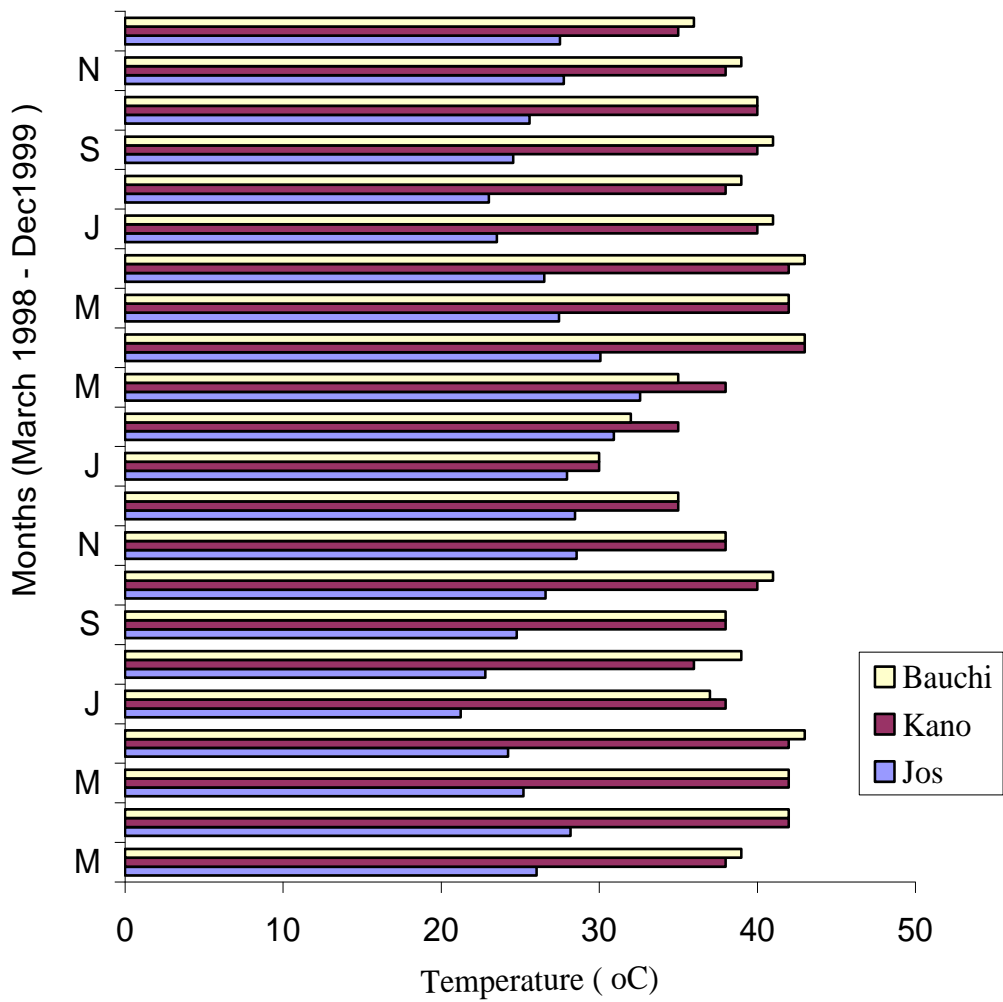
Figure 2. Mean Temperature (1998 to 1999) for Jos, Bauchi and Kano.
