STUDIES ON THE PREVALENCE OF SALMONELLAE IN FRESH WATER FISHES IN SOME PARTS OF NORTHERN NIGERIA

This thesis was submitted to the post graduate school as part fulfilment of the requirements for the award of a Master of Science degree

BY

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THESIS APPROVAL

This thesis by Ediga B. Agbo meets the regulations governing the degree of Master of Science of Ahmadu Bello University and is recommended for acceptance in partial fulfilment of the requirements for the degree.

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DECLARATION

I hereby declare that the work presented in this thesis was carried out by me under the careful supervision of Doctors A. A. Ahmad and J. Adekeye in the Laboratory of the Department of Microbiology, Ahmadu Bello University.

No part of this work has been submitted for any degree or qualification before.

The works of other workers were referred to during this work and they are duly acknowledged.

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CERTIFICATION

This thesis entitled "Studies on the prevalence of Salmonellae in fresh water fishes from some parts of Northern Nigeria" meets the regulation governing the degree of Master of Science Ahmadu Bello University Zaria and is approved for its scientific knowledge and literary presentation.

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Dedicated to my father Mr. Boniface Adeka Agbo.

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A B S T R A C T

The intestinal contents of a total of 500 fishes were examined for the prevalence of Salmonella. Six different types of fish were examined. These are (with local names in brackets) Tilapia sp (Karfasa), Alestes sp (Jan Wutsiya), Clarias sp (Tarwada), Synodontis sp (Kurungu), Labeo sp (Dubi) and Lates sp (Giwan ruwa). Samples were purchased from four towns in Northern Nigeria viz:- Kano, Kaduna, Funtua and Zaria.

Four (0.8%) of the fishes gave positive isolates of the organism and these were identified as S. tennesse, S. middlsbrough and a new serotype S. maska. Three of the isolates were from Tilapia sp while the fourth was from Synodontis sp. No other species was positive for the organism. None of the fishes sampled from Zaria was positive for Salmonella whereas two from Funtua, one from Kano and one from Kaduna were positive for it.

The isolates were susceptible to 10 of 11 antibiotics tested for and resistant to 1.

Infective dose $_{50}(IF_{50})$ for infection of fishes with Salmonella was found to be 30 organisms/Ml. Five fishes infected with Salmonella retained the organism after refrigeration at $4^{o}C$ for three weeks and only two out of fifteen stored at - $3^{o}C$ were negative for the organism after four weeks. Live fishes infected with Salmonella organism rapidly lost it and by the 24th day after infection were completely negative for the organism.

CHAPTER ONE

INTRODUCTION

Fish is an important part of our food as a source of protein and is consumed by a large population of people all over the world for its nutritional importance. Fish is consumed in various forms such as smoked fish, dried or fresh fish which is either cooked or fried before consumption.

The consumption of fish has increased around the world and this is largely due to increased population and the consequent high demand for fish which is a source of good protein and for its highly vital source of unsaturated oils.

Fishes around the country are usually caught in rivers and other water bodies which receive a lot of pollutants from human and animal waste materials. Some fish ponds are also fertilised with chicken droppings to promote the growth of planktons which are consequently fed upon by the fish.

Fishes being filter feeders, the type of organisms present on them and within their guts is determined by their environment (Wyatt et al., 1979; Bryan, 1980).

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The aim of this study has been to examine the potentials of fresh fish as a possible vehicle for the transmission of Salmonella organisms. A quantitative enumeration has not been carried out because the presence of just one Salmonella organism is enough to initiate human infection and as contended by the U.S. food and drug administration, any food contaminated with the organism is adulterated under the terms of the food, drug and cosmetic act (Bruch, 1968).

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CHAPTER TWO

REVIEW OF LITERATURE

2.1 THE GENUS SALMONELLA

Cultural Characteristics and Microscopy

As described by LeMinor and Rhode (1974) the genus consists of small gram negative rods, non-sporulating and motile by means of peritrichous flagellation. Non-motile mutants may occur such as S. gallinarum and S. pullorum which are always non-motile.

Colonies are generally 2-4 mm in diameter but aberrant strains exist (S. abortus-equi, S. typhi-suis and S. abortus-ovis) which produce colonies only 1 mm in diameter. Most strains grow on defined media with no need for growth factors.

The Salmonella subcommittee of the international society of microbiologists gives the generally accepted description of Salmonella as:-

"A large genus of serologically related gram negative, non-sporing rods 0.4 - 0.6 x 1.3 microns in usual dimensions, but variants may show short filaments. With certain exceptions, there is motility by peritrichous flagellation. They are aerobic and will give negative results for the following tests - fermentation of sucrose,

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lactose and dulcitol, gelatine liquefaction and production of indole or the urease enzyme. No acetyl-methyl carbinol is formed. Glucose is utilised with or without gas formation. Citrate is utilised and in triple sugar iron agar gives alkaline slant, acid butt and hydrogen sulphide."

Some rare species may not conform to all the biochemical tests.

Salmonella is indistinguishable from E. coli under the microscope or in ordinary nutrient media. But this is achieved by serology and biochemical tests. Based on biochemical tests, Kauffman (1960, 1963, 1964) made sub-divisions into subgenera which were later confirmed by Rhode (1965, 1966, 1967).

Distribution

Salmonella are widely distributed geographically with man and animals being the primary reservoirs (Smith, 1967). All species of Salmonella may be presumed pathogenic for man, animals or both. The best known species so far is S. typhi which causes typhoid fever. Its serological and biochemical difference makes a seperate description for it necessary. Citrate is not used by S. typhi and sugar fermentation is not accompanied by gas formation. Other serotypes such as S. paratyphi A also produce such variant strains (Kauffman, 1963).

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Salmonella have no respect for international boundaries and are found virtually everywhere in the world. Some species or strains are host specific while it has been demonstrated that the non-host specific types can in fact b e haboured and diss eminated by animals not normally parasitised by them (Christie, 1969, Lawson et al 1974). The potential danger of Salmonella to public health and the attendant slow down in the economy is highlighted by the ubiquity of the organism, its lack of host specificity and the abundance of reservoirs (Morse, 1974).