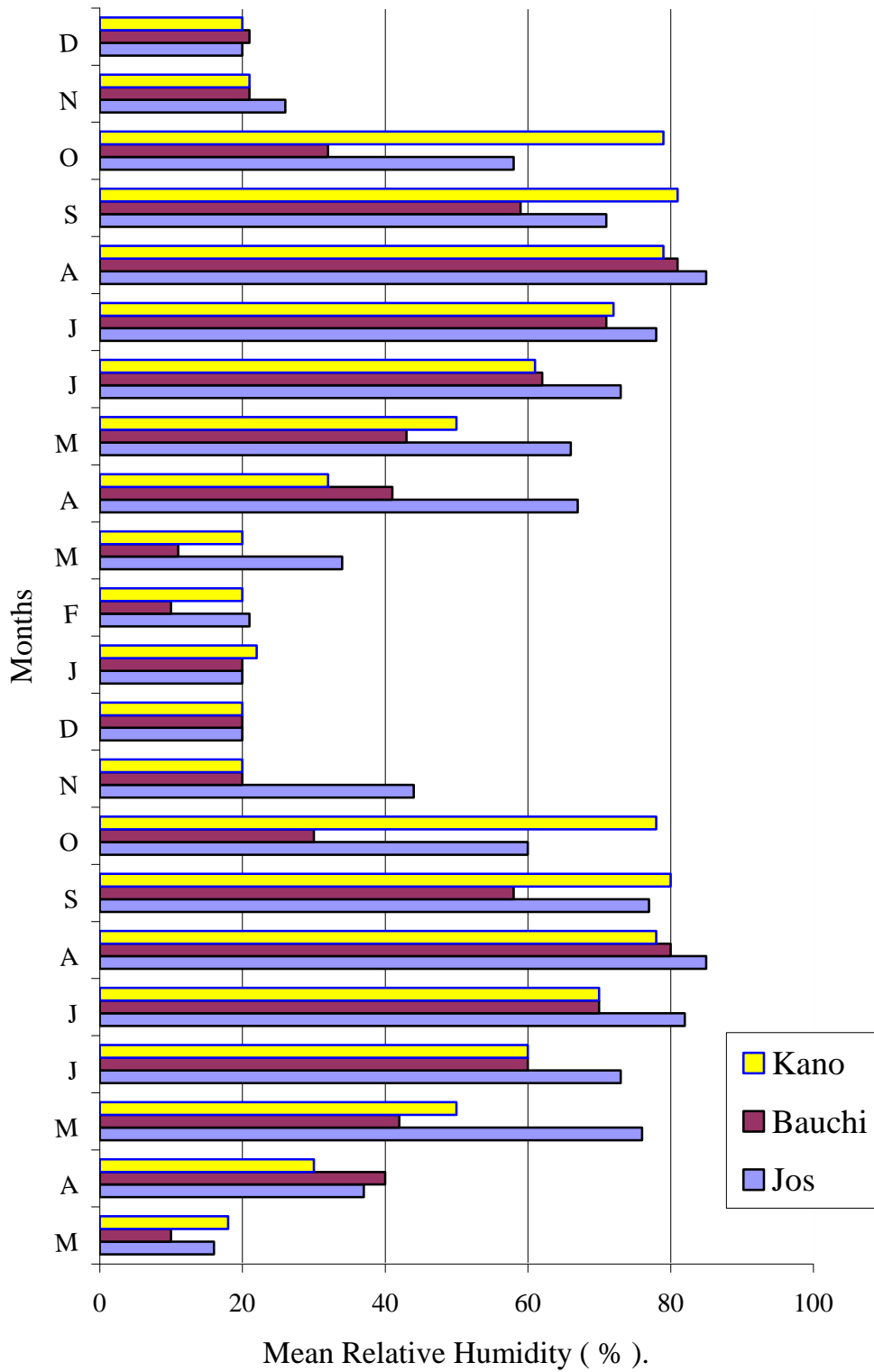
Figure 3. Mean Relative Humidity (March 1998 to December 1999) for Jos, Bauchi and Kano.