Poultry are the major carriers. Other well known carriers are pigs and cattle. The animals need not be sickly to be a carrier and foods from them are the major vehicles of transmission (Olayemi, 1978, Kwaga 1984, Edel et al 1977).

There is the high need for a constant survey of incidence of Salmonella among livestock and this is substaintiated by the following reasons:-

(i) There is the increase in the isolation of the so called exotic strains on which evidence already exists that they can be transferred from feeds through livestock and their products to man.

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(ii) There is also the danger that a new serotype which has hitherto been undiscovered can suddenly become endemic in an area and cause a serious problem (Clark et al 1973).

(iii) Studies of isolations of Salmonella from different sources are important for monitoring the spread of Salmonella serotypes. This argument is vital to the success of any public health programme.

Discovery

After studying epidemiological evidence available, Budd (1956) suggested the infectious nature and transmission of typhoid fever. The typhoid germ was first seen by Eberth in 1880 and the first isolation was by Gaffrey in 1884 (Burrow 1973). The first isolation of a non-specific Salmonella was by Salmon and Smith (1885) when they isolated Salmonella cholera suis from swine afflicted with clinical hog cholera (Gutzman et al 1976). Other isolates were subsequently recovered from various sources, S. enteritidis from infected beef incriminated in an outbreak of gastroenteritis (Gaertner, 1888), S. typhimurium from mice (Loefler 1892) and S. typhimurium from man and animal flesh suspected

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of transmitting the organisms (Ewing and Ball, 1966; Burrows, 1973; Difco Manual 1976).

2.2 CLASSIFICATION

Classification of Salmonella has been quite confusing with the main organisms not following the usual rules of nomenclature. The confusion is not helped by the fact that members are closely related biochemically and morphologically. No general agreement exists as regards taxonomy of Salmonella and most workers prefer to regard serotypes as sub-specific entities.

Various methods of naming have been advocated but only two have come close to universal acceptance viz:- the Kauffman - White Scheme (Taylor, 1967) and Ewings proposal (Ewing, 1966). White carried out extensive studies on the serotyping of Salmonella organisms. This was later extended by Kauffman who published the Kauffman - White scheme (Kauffman 1975). This scheme was favoured by the International Society of Microbiologists and accords a specific status to each antigenically distinguishable Salmonella type. Kauffman made subdivisions into four sub-genera 1 - IV (Kauffman 1975).

In his proposal, Ewing (1966) recognised only three species viz:-

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- S. typhi - the type species
- S. choloerasuis

S. enteritidis to which all other serotypes of Salmonella belong except the first two above.

However, the method which has received wider acceptance so far is naming the organisms after the places from which they were isolated.

Antigens of Salmonella

Salmonella has several antigens but the ones of importance in classification of the organism are the Flagella (H) antigen, the somatic (O) antigen and the capsular (Vi) antigen.

(a) The O antigen has a phospho-lipid-polysaccharide complex and has a specificity which depends on the nature of the terminal groups. It is resistant to alcohol or acid (Kauffman, 1975; Buxton and Frazer, 1977).

(b) H antigen is a heat-labile protein complex which has a specificity determined by the amino acid content and the order in which they occur.

(c) The K antigen is a group of antigens comprising the Vi and two others '5' and 'M') which have been described by other workers (Edwards and Ewing, 1972; Poelma and Silliker, 1976). K antigens are either envelope or capsular antigens

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made of polysaccharides. They have the capacity to completely mask the O antigens from the action of homologous antisera, a situation which is removed by heating the cell suspension. This action is the effect of the Vi antigen and it has been suggested that it has some protective effect against phagocytosis of the cell. The antigen is non-toxic on injection and bears no relationship to the pathogenicity of the organism (Wilson and Miles, 1956).

Other antigens which have been described for Salmonella are the 'Fimbral', 'X' and T antigens (Edwards and Ewing, 1972; Stewart, 1968; Buxton and Frazer, 1977). The 'X' antigen is thermostable and is responsible for cross agglutination between certain unrelated Salmonella serotypes (Edwards and Ewing, 1972). The 'Fimbral' antigen has the same effect the Vi antigen exerts on the O antigen (Kauffman, 1975).

The Kauffman - White Scheme

In this scheme, the organisms are identified by the numbers and letters given to the different O, H and Vi antigens possessed by the organism. The representation only shows those antigenic properties of primary diagnostic importance and is not a complete record of the antigenic complexity of Salmonella (Kauffman, 1966).

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There are two forms of flagellar (H) antigens viz:

(a) Specific phase (Phase 1) possessed by only a few species and

(b) Group Phase (Phase 2) possessed by a wider group of the organism.

The Kauffman White scheme for naming is made up of the three parts showing the O, Phase 1 and Phase 2 antigens respectively. Each part is separated by a colon and the component s of each part by commas. Parenthesis are used to denote incomplete antigens while antigens which may or ma y not be present are enclosed in brackets. A dash is usually substituted for a missing antigen.

In designating the antigens, arabic numerals are used for the O antigens numbering them 1 to 67 with a few missing. Phase 1 H antigens are denoted with roman numerals a - z except J and beyond z, we have Z_1, Z_2, Z_3 etc. Phase 2 H antigens are denoted with arabic numerals 1, 2, 3 . . . . . .

Antigenic Variations

For better practical work and interpretation of results, it is important to understand the variations in the antigenic structure of Salmonella (Stewart 1968; Kauffman 1975). A few types of variations have been described by various authors (Wilson and Miles, 1955;Le Minor and Rhode, 1974; Burrows, 1973;

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Kauffman, 1975; Edwards and Galton 1967; Stewart, 1968).

(1) Phase Variation:- This is a fluctuating, completely reversible immunological variation. In this, the H antigens can be separated into relatively stable components. The variation is a qualitative change of the flagella antigen, a saltative oscillation of the antigenic complex between two serologically contrasting states showing a kind of resonance which consists of alternate expression of one or the other of these two genes between the loci of Phase 1 and Phase 2. Three types of Phase variations have been described viz:-

1. (a) Specific - nonspecific phase variation of Andrews

   (b) α - β Phase variation of Kauffman and Mitsui

   (c) Phase variation of Edwards and Brunner.

2. Form or O antigen development caused by absence or poor development of O antigen components.

3. VW or Vi antigenic variation involving less of Vi antigen.

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4. S-R antigenic variation which is the loss of O antigen that confers smoothness to the colonies. This is manifested by a change in colonial appearance from smooth to rough forms. Loss of virulence and autoagglutination in saline solution accompanies this variation.
5. OH - O variation:- In this, the organism loses the flagella irreversibly and along with it, the antigens. There is an attendant complete or incomplete loss of motility.
6. M - N variation: Alternation between the mucoid and non-mucoid form.

Genetic changes can also lead to antigenic variation. Variations mediated by bacteriophages such as transduction or lysogenic conversion also occur. Each lysogenised strain undergoes antigenic change. These changes are brought about by the action of phages on the cell's genetic structures. Other variations mentioned are transduction, conjugation and those involving the T and 'X' antigens.

Serotyping

This is the serological identification of Salmonella strains by the use of antisera against appropriate antigens.

In serotyping Salmonella, the initial test is to identify the somatic (o) antigens with the use of

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polyvalent antisera against the main O groups. The H antigens are then identified using tube agglutination techniques.

There are three stages to the serological identification of Salmonella (Edwards and Ewing, 1972; Ewing, 1969; Ewing 1972; Ewing and Martin 1974).

The first stage involves complete identification of certain important species and serotypes of Salmonella. These are:-

S. typhi

S. enteritidis ser. paratyphi A

S. enteritidis ser. Paratyphi B

S. cholera - suis

S. enteritidis Ser typhimurium.

Only a few antisera are required for this first stage. These are:- a polyvalent antiserum against A through E inclusive (serogroup E agglutinates all members of the E complex), antisera for flagella antigens a, b, c, d, i and 1 complex, single factor H antisera for factors 2 and 5 and Vi antiserum produced with Citrobacter, 029 Vi antigen (Ewing, 1972).

The second stage is intermediate and helps in the complete identification of the 50 or 60 commonly occuring serotypes of S. enteritidis including bio-serotype paratyphi A. The species S. cholera suis and S. typhi are identified completely (Edwards, 1962;

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Ewing, 1966, 1969; Martins and Ewing, 1969). Fifty one unabsorbed and thirty two absorbed antisera are required for stage 2. These antisera also allow for partial characterisation of serotypes not listed along with the 50 or 60. Stage two may be modified by the addition of antisera for complete serotyping of particular strains that may become epidemic or endemic in an area or serotypes that are of special interest.

The third stage involves complete identification of all known serotypes of Salmonella including new serotypes. This requires the use of a vast number of antisera and is best restricted to a few laboratories which are best equipped for this purpose and may serve as reference centres. Emphasis should be given to complete identification of Salmonella isolates regardless of the stage of serological identification adopted (Ewing, 1972).

Phage Typing

This is among several methods now used to classify, differentiate and identify Salmonella. It was first developed for Salmonella cholera suis but has now been extended to other serotypes such as S. typhimurium, S. paratyphi A, B and C, S. dublin, S. enteritidis and S. gallinarum (Wilson and Miles 1955; Stewart, 1968; Edward and Galton, 1967; Bryan et al 1979). Phage typing depends on the sensitivity of cultures to a

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series of bacteriophages at appropriate dilutions. Phage typing of S. typhi and other Salmonellae which have the Vi antigen are based on a series of adapted phages from phage Vi II of Cragie and Yen (1938) S. paratyphi B (Felix and Callow, 1943) and S. typhimurium (Anderson et al 1977). Phage typing employs a series of phages and analogous methods have been developed for other Salmonellae some of them making use of the lysogenicity of the strains. However, not all strains of Salmonella are typable by bacteriophages (Wilson and Miles 1955).

2.3 SALMONELLA IN FEEDS

Animal feeds have been identified as a major source of Salmonella infection to animals (Kwaga, 1984). This can aid the creation of the healthy carrier state in animals and also contribute to contamination of foods of animal origin. Since contamination of feeds originates primarily as a result of the incorporation of items of animal origin such as bone meal, meat scraps and fish meal (Edwards and Galton, 1967; Rosted et al, 1975; Bryan, 1981) it is important to consider the high risk of contamination of fish ponds using chicken droppings as fertilizer since poultry have been recognised as a major reservoir of Salmonella (Edel et al, 1977).

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2.4 SALMONELLA AND THE ENVIRONMENT

One of the striking features of Salmonella is the fact that the organism has succeeded in adapting itself to a wide range of hosts. In favourable conditions outside the host, they can survive for a considerable length of time and resume multiplication as soon as conditions become amenable. Acording to Morse (1974), environmental factors exert a profound effect on the occurrence, epidemiology, diagnosis, course, prevention, therapy and control of Salmonellosis. The organism is excreted in feces of man and animals and through this, can readily contaminate foods, animal feeds, water and water ways (Bowmer, 1964). It is pertinent to note here that sewage waters draining into water channels pose a high risk as vehicle of transmission of Salmonella organisms.

Cherry et al (1975) suggested that Salmonella may exhibit a free living state, but this information is inconclusive Factors affecting the growth and destruction of Salmonella can be reviewed briefly here. Like all other Gram negative rods, Salmonella will grow on a wide range of culture media producing visible colonies within 24 hours. PH optimum is around neutrality with the range falling between 4.0 and 9.0. They grow best at PH 6.6 - 8.2 (Diesch, 1978). The temperature range is between 5.3 centigrade and 45

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centigrade. The optimum is 37 centigrade. Salmonella will not grow below a water activity (aw) of 9.4 at neutral PH. Also, they will not survive in an environment of high salt concentration.