Table 10. Number of Sampled Doctors in Jos, Bauchi,and Kano who were Awareof *Listeria monocytogenes.*

S/no	Awareness category	Experimental Towns			
		Jos	Bauchi	Kano	Total
1.	Number that participated	38	30	32	100
2.	Number handled meningitis	38	30	32	100
3.	Number handled spontaneous abortion	25	18	28	71
4.	Number aware of *L. monocytogenes*	38	30	32	100

4.2 FORMULATED PLATING MEDIUM FOR *LISTERIA MONOCYTOGENES.*

There was a profuse growth of *L. monocytogenes* on brain heart infusion agar used as a control medium. Colistin selective agar (CSA) supported the growth of *L. monocytogenes* and most of the gram positive organisms. However, colistin – cefuroxime selective agar inhibited these organisms, except *L. monocytogenes* (Table 11).

4.2.1. Comparative Cost Analysis

The cost of current *Listeria* selective agar, which averaged N18, 000.00 per 500 g currently, may be prohibitive for most laboratories (Appendix 4). The formulated plating medium was estimated at about N6, 500.00 only per 500 g.

Table 11. Growth of *Listeria monocytogenes* and other Bacteria on Media Incorporating Combinations of Selective Agents.

Species of microorganisms	Growth Media		
	CSA[a]	CeSA	CceSA
Candida albicans	-	-	-
Escherichia coli	-	-	-
Klebsiella spp	-	+[b]	-
Listeria monocytogenes type 4b (SLCC2379)	++	++	++
Proteus mirabilis	+	-	-
Streptococcus agalactiae	++	-	-
Staphylococcus aureus	+	-	-
Enterococcus faecalis	+	-	-
Streptococcus pneumoniae	+	-	-
Streptococcus pyogenes	+	-	-

Key: [a]CSA, CeSA and CceSA are colistin, cefuroxime, and colistin – cefuroxime selective agar, respectively.[b]+++, ++, +, and - are profuse, moderate, scanty and no growth, respectively.

SUMMARY OF RESULTS.

1.   Bacterial meningitis occurred throughout the year.

2.   *Neisseria meningitidis* was the most prevalent pathogen associated with meningitis cases.

3.   *Streptococcus pneumoniae, Esherichia coli, Staphylococcus aureus* and *Listeria monocytogenes* were also isolated*.*

4.   *L. monocytogenes* types 4b and 1a were the prevalent serotypes.

5.   *S. agalactiae* and *L. monocytogenes* were also associated with spontaneous abortion in the study towns.

6.   A locally fermented cow milk product, "nono" was free from contamination by *L. monocytogenes*

7.   A combination of cefuroxime and colistin in Brain heart infusion broth base medium at pH 7.4 was selective for *Listeria.*

CHAPTER FIVE

DISCUSSION

5.1 SAMPLE COLLECTION.

The 300 CSF, 300 placenta swabs, 300 blood and 300 "nono" samples collected helped to give insight into the burden of bacterial meningitis and spontaneous abortion. For example, the CSF samples collected showed that there were more cases of meningitis between the months of March and May (Figure 1). This coincided with the peak period of meningococcal meningitis (Lapeyssonnei, 1963). Although samples were still collected for the other months, there was a decline towards the end of the year. This showed the endemic nature of bacterial meningitis (Ejembi et al.1998; Campagne et al.1999 and Greenwood, 1999).

5.2 EXAMINATION AND CHARACTERISATION OF ISOLATES.

The examination of cultures revealed that the bacterial pathogens isolated from the CSF and Placenta swab and blood, were significant, as the micro-organisms isolated stem from specimens taken from the patients. This agrees with one of Kock's postulates, that the organism should be grown artificially in pure form from the tissue (Potter et al., 1968). In addition, it met the case definition (Lwin et al., 1991). Cerebrospinal fluid is normally a sterile fluid. Any organism isolated in pure form in a case of an infection, is considered the cause of the disease condition. This was the situation with the CSF samples collected from patients in the study towns.

These organisms couldn't have been isolated without the use of more than one culture medium. This procedure enhanced the isolation of the causative organisms. It is suggested that hospital laboratories should not depend on a single culture medium for CSF or placenta swab, especially as these samples are

not easy to collect. In most cases, the volume of CSF sample is small (Cheesbrough, 1984). The combination of colony morphology, motility, Gram stain and carbohydrate fermentation reactions, along with the detection of catalase and coagulase enzymes, helped in characterising the various bacteria isolated (Cowan & Steel 1992). Record of such characteristics of each bacterium should be kept, as well as that of the isolate. This could help in epidemiologic studies, especially where similar bacterium was isolated.

A direct Gram stain was carried out on the CSF samples. Although this would not identify a specific bacterium, the presence of any Gram positive or Gram negative bacterium may give an idea of the causative agent. Consequently, prompt antibiotic treatment could be commenced, pending the outcome of cultural and antibiotic sensitivity tests, which can take 3 days or more. The rural areas would benefit more from this, especially as they may lack manpower and facilities in the laboratory.