2.5 SALMONELLA IN FISHES

Much work has not been carried out on the prevalence of Salmonella in fresh water fishes. In fact, materials documented in literature to that effect are very rare. In view of this, Betty Hobbs (1954) suggested that fresh fish could be regarded as one of the safest articles of our dietary. This suggestion was later found to be based on the fact that fresh fishes sold in the U.K. were caught from unpolluted river waters. Evidence contrary to this first appeared when an outbreak of Salmonella infections was traced to direct contact with contaminated fresh fish. The source of contamination was however not acertained (Floyd and Jones, 1954).

Salmonella has been reported in fresh and marine species of fresh fishes (Andrews et al, 1977; Arcisz, 1950; Floyd and Jones, 1954; Cibbons, 1934; Cohar, 1934; Gulasekharan et al 1956) and this is generally associated with fecal contamination of the water (Wyatt et al 1980). In fact, research has shown that the presence of Salmonella in live fish is directly related to the level of Salmonella in water (Wyatt

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et al, 1979) and the level of Salmonella in water depends on cases of salmonellosis in the population with the attendant discharge of the organism into water or sewage which eventually pollute the water (Bryan, 1980). Fishes being filter feeders, they usually acquire Salmonella from polluted water, but sometimes they are introduced onto the body surface by handlers.

Filter feeders accumulate microbes from their environment in their guts. These are not necessarily killed or digested but remain viable within the gut (Rowse and Fleet, 1983).

Salmonella are common in animal feces and wastes from slaughter-houses and poultry processing plants. These organisms sometimes reach fresh waters from which fishes are caught. Fishes acquire Salmonella while in polluted water or consequently during storage after being caught (Bryan, 1980).

Although much work has not been documented on the prevalence of Salmonella in fishes, it is important to note here that improper storage of fish after being caught could aid the growth of Salmonella to populations that could cause disease or contaminate processing surfaces, kitchen utensils and the hands of handlers. The few cases of isolation of Salmonella from fish available heightens this observation.

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Floyd and Jones (1954) isolated several Salmonella and Shigella organisms from fresh fishes sold in a Cairo market.

There has been a few reported cases of Salmonellosis associated with fish and shell-fish (CDC, 1971, 1972, 1974, 1976) and these do not accurately reflect the importance of Salmonella in these commodities (D'Aoust et al, 1980). The importance of fish and shell-fish as vehicles of human infections and the ability of pathogens to survive long periods of storage in frozen shell-fish products is well documented (Brown and Dorn, 1977; Raj and Liston, 1961).

Routes of transmission of Salmonella within and between groups of mammals, birds and reptiles have been established (McArdle, 1968) but as yet, no species has been reported to be pothogenic to fishes (Shewan, 1962). In spite of this, the possibility of the presence of Salmonella in fish continues to receive attention and the isolation of coliforms from fish only increases the belief that Salmonella could be present. High levels of coliforms have been reported in fresh fishes from Kenya (Binta et al 1982). It was observed that this poses a high risk to public health in view of the consumption of raw or undercooked fish. S. typhi has been isolated from raw fish in Canada

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(Gibbons, 1934). Conclusive evidence of the survival of Salmonella in fresh fishes were reported when studies showed that fish could elicit humoral responses to the presence of Salmonella organisms (Baker and Smitherman, 1983). The investigators observed an elevation of serum antibody of the fish Tilapia aurea against Salmonella typhimurium in a fish pond. However, there was no suggestion that the Salmonella was pathogenic to the fish. The investigators also reported that 95% of Salmonella taken into the guts were lost through gradual movements of the gut contents within 16 days and the organisms completely disappeared from the fish within 32 days.

The presence of Salmonella in raw foods such as catfish, red meats, poultry and frog legs is of concern because of the potential of cross contamination of foods consumed raw and/or its survival in food that receives marginal heat treatment (Wyatt et al, 1980). As an example the cutting surfaces and kitchen utensils used in cutting fish could easily cross contaminate ingredients for salad in the kitchen. Over 48% of finished products have been shown to be contaminated with Salmonella in a catfish processing plant (Wyatt et al 1986).

So far, the available literature suggests that more work needs to be carried out on the prevalence

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of Salmonella in fresh fishes.

2.6 ANTIBIOTIC SENSITIVITY PATTERN OF SALMONELLA

The use of antibiotic therapy has aided greatly in combating several diseases of microbial aetiology. Antibiotics have been used for curing diseases, prophylaxis and growth promotion in animal husbandry and even in plant protection in agriculture (Gutzman et al 1976; Linton, 1977).

As the use of antibiotics become more extensive, there is the gradual increase in resistance by the microbes to these antibiotics. This has already given rise to serious clinical problems. The result is that, for organisms like Salmonella, despite the fact that a lot of work has been done on the drug therapy of Salmonellosis, the organism has developed and continues to maintain resistance to a relatively large number of antibiotics (Kwaga, 1984).

Antibiotic management of salmonellosis is not without its problems. Apart from creating resistant strains, normal intestinal flora are suppressed allowing Salmonella to have a clear field in which to establish a carrier state (Gutzman et al, 1976; Gibson, 1961; Schroeder et al, 1968). Although several drugs have been reported to be effective against Salmonella, chloramphenicol still remains the most effective (Schroeder et al, 1968; Neu et al,

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1971; Hummel, 1979; Timoney, 1978).

Salmonellae are largely dependent on the resistance transfer factor as a means of developing resistance to antibiotics. This is the transfer of the R - (resistance) - factor between bacterial cells by cell to cell contact (Baldwin, 1970) and has been described with several names such as infectious drug resistance, contagious drug resistance or simply, R - factor resistance. Prior to the discovery of the transferable nature of resistance to antibiotics, this phenomenon was credited to chromosomal mutation and selection (Smith, 1966) and since the discovery of the transferable nature was made in Japan in 1959 (Smith, 1966), several workers have had results which agree with this discovery (Anderson and Datta, 1965; Gill and Hook, 1966; Okubadejo, 1967; Marsik et at, 1975; Pocurull et al, 1971; Neu et al, 1975).

Resistance transfer factor is controlled by extrachromosomally located genes or plasmids attached to transfer or sex factor as an episome. There are two independent components to the R-factor. One codes for transfer of the R-factor through conjugation while the other codes for the actual resistance of the organism to antibiotics. Both are DNA and can occur independently in different bacterial cells (Anderson, 1965). The R-factor occurs more frequently when

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infecting bacteria are able to colonise the gut (Linton, 1977).

The resistance transfer factor RTF and the R-factor behave independently of each other and only become associated when found in the same cell. In the absence of the RTF, the R-factor cannot be transferred by conjugation and without the R-factor, the RTF cannot impart resistance. The principal activity of the RTF is to potentiate the formation of conjugation tubes (Pilli) between the donor and recipient cells (Anderson, 1965; Baldwin, 1970) through which the episomes are transferred (Smith, 1966, 1967). The actual transfer takes about one to three minutes after formation of the pilli and the organism becomes fully resistant about twenty minutes later (Watanabe, 1963; Smith, 1967).

Various theories have been advanced to explain the mechanism by which the R-factor mediates the resistance of the organism to drugs. These include non specific alteration of the bacterial cell wall to permeability to the drugs and production of the penicillinase enzyme which destroys the penicillin molecule (Anderson and Datta 1965).

Resistance factors and RTFs occur both among pathogens and non-pathogens, especially those inhabiting the alimentary tract It is possible for

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transfer to occur between two different types of bacteria, provided they are both gram negative because Gram-positive organisms have neither been shown to possess RTF or R-factor nor have they been reported to be able to conjugate, even among themselves (Smith, 1966).

Transfer of resistance occurs both in the presence or absence of antibiotic theraphy, but the incidence is higher during antibiotic usage (Baldwin, 1970). The presence of antibiotics probably promotes the formation of R-factors (Anderson, 1965).

2.7 PATHOGENICITY

Salmonellae have demonstrated the ability to infect a wide range of hosts including man and animals. Based on the type of host specificity shown, the organism has been grouped into three (Prost and Rieman, 1967):-

(a) Salmonella pricipally adapted to man causing typhoid fever, S. typhi and S. paratyphi.

(b) Those principally adapted to animals, e.g. S. abortus-equi, S. abortus-ovis and S. cholera suis.

(c) Non-host specific Salmonella. This group contains a large number of Salmonella which occurs mainly in animals. The group is of

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particular concern because members have been reported to cause acute septicaemic diseases in young individuals and have also been incriminated in various cases of human gastroenteritis. Of major concern is the fact that members can infect both man and animals depending on in which host they suddenly find themselves in conducive environment (Prost and Rieman, 1967).

Salmonellosis is a foodborne infection resulting from ingestion of foods contaminated with the organism. There is an incubation period of 12 - 36 hours, although a short time of 5 has been reported while the upper limit is about 72 hours.

Symptoms include nausea, vomiting, headache, chills and diarrhea. Muscular weakness and faintness are also observed. These symtoms are presumed to result from the action of endotoxins liberated from the cells by the action of low stomach pH and proteases.

The disease syndromes arising from Salmonellosis are divided into three distinct clinical types:-

(a) Typhoid fever: Most severe form and results from infection with S. typhi. This type specifically affects man.

(b) Paratyphoid fever: A milder form of typhoid fever caused by S. paratyphi A, B and C.

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Federal Government has responded positively to this by the recent programmes aimed at producing more fish in the country and encouraging individuals to raise their own fish.

Fishes around the country are usually caught from rivers and these rivers receive a lot of pollutants from human and animal waste materials. Some fish ponds are also fertilised with animal droppings such as the Maska fish farm in Funtua which is fertilised with chicken droppings. The risk of spreading Salmonella by fecal contamination of water cannot be over-empasised and the potential of chickens as carriers of Salmonella organisms are well documented (Edel et al, 1977).

This study into the prevalence of Salmonella in fresh fishes was necessary to give an insight to the role of fish as a vehicle for transmission of Salmonella in some parts of Northern Nigeria.

As a means of producing more fish by investing less, the recent trend which is fast gaining acceptance around the world is to introduce fish cultures into waste water treatment ponds. This achieves the double effect of raising fish for consumption and aerating the pond due to removal of plankton by the growing fish cultures (Edwards and Densem, 1980; Schroeder, 1975).

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A major arguement in favour of this method is that it is cost limiting and depends solely on availability of land through which the sewage can be channelled (Schroeder, 1975). Fishes raised in such ponds could carry such human pathogens as enteric viruses and Salmonella (Shewan 1962) and if a means is not derived to monitor the possible effects of this method, what started as a drive towards a better economy could well end up as economic suicide with the human population being afflicted.

Workers who have monitored the state of salmonellosis in recent times agree on one point summarised by Silliker (1982) as "the problem is a little greater than it used to be, certainly not less." The Phrase, "this is the tip of the iceberge" has become a common way of describing epidemics of Salmonellosis reported in journals.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Fish Sampling

Six different types of fishes were examined. They were (with local names in brackets) Tilapia (Karfasa), Alestes (Jan Wutsiya), Clarias (Tarwada), Synodontis (Kurungu), Labeo (Dubi) and Lates (Giwan ruwa) species. Fishes were purchased from four different geographical locations in the Northern part of the country viz:- Kaduna, Kano, Funtua and Zaria. Fishes from Funtua were purchased from the Federal Government fish farm at Maska while other samples were fresh fish purchased directly from the markets and fishermen. All the samples obtained from Funtua and some from the other locations were transported alive to the laboratory. Special care was taken to ensure that all fishes examined were caught on the same day.