5.2.1 **Isolates from CSF.**

The frequency of occurrence of *Neisseria meningitidis* was highest in all the meningitis positive cases. This agrees with the findings of Emele and Anyiwo (1994). These workers reported that *N. meningitidis* was the main causative organism in three consecutive epidemic out-breaks in Sokoto, Nigeria. A similar observation was made by Campagne et al. (1999) in Niamey, near Nigeria, and within the meningitis belt. *N. meningitidis* was the main organism responsible for the meningitis epidemic during the hot-dry season.

The World Health Organization (WHO) has sponsored researches in the study of this pathogen that have resulted in the administration of vaccines in order to prevent the spread of the disease (Greenwood, 1999). This is not

applicable to the other pathogens that are associated with the disease. The lack of detailed research information about these associated organisms has made the management of the meningitis disease difficult. The results show that the meningococcal meningitis causative organism and the associated organisms occur throughout the year and are therefore endemic. Thus, *N. meningitidis* may have attracted attention because of its ability to flare up to epidemic proportion in the hot-dry season, a period that favours its spread (Greenwood, 1987). Other endemic pathogens include *S. pneumoniae*, *E. coli*, *S. aureus* and *L. monocytogenes*. These pathogens were found to be associated with cases of meningitis, as they were isolated in pure form from the CSF samples. In the present study, *S. pneumoniae, E. coli, S. aureus* and *L. monocytogenes* were found to be the main organisms associated with (endemic nonepidemic) meningitis. However, the role of other species of organisms, such as *Enterococcus faecalis, Streptococcus pyogenes, Proteus mirabilis* and *Haemophilus influenzae* should not be overlooked. These microorganisms may have the potential of assuming epidemic proportions when the environmental conditions are right for meningitis disease development (Chiwuzie et al. 1995).

L. monocytogenes* was the fifth common endemic pathogen associated with 5.58% of the meningitis disease cases in the study towns. This observation is serious since the organism is intracellular and has the potential to claim the lives of mother and child (fetus), as well as that of non-pregnant adults as reported by Gerhard et al. (1993). Other endemic pathogens reported to be responsible for bacterial meningitis include *S. pyogenes*, *E. faecalis*, *H. influenzae* and *P. mirabilis*. These pathogens were associated with a total of 45.58% of the meningitis cases in the adults, children and neonates during the

present study. Stone (1990) reported that these pathogens are opportunistic organisms and, consequently, their control could be very vital in the management of meningitis. Furthermore, *S. pneumoniae*, *E. coli* and *S. aureus* are regularly associated with the disease and should be given similar attention as the traditionally accepted causative organism, *N. meningitidis*. These microorganisms are therefore of public health importance and should be treated as such. This would go a long way in controlling the disease.

Occurrence of meningitis by age group

A break down of the ages of the patients randomly sampled, gave a qualitative insight to the age group that were susceptible to meningitis. Neonates (≤1 month), children under two years and adults (≥30 years) were infected. Christophe, Sophie, Renaud and Claude (1999), also reported that age was a major risk factoring bacterial meningitis.

Meningococcal meningitis appeared to affect young children (5 - 9 years) more as reported by Bell, Brown, Halliday, McClure, and McCreid (1986). Infection by *Streptococcus pneumoniae* affected neonates, children and older adults. It was the leading non epidemic bacterial agent after *Neisseria meningitidis* (Akpede et al., 1994; Ozumba, 1995 and Heikki, 2001). This finding varied from that by Airede (1993) in which *Staphylococcus aureus* and *Klebsiella* spp were reported to be the common aetiologic pathogens in neonatal bacterial meningitis in the middle belt of Nigeria.

Escherichia coli, *Staphylococcus aureus* and *Listeria monocytogenes* were other leading bacterial pathogens. While *E. coli* affected patients below 14 years, S*. aureus* affected almost all patients. Infection by *L. monocytogenes* affected the two extremes of the age ranges (≤11months and ≥ 30 years). Compared to

Neisseria meningitidis and other pathogens, *L. monocytogenes* accounted for 5.58% of the cases. This is of importance as the organism has been reported to be intracellular. It has the potential to claim the lives of patients, in this case, patients who were ≤11months and ≥30 years (Gerhard et al*.* 1993). The impact of the other bacterial agents, such as *Streptococcus pyogenes, Haemophilus influenzae, Proteus mirabilis* and *Enterococcus faecalis* should not be over looked. Stone (1990) and Nicoll and Brown (1994) reported that these pathogens are opportunistic organisms and, consequently, their control could be very vital in the management of meningitis. Since the impact of life- threatening bacterial meningitis, especially in children across Africa, and Nigeria in particular is not quantified (Heikki, 2001), it has become imperative to advise on the combination of vaccines against the incriminated species of microorganisms (Mascola et al.1991; Blakebrough et al*.* 1998 and Greeenwood, 1999).

Distribution of meningitis pathogens in the study towns.

Neisseria meningitidis, Streptococcus pneumoniae, Escherichia coli, Staphylococcus aureus and *Listeria monocytogenes* were the five most frequently isolated pathogens in the three study towns. These pathogens, along with the others, occurred in all the study towns. This showed that the study towns share common bacterial agents of meningitis. Thus, control, treatment and vaccination, as well as epidemiological studies can be effectively coordinated.

White blood cell count.

White blood cell count (WBC) in CSF samples served as an early indicator of bacterial meningitis infection prior to the isolation of the causative pathogen. This study showed that 82.33% of the cases had WBC count >5 cells / ml, while 17.67% had WBC count <5 cells / ml. This finding confirmed earlier reports by

Onyemelukwe et al. (1983) and Bell et al. (1986) in which WBC counts >5 cells / ml were diagnostic of bacterial meningitis in CSF tested patients. White blood cells are dispatched by the body's immune system to engulf and destroy invading bacterial agents. Their presence in the CSF in increased number is diagnostic of infection (Weir 1973; Jurado et al. 1993; Shindang, 1998).

Meteorological data of the study towns.

Except for *N. meningitidis*, the seasonal distribution of the meningitis pathogens may not have been determined by the meteorological factors, essentially high temperatures and low relative humidity (Emele & Anyiwo, 1994; Campagne et al., 1990; Greenwood, 1990). Thus, it is possible that *L. monocytogenes* infection may not be associated with the seasons (Schlech et al., 1983; Smith & Archer, 1988).

5.2.2 Isolates from Placenta swabs and Blood.

Eight species of pathogenic bacteria were isolated from the cases of spontaneous abortions. As far as the medical records show, this is the first time attempts have been made to isolate the species of microorganisms associated spontaneous abortions in the study towns. The organisms included *E. faecalis*, *P. mirabilis*, *E. coli*, *L. monocytogenes*, *S. aureus*, *S. agalactiae*, *C. albicans and S. pyogenes.* Gerhard et al. (1993) and Claire et al. (1996) reported that the pathogenicity of *Listeria* is exhibited by its ability to penetrate and replicate in a number of cells of the body including phagocytic cells from where it escapes with the aid of its listeriolysin O antigen. Consequently, it infects other cells, thus promoting cell-to-cell spread Kocks et al. (1992). The general effect of this process is the colonization of the placenta leading to a painful contraction of the

uterus and dilation of the cervix that may eventually lead to abortion as reported in typical cases by Khong et al. (1986).

The study carried by Onyemelukwe et al. (1983) did not include blood and placenta samples of pregnant mothers in Zaria. These workers isolated *L. monocytogenes* from vaginal swabs of non-pregnant adults. There are no known records of isolates of *Listeria* from the blood and placenta of mothers in the experimental areas. The present study is therefore the first study that isolated *L. monocytogenes* from the placenta and blood of patients in the experimental areas. Khong et al. (1986) and Sirry et al. (1994) reported perinatal listeriosis and meningo-encephalitis caused by *L. monocytogenes* in pregnancies.

Although eight species of bacteria were isolated, only two species, *L. monocytogenes* and *Streptococcus agalactiae*, can be said to cause spontaneous abortion in the patients. These organisms met the criteria, as they were isolated from blood and placenta (Jensen & Andersen, 1979; Shiers, 2001 and Porent, 2005). The role of the other bacterial pathogens in spontaneous abortion is in doubt as they were isolated from placenta swab only (Dashner, 1997).