3.2 ISOLATION OF SALMONELLA FROM FISH SAMPLES

Samples of fish were t r a n s p o r t e d to the laboratory immediately after purchase and two methods were employed to examine for the prevalence of Salmonella in their guts. Using sterile surgical materials (scissors and forceps) the samples were cut open by making a long slit on the ventral aspect of each fish. The intestinal contents were then removed and put into enrichment

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broth thus.

(a) Method 1

Intestinal contents of the fish were teased out into Selenite F broth for enrichment (Leifson, 1936). The broth was then incubated at 37°C for 24 hours. Growth from the broth culture was streaked on desoxycholate citrate agar (DCA) and then was incubated at 37°C for 24 hours.

(b) Method 2

Intestinal contents were teased out into tetrathionate broth for enrichment (Kauffman, 1935). This was incubated at 37°C for 24 hours and then streaked on brilliant Green agar (BGA) before incubating for 24 hours at 37°C.

All media were prepared according to the instruction of the manufacturers and surgical materials were sterilised by dipping in methylated spirit and flaming twice before each successive use.

3.3 PRELIMINARY IDENTIFICATION OF ISOLATES

Colonies which showed cultural characteristics of Salmonella i.e. Pale/Colourless, black centered colonies on DCA and pink opaque colonies surrounded by red halo on BCA were subjected to preliminary biochemical identification tests by inoculation of

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Triple sugar iron (TSI) agar and urease agar (Ewing, 1972). The TSI agar was inoculated by stabbing the butt and then streaking the slant while urease agar was streaked on the surface only. Inoculated test media were incubated at 37°C overnight and observed for reactions typical of Salmonella viz:- alkaline slant over acid butt on TSI agar and a negative result on urease agar slant. Such colonies were then preserved on nutrient agar slants for further studies.

3.4 BIOCHEMICAL IDENTIFICATION

All isolates which had typical Salmonella reactions on TSI and urease agars were tested according to the methods of Ewing (1972), Cowan and Steel (1974) and Mc Faddin (1977). The tests performed were:- test for production of indole, methyl red test, Voges-proskauer test, production of hydrogen sulphide, motility, triple sugar iron agar (TSI), citrate utilisation, lysine decarboxylase ornithin decarboxycase, acid and gas from glucose, lactose fermentation, sucrose fermentation, nitrate reduction test, potassium cyanide utilisation, mannitol autilisation and gelatin liquefaction.

The reactions of a typical strain of Salmonella are illustrated in appendix A. Isolates which gave similar results were identified as Salmonella.

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3.5 SEROLOGICAL CONFIRMATION OF ISOLATES

Isolates biochemically identified as Salmonella were confirmed serologically by the slide agglutination technique. A polyvalent antiserum against groups A through I was used for this method and it was kindly provided by Dr. A. Adesiyum of Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

Before being tested, isolates were transferred onto plates of DCA and incubated overnight at 37°C. A drop of antiserum was placed on a microslide and using a wire loop, a pure colony of isolate was mixed thoroughly with the serum and observed for agglutination. Those showing agglutination were confirmed as Salmonella organisms.

3. 6 DETERMINATION OF ANTIGENIC FORMULAE

Isolates serologically identified as strains of Salmonella were kindly confirmed and their antigenic formulae determined by Professor L. LeMinor of the Salmonella reference laboratory, Institute Pasteur, Paris, France.

3. 7 REACTION OF ISOLATES TO ANTIMICROBIAL AGENTS

The reaction of all isolates to 11 different antimicrobial agents was examined using the method of Kirby - Bauer (1966) as modified by Olayemi (1978). The antimicrobial agents used were as follows:- cotrimoxazole (25 mg) chloramphenicol (50 mg) ampicillin (25 mcg) nitrofuratioin (200 mcg),

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tetracycline (50 mcg), nalidixic acid (30 mcg), gentamycin (10 mcg) sulphadiazine (300 mcg), cephaloridine (5 ug), kanamycin (5 ug), and streptomycin (10 ug).

A pure colony of each isolate was inoculated into 5 ml. of tryptic soy broth and incubated for about 18 hours until turbidity approximated that of barium sulphate standard (0.5 ml of barium chloride - $BaCL_2 \cdot 2H_2O$ - Solution in 99.5 ml of 0.36N Sulphoric acid). Using sterile cotton swabs, petri dishes containing 20 ml of Mueller Hinton agar were inoculated by dipping swabs in the broth culture, draining off excess culture by pressing swabs to the side of the test tube and then spreading the agar surface in three directions rotating the plate 60^{o} approximately in between streakings. Each plate was left for about 5 minutes at room temperature before multodiscs containing the antimicrobial agents were applied. Plates were left again at room temperature for about 5 minutes to allow multodiscs to adsorb well onto the agar surfaces before finally being inverted.

Plates were then incubated at $37^{o}C$ overnight and results observed. Measurement was taken of the diameter of the zones of inhibition of the organisms and results were interpreted based on the table

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each fish were collected aseptically and cultured for Salmonella using the technique of enrichment in tatrathionate broth followed by plating on brilliant green agar. The procedure was repeated three times.

At the end of this screening, 18 Salmonella free fishes were divided into 5 groups of 3 each. Then the suspension of the inoculum was made by growing Salmonella tennessee in tryptic soy broth until turbidity approximated that of McFarland scale No. 1 (300 cells/ml). This suspension was then used to label four of the 5 groups of fishes by adding the suspension directly into the tank water to achieve the final dilutions of 2, 5, 50 and 250 cells/ml respectively. The fifth group was used as control and tryptic soy broth free of Salmonella was added.

After inoculation, fishes were left in the tanks for 6 hours before the water in the tanks was changed. Subsequent changes of the water was done at a 12 hourly interval. The fecal contents of all fishes were examined for presence of Salmonella organisms after days 1, 4, 8 and 11 respectively. The same method mentioned above was used for checking infection of experiment fishes. The number of fishes per group which had the organism were tabulated after each sampling.