Occurrence of spontaneous abortion pathogens in age groups.

A view of the age groups affected showed that *L. monocytogenes* and *S. agalactiae* affected patients in the age group, 20-24, 25-29, 30-34 and ≥ 40 years, respectively. The age range 30-34years suffered more from spontaneous abortion caused by both *L. monocytogenes* and *S. agalactiae*.

5.2.3 Isolates from Nono Samples.

Two species of bacteria, *Enterococcus faecalis* and *Streptococcus agalactiae,* were isolated from the "nono" samples. *Listeria monocytogenes* was not isolated.

The methods of isolation used such as cold enrichment procedure in a nutrient broth would have assisted any "injured" listerial cell present to grow. The isolation of these Streptococcal species agrees with the findings by Benkerroum et al. (2002) in which starter culture used for fermentation of yogurt were also Streptococcal species - non medical importance. They were also reported to produce bacteriocins that suppress the growth of listerial cells. It is therefore possible that the presence of *E. faecalis* and *S. agalactiae* in nono suppressed the growth of *L. monocytogenes* (Akpavie & Ikheloa, 1992; Barbuddhe et al. 1994; Meydari & Woel-Kyu 2000).

Though "nono" may appear safe from *Listeria*, it may not be free from contamination by *E. faecalis* - faecal marker of water pollution, and *S. agalactiae* - shed in milk in cases of mastitis in cattle. This perhaps may explain why listeriosis is not a common disease recognized by health workers, like malaria or gastroenteritis in the study areas.

5.2.4 Awareness survey.

The awareness survey conducted amongst medical officers and hospital bacteriologists on *L. monocytogenes* showed that the awareness level of this pathogen was low. Riviera et al. (1993) reported that this disease is rare and may not be noticeable unless there is an outbreak. Sporadic cases can only be noticed where there is liaison between the clinician and the hospital bacteriologists (Khong et al. 1986). This would help in creating awareness on listeriosis.

5. 2. 5 Serological Analyses.

Serological investigation on the *L. monocytogenes* isolates revealed that serovars 1a and 4b were responsible for the cases of *L. monocytogenes*

meningitis and spontaneous abortion in the study towns. The results obtained confirm the earlier findings of Onyemelukwe et al. (1983), Sirry et al. (1994) and Elcuaz et al. (1996). The occurrence of types 1a and 4b of *L. monocytogenes* in the study towns suggests the pathogenic potential of the said strains of microorganisms (Ozturk, 1990). These findings reveal also the significance of the geographical distribution of these serovars. This would help in detecting the sources of listeriosis epidemic as well as in the development of appropriate control measures.

Serovar 4b had a higher incidence of occurrence than 1a (Bortolussi et al.1984; Hof, 1984; McLauchlin, 1990 and Michal et al. 2004). Bauchi had the highest incidence of 4b, while Kano had the highest incidence of 1a. Consequently, a data bank of these serovars, if maintained, can be used to trace listeriosis outbreaks.

5.2.6 Statistical Analysis.

The bacterial organisms isolated occurred in all the study towns. This suggests that the organisms are endemic. There was a significant difference in the distribution of the isolated meningitis pathogens in Jos, Bauchi and Kano at $p > 0.05$ ($\chi^2 = 14.92$, df = 2). This will help in epidemiological studies (Zaki et al., 1990; Ejembi et al., 1998 and Campagne et al., 1999) of listeriosis in general and meningitis in particular. Furthermore, it would also help in the development of appropriate vaccines.

The pH of nono sampled in the study towns showed that it was acidic. This finding agreed with that by Shehu and Lamido (1994). In addition, Siraqusa and Johnson (1988) reported the suppression of growth of *L. monocytogenes* at low

and high inoculum levels at pH 4.1 by direct plating. This may probably explain why *Listeria* was not isolated.