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The remaining 5 fishes in each tank were left alive and were continuously examined for the persistence of the Salmonella organisms.

Fishes kept at $4^{o}C$ were cut open after 1 week and intestinal contents were examined for survival of the organisms. They were returned to the refrigerator and investigated twice more at 1 weekly interval. The samples stored at $-3^{o}C$ were cut open at the end of 4 weeks and intestinal contents were examined for the survival of the organisms in their guts. All the samples in all cases were enriched in tetrathionate broth followed by plating on BGA and then isolates were subjected to biochemical tests.

CHAPTER FOUR

RESULTS

The intestinal contents of a total of 5 00 fresh fish samples were examined for the prevalence of Salmonella organisms. Table 1 illustrates the different areas from which samples were purchased, the distribution of samples by species and the total number of samples examined from each area.
Out of a total of 500 of them, four (0.8%) were positive for Salmonella. Three (1.8%) of the Tilapia sp yielded Salmonella while only one (1.1%) synodontis sp was positive for it. No other fish species was positive for the organism. No sample from Zaria was positive, but one (0.8%) from Kano, two (4%) from Funtua and one (0.7%) from Kaduna were positive for it.

The four isolates were identified as three different serotypes (S. tennessee, S. middlesbrough) one of which is a new one (S. maska). The antigenic formulae of the serotypes are as given on table 2.

The use of enrichment of gut contents of fish samples in tetrathionate broth followed by plating on Brilliant green agar yielded three isolates whereas the use of enrichment in selenite broth before plating on Desoxycholate citrate agar for the isolation of the same organisms from the same

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fish yielded only one isolate.

All the four isolates were susceptible to ten of the eleven antiobiotics tested for. The four of them were resistant to sulphadiazine. The minimum inhibitory concentration (MIC) for tetracycline was found to be 3.125 ug/ml for the four isolates while for chloramphenicol, the highest MIC recorded was 50 ug/ml, the rest being 25 ug/ml. MIC for streptomycin was not higher than 50 ug/ml too.

Table 3 shows that only one out of three fishes became infected at concentration of 10 organisms/ml but at 50 organisms per ml all the three experimental fishes became infected. Infective dose 50 (IF_{50}) was estimated to be 30 organisms/ml and this concentration was used for the next experiment.

All infected fish kept at $4^{o}C$ still had their organisms when examined three weeks after refrigeration. At $-3^{o}C$ two fishes had lost their organisms after four weeks.

Salmonella

Table 4 shows that fishes lose organisms very rapidly after infection. Most fish became negative between sixteenth and twentieth days post infection irrespective of the type of Salmonella organisms. By the twenty fourth day, all infected fishes were free of Salmonella

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TABLE 1: NUMBER OF SAMPLES FROM DIFFERENT LOCATIONS WITH PERCENTAGE OF POSITIVE ISOLATES

Type of fish	Kaduna	Kano	Funtua	Zaria	Total
Tilapia	27	40 (2.5)*	50 (4)	50	167 (1.8)
Alestes	21	29		40	90
Clarias	28	29		41	98
Synodontis	37 (2.7)	19		31	87 (1.1)
Labeo	17			19	36
Lates	7			15	22
Total	137(0.7)	117(0.8)	50(4.0)	196	500 (0.8)

*Percentage positive for Salmonella

TABLE 2: TYPE AND NUMBER OF FISHES SAMPLED WITH SEROTYES OF POSITIVE ISOLATES

Type of Fish	No Sampled	No Salmonella Positive	Serotype Isolate	Antigenic Formula
Tilapia	167	3(1.8)*	S.tennessee	$6.7:Z_{29}$:-
			S.tennessee	$6.7: Z_{29}$:-
			S. maska	1,4,12,27:
			Z_{41}: e nz_{15}	
Alestes	90	-		
Clarias	98	-		
Synodontis	87	1(1.1)	S.middles-brough	$1,42:i:z_6$
Labeo	36	-		
Lates	22	-		
Total	500	4(0.8)		

* Percentage positive for Salmonella

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TABLE 3: DETERMINATION OF INFECTIVE DOSE OF SALMONELLA IN FISHES

No of Salmonella per ml of water	No fishes tested	No. of infected fishes in various days post Inoculation			
		Day 1	Day 4	Day 8	Day 11
2	3	0	0	1	0
10	3	0	1	1	1
50	3	0	3	3	2
250	3	0	3	3	3
Control	3	0	0	0	0

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TABLE 4 PERSISTENCE OF SALMONELLA IN LIVE FISHES AFTER INOCULATION

Organism	No. of fishes tested	No. of fishes showing persistence of Salmonella						
		Day 1	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24
S. maska	5	0	4	5	5	2	0	0
S. tennessee	5	0	3	5	4	4	1	0
S. middlesbrough	5	0	3	5	5	3	1	0
Control	5	0	0	0	0	0	0	0

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CHAPTER FIVE

DISCUSSION

The isolation rate in 500 fish samples was 0.8%. This differed from the results of Fajardo and Marth (1979), D'Aoust et at, (1980), Binta et al (1982) and Saddik et al (1985) who did not isolate any Salmonella. Similarly, the isolation rate in this study was much lower than the results of Floyd and Jones (1954) and Gulasekharam et al 1956) with the rates 11.7% and 6.2% respectively.

The 0.8% isolation rate shows that the prevalence of Salmonella in fresh fishes examined is very low. Since the level of Salmonella in water depends on the level of fecal contamination of that water (Wyatt et al 1979, 1980) then the present result shows that cases of pollution of the water with Salmonella containing fecal matter are very minimal. On the other hand, the low isolation rate could have been as a result of fish acquired Salmonella organisms being lost from the body after a period of between 16 to 32 days (Baker and Smitherman, 1983).

Isolation of Salmonella in this study confirms the presence of Salmonella in the guts of live fishes and agrees with the report of Gulasekharam et al (1956) that in such a case, the organisms entered fishes from polluted waters. That no other species of

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fish was positive for Salmonella could mean that the organism probably has a lower survival rate in those fishes than in the ones which gave positive isolates.

Serotyping of the isolates shows the diversity of Salmonella serotypes in nature. Three different serotypes were identified. This confirms Spiller's (1967) report that in terms of number of times of isolation, no one serotype enjoys the superiority of the highest number relative to other serotypes in Africa.

The finding of a new serotype in the four isolates points more to the diversity of Salmonella serotypes as new serotypes continue to be discovered with the progression of studies on Salmonella. Investigations show that S. tennessee and S. middlesbrough are being reported for the first time in this country.

S. tennessee was isolated from two fishes brought from Kano and Funtua. This suggests that the serotype is probably widespread in the country. No particular reason can be advanced for the presence of S. maska in Funtua since Salmonella organisms are ubiquitous.

That no sample from Zaria was positive could be due to the fact that Zaria Dam from which the fishes

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were caught has a very minimal amount of fecal contamination. Zaria municipal water supply is obtained from this Dam. Hence it is jealously protected and permits are even needed before fishing is allowed.

Since running water purifies itself, this could be the reason for the low rate of isolation from the other areas from which samples were purchased. Except from Funtua, fishes from Kano and Kaduna were caught from Rivers Hadejia and Kaduna respectively. The 4% isolation rate from Funtua could be attributed to the source of the fishes. Funtua samples came from a fish pond fertilised with chicken droppings. The potentials for poultry as Salmonella carriers have been well documented (Edel et al 1977) and this could be responsible for the introduction of Salmonella into the fish ponds.

This study also shows that enrichment in tetrathionate broth followed by plating on Brilliant green agar is apparently superior to enrichment in Selenite broth before plating on Desoxycholate citrate agar for isolation of Salmonella organisms from the intestinal contents of fresh fishes. This confirms an earlier report on enhanced recovery of Salmonella using tetrathionate and Brilliant green enrichment (D'Aoust et al 1980).

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The fact that all isolates were resistant to sulphadiazine shows only one pattern of resistance. This differs from the results of Olayemi (1978), Sojka and Wray (1980) and Kwaga (1984) in that they all observed multiple patterns of resistance. The single pattern of resistance is probably due to the few number of isolates examined.

The MIC observed for the three antibiotics showed that the isolates were susceptible to the antibiotics tested.

This study has shown that even with low contamination of water (2 organisms/ml), Salmonella can still appear in the gut contents of fishes and with an increase of level of contamination to about 30 organisms per ml, at least 50% of fishes would be infected.

The studies show that once contaminated by Salmonella, fishes retain the organism even at low temperatures and the organisms will survive in temperatures as low as $-3^{o}C$ up to four weeks.

In live fishes, the studies showed that once infected with Salmonella, the organism starts appearing in fecal contents on the fourth day and the organism is rapidly lost after infection provided the water is pure. The disappearance of the organisms from the fecal contents of fishes by the twenty

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fourth day agrees with the results of Baker and Smitherman (1983) who reported complete loss of Salmonella organism from Tilapia aurea within 16 to 32 days.

The results of the study show that when infected with Salmonella organisms, fishes do not retain them indefinitely but lose them as quickly as possible. This also means that depending on the frequency of contamination of the water, fishes can either have Salmonella present in them for a considerable length of time or go through a cycle of infection and purification with the organism.

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CONCLUSION

1. Four isolates of Salmonella were obtained from the intestinal contents of fresh fishes.
2. The prevalence of Salmonella in fishes from the areas studied seems to be very low.
3. The prevalence of Salmonella in fish depends on the contamination of water with Salmonella organisms.
4. Salmonella serotypes in the environment seem to show complexity in number of serotypes.
5. Salmonella will survive in fishes stored at low temperatures up to four weeks.
6. Once infected with Salmonella, fishes lose the organisms very rapidly and by the twenty fourth day are completely free of the organism.

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APPENDIX A

TABLE 5: SOME BIOCHEMICAL CHARACTERISTICS OF A TYPICAL STRAIN OF SALMONELLA

	TEST	RESULT
1.	$K/_A$ on TSI	+
2.	Urease	-
3.	H_2S	+
4.	Motility	+
5.	Indole	-
6.	Citrate (Simmon's)	+
7.	Methyl red	+
8.	Vosges proskauer	-
9.	Lactose fermentation	-
10.	Glucose fermentation	+
11.	Sucrose	-
12.	Lysine decarboxylase	+
13.	Mannitol	-
14.	Dulcitol	+
15.	Maltose	+
16.	Nitrate	+
17.	Potassium cyanide	-
18.	Gelatine	-

APPENDIX B

Some Diagnostic characteristics of S. maska

Test	Result
Mobility	+
Oxidase	-
KCN	-
Voges - Proskauer	-
Methyl red	+
Pigment	-
SUGAR METABOLISM	
Xylose	+
Arabinose	+
Adonital	-
Rhamnose	+
Glucose (gas)	+
Mannitol	+
Sorbose	-
Sorbitol	+
Dulcitol	+
Lactose	-
ONPG	-
Maltose	+
Saccharose	-
Trehalose	+
Inositol	+
Salicine	-
Raffinose	-

Test	Result
Cellobiose	+ (gas-)
Glycerol	+ (gas-)
Melibiose	+
CATABOLISM OF SOME ORGANIC SUBSTANCES	
Gelatinase film	-
Lysine decarboxylase	+
Thymine deaminase	-
Urease	-
Ornithine decarboxylase	+
Phenylalanine deaminase	
Arginine dihydrolase	+
Indole	-
ACTION ON ORGANIC ACIDS	
Simon's citrate	+
Malonate	-
Mucate	+
Tartrate	+
Galactouronate	-
ENZYMES	
Nitratase	+
γ Glutanyl transferase	+
β Glucoronidase	-
Phage	0/+

Curtesy of Pr L. Le Minor. Institut Pasteur, Paris, 1986.