5.2.7 Meteorological data.

Temperature and relative humidity play a role in the spread of the meningitis pathogens. These factors contribute to conditions leading to susceptibility of infection especially with *N. meningitidis* as reported by Emele and Anyiwo (1994). Low mean temperatures recorded for Jos may explain the comparatively low occurrence of *N. meningitidis. L. monocytogenes* is not reported to be spread through the droplet route. Thus temperature and relative humidity in the study towns may not affect person to person spread of listeriosis (Lapeyssonnei, 1963; MacCowan et al., 1994).

5.3 FORMULATED *L. MONOCYTOGENES* MEDIUM.

The result of the action of colistin and cefuroxime on the microorganisms is important. All the gram-negative, as well as the gram-positive organisms challenged, except *L. monocytogenes*, were inhibited. This finding agrees with that by Golden et al. (1990) in which direct plating procedures without prior enrichment can be successfully used to isolate *L. monocytogenes* from samples with large background microflora..

The problem bacteriologists' face in isolating *Listeria* is the presence of contaminating Gram -positive and Gram-negative bacteria, as well as that of fungi. These organisms are present in food, stool and placenta material, among others. The tiny nature of the colonies may cause their being mistaken as contaminants (Kokubo et al., 1990). These factors, along with the high cost of *Listeria* broth and solid media make routine isolation of *Listeria* rather expensive (Smith & Archar, 1988). Further more, Brain heart infusion agar used as basal

medium for the formulation of the *Listeria* medium in this study contained glucose. This is considered the best energy source for the growth of *Listeria* (Swaminathan et al., 1988; Lachica, 1990). Most published formulation on plating media does not contain added carbohydrate utilizable by *Listeria.* Furthermore, the cephalosporins are tolerated by *Listeria* at the concentrations used (Curtis & Lee, 1995).

Conclusion.

The picture of the involvement of *L. monocytogenes* and other microbes in spinal meningitis, spontaneous abortion and food contamination has been presented. *L. monocytogenes* has been incriminated by this study to be associated with bacterial meningitis and spontaneous abortion. Other species of microorganisms, such as *N. meningitidis, S. pneumoniae, E. coli* and *S. aureus*, were associated with the infection. *S. agalactiae* was associated with cases of spontaneous abortion in women in the study towns. It was observed that nono, a widely consumed fermented cow milk product, was free from *Listeria* contamination, but could be contaminated by other species of pathogenic organisms, such as *E. faecalis* and *S. agalactiae* which were also associated with some cases of meningitis.

The isolation of serovars 4b and 1a of *L. monocytogenes* suggests that these strains of organisms could be responsible for the cases of listeriosis in the study towns. This agrees with the findings of Bortolussi et al. (1984) and Hof, (1984) who reported the preference of *L. monocytogenes* serovar 4b for the cells of the central nervous system and placenta. The cost of the available commercially prepared *Listeria* enrichment broth and *Listeria* selective agar may be prohibitive for most hospital laboratories. Consequently, the use of a simple

Brain heart infusion agar that contains colistin and Cefuroxime with a pH of 7.4 may be more cost effective.

CONTRIBUTION TO KNOWLEDGE

a. This study has shown that *Listeria monocytogenes* is associated with cases of bacterial meningitis and spontaneous abortion in the study towns.

b. The study has also shown that serovars 4b and 1a of *L. monocytogenes* are present in the test cities and may be responsible for some of the meningitis and spontaneous abortion infections.

c. The study has also shown that *S. pneumoniae, E. coli*, *S. aureus, E. faecalis*, *P. mirabilis* and *S. pyogenes* are endemic in the study towns.

d. *L. monocytogenes* and *Streptococcus agalactiae* were associated with cases of spontaneous abortion.

e. Judging from the results obtained from the present study, it appears that nono is safe from *Listeria* colonization, perhaps due to its pH value.

f. A medium containing colistin (1 mg / 100 ml) and cefuroxime (2 mg / 100 ml) in brain heart infusion agar favoured the growth of *L. monocytogenes* and inhibited the growth of contaminants.

RECOMMENDATIONS

a. A more detailed survey should be carried out on the roles of micro organisms associated with listeriosis in Nigeria and, indeed, West Africa. This will help identify the species and serovars of microbes that cause meningitis.

b. A capsular vaccine combination against *Streptococcus pneumoniae, Escherichia coli,* Staphylococcus *aureus* and *Listeria monocytogenes* should be included in the current immunization drive against meningitis in northern Nigeria.

c. Better and faster diagnostic facilities should be introduced in the Nigerian health facilities in order to enhance the diagnosis of the infection.

d. A disease surveillance centre should be set up in the Health Ministry of each northern Nigerian state in order to help the monitoring of the trend of this infection.

e. Areas for further studies should include host /*Listeria* spp interractions and infection preference of the pathogen for the central nervous system and placenta.

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Appendix A1. Consent Form Signed by Placenta Swab and Blood Samples Donors.

S/no.	Conditionality
1.	We are carrying out a study on a bacterium called *Listeria monocytogenes*. The disease it causes is generally called listeriosis.
2.	The disease affects adults, children and pregnant women. It is characterized by meningitis and premature abortion (miscarriage). The danger with this disease is that it is not common but when it infects people, it can lead to an epidemic where many infected people may die. Many people may have died or lost their pregnancies as a result of infection by *Listeria*. If it is detected on time, it could be cured. Your present condition may have been caused by this bacterium and we need to carry out a test in order to diagnose it. To do this, we would like to use a sterile swab to collect a sample of the aborted placenta.
3.	This will not hurt you nor would it cost you anything. No one will be informed of the result except your doctor. We will therefore need your consent.
3.	I agree that JOHN SHINDANG of University of Jos test portion of my aborted placenta in order to isolate *Listeria monocytogenes.*

…………………………….. ……………..
Name of Patient / Sign Date

Appendix A2. Questionnaire Distributed to 100 Medical Doctors in the study towns of Jos, Bauchi and Kano on the Role of *Listeria monocytogenes* in Meningitis and Spontaneous Abortion.

S / n	Category

1. Serial No:

2. Profession:

3. Have you ever handled these medical cases? (Tick):

   Meningitis [   ]   Spontaneous abortion [   ]

4. Have you ever heard of *Listeria monocytogenenes*?

   Yes   [   ]   No   [   ]

5. If yes, have you ever requested or diagnosed a case of *Listeria monocytogenes* infection?

   Yes   [   ]   No   [   ]

6. Has the laboratory in your hospital isolated *Listeria monocytogenes*?

   Yes   [   ]   No   [   ]

7. If yes, how many times?

8. What age group is commonly affected?

   Meningitis   [   ]   Spontaneous abortion   [   ]

Appendix A3. Questionnaire Distributed to 100 Medical Laboratory Scientists in the Study Towns of Jos, Bauchi and Kano on the Role of *Listeria monocytogenes* in Meningitis and Spontaneous Abortion.

S / n   **Category**

1. How many of the under mentioned cases have you carried out Lab diagnosis on last few years? (Tick):

   Meningitis [   ]   Spontaneous abortion [   ]

2. Commonest bacteria isolated (specify)………………………………..

   …………………………………………………………………………

   …………………………………………………………………………

   …………………………………………………………………………

3. Have you ever isolated *L. monocytogenes* from:

   a. CSF [   ]   b. Placenta swab   [   ]

   c. Blood [   ]

4. a. Is *L. monocytogenes* commonly isolated routinely in your Lab?
   If no, give reasons.

   ………………………………………………………………………

   ………………………………………………………………………

   ………………………………………………………………………

   b.   How did you confirm that what you isolated was *L. monocytogenes?*

   ………………………………………………………………………

   ………………………………………………………………………

5. What problems of isolation of *L. monocytogenes* did you experience?

   ………………………………………………………………………

   ………………………………………………………………………

Appendix A 4. Comparative Cost Analysis of some Agar and Antibiotics.

S / no.	Agar and Antibiotics	Quantity	Cost (naira)
1.	PALCAM agar	1 x 500 g	20 000.00
2.	Oxford listeria agar	1 x 500 g	18 000.00
3.	Listeria enrichment broth	1 x 500 g	15 000.00
4.	Formulated listeria selective agar:		
	a.BHI agar	1 X 500 g	4500.00
	b. Colistin	1 g vial	800.00
	c. Cefuroxime	750 mg vial	1 200.00

Source: Medicom, Murtala Mohammed way, Jos.