Methods for Serotype Classification of *Haemophilus paragallinarum* Field Isolates

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg from May 1996 to February 1998 under the supervision of Dr T.H.T. Coetzer and co-supervision of Dr R. F. Horner.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

Kerry Taylor
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ABSTRACT

Historically, the causative agent of infectious coryza has been identified as the NAD requiring bacterium *Haemophilus paragallinarum* and the implementation of an intensive vaccination program led to the effective control of this contagious upper respiratory infection. More recently, however, a decline in the protective capacity of a vaccine conditioned immune response was noted, with a number of contributing factors, including the emergence of a fast-growing NAD-independent bacterium, which has largely replaced the traditional NAD-dependent variety. As such, accurate, reproducible methods for determining and continually monitoring the type of infecting bacteria was necessitated. To address this need, strains of *H. paragallinarum* were evaluated according to both their phenotypic and their genotypic properties, in a combination serodiagnostic approach.

A data bank of NAD-dependent *H. paragallinarum* reference strain and field isolate serovar-specific fingerprints was established on both a whole cell and outer membrane protein level. Visual comparative analysis of the qualitatively and quantitatively similar outer membrane protein patterns of all strains of NAD independency studied with the formulated data bank, indicate that the NAD-independent strains displayed profiles typical of serovar C-3. The outer membrane proteins have been identified as putative virulence determinants and, as such, were characterised according to their surface location, susceptibility to heat modification, functional role as endotoxins, sequence homology to structural membrane counterparts, and finally, their ability to induce an immune response. These studies represent novel efforts and form the foundation for identifying those antigens responsible for maintaining an infection in the host milieu. Ribotype analysis served as an adjunct to phenotypic observations, with the local NAD-independent field isolates being identified as serotype A. These contradictory outcomes call for the creation of a set of reference strains specific for NAD-independent isolates. The identification of restriction fragment length polymorphisms in the conserved 16S rRNA gene sequences indicate the potential application of this method for type assignment, requiring the recognition of a battery of versatile restriction enzymes to generate serovar-specific polymorphic profiles. The complexity of serotype allocation demands that a combination approach in which genotypic analyses complement phenotypic-based methods of haemagglutination inhibition and outer membrane protein profiling. The groundwork for implementation of such a system has been accomplished.

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LIST OF ABBREVIATIONS

A_{280}	absorbance at 280 nm
ABTS	2,2′-azino-di-(3-ethyl)-benzthiozoline sulfonic acid
ARDRA	amplified rDNA restriction analysis
AZT	anilinothiazoline
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bis	*N*, *N*'-methylenebisacrylamide
BRENDA	bacterial restriction endonuclease DNA analysis
BSA	bovine serum albumin
BSA-TBS	bovine serum albumin dissolved in Tris-buffered saline
c	concentration
C-terminal	carboxy terminal
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CPD	critical point drying
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytosine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
DIG	digoxigenin
dist. H_2O	distilled water
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate(s)
DTT	dithiothreitol
dTTP	thymidine-5'-triphosphate
E	extinction coefficient
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	relative centrifugal force
h	hour(s)
HA	haemagglutinin(s)

HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HPLC	high-performance liquid chromatography
HRPO	horseradish peroxidase
IgG	immunoglobulin G
IgY	immunoglobulin Y
kDa	kilodalton(s)
l	light path
LPS	lipopolysaccharide(s)
M_r	relative molecular weight
min	minute(s)
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NBT	nitroblue tetrazolium
N-terminal	amino terminal
OMP	outer membrane protein(s)
ON	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-Tween	Tween 20 diluted in phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PTC	phenylthiocarbamoyl
PVDF	polyvinylidene difluoride
®	registered trademark
REA	restriction endonuclease analysis
RFLP	restriction fragment length polymorphism(s)
RNA	ribonucleic acid
rrn	rRNA operon
rRNA	ribosomal RNA
RT	room temperature
s	second (s)
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
TBS	Tris-buffered saline
TEMED	*N*, *N*, *N'*, *N'*-tetramethylethylenediamine
TMB	test medium broth
TM/SN	test medium agar supplemented with chicken serum and NAD
tricine	*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
UV	ultraviolet
WCP	whole cell protein(s)

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CHAPTER 1

Introduction

Infectious coryza is a disease of poultry caused by the bacterium *Haemophilus paragallinarum.* Flocks of chickens provide the natural host for *H. paragallinarum,* however, infection in pheasants (Delaplane *et al.*, 1935), Japanese quail (Reece *et al.*, 1981) and single cases in guinea fowl (Yamamoto,1972) have been diagnosed. The effects of infectious coryza are observed globally, with cases reported from Argentina (Terzolo *et al.*, 1993), Brazil (Blackall *et al.*, 1994) and the United States of America (Page, 1962), through Germany (Hinz, 1973) to China (Chen *et al.*, 1993), Japan (Kume *et al.*, 1978), Malaysia (Mohd Zain and Iritani, 1992), Indonesia (Takagi *et al.*, 1991b) and Australia (Thornton and Blackall, 1984). The first serious outbreak in South Africa occurred in 1968 on a multi-age farm of approximately 10 000 commercial layers (Coetzee *et al.*, 1982). As the bacterium is rapidly disseminated within a flock, especially by the introduction of replacement pullets carrying infectious coryza (Yamamoto and Clark, 1966), it has since spread country-wide with important economic ramifications. The greatest economic losses result from an increased number of culls and a marked reduction (10-40%) in egg production (Arzey, 1987; Sarakbi, 1987). By the mid 1970's infectious coryza was one of the most feared economic threats egg producers were facing due to the high exposure risks and inefficient control measures (Coetzee *et al.*, 1982). Infectious coryza has since established itself in Kwazulu-Natal, with fresh incidents of the disease being reported weekly, rendering this infection historically as one of the biggest problems on a multi-age farm (Sarakbi, 1987). The need for highly efficient and highly effective control measures thus became apparent.

Immunization against infectious coryza has been proposed as an economically-viable control procedure in endemic areas (Sarakbi, 1987). However, immunity induced by bacterins is serotype-specific (Yamamoto, 1991). The efficacy of infectious coryza vaccines may thus be monitored by the determination of the serotype of the infecting bacterium and its subsequent inclusion in existing vaccines. Several techniques of variable reproducibility and reliability have been proposed for the classification of *H. paragallinarum.* Due to their lack of reliability, new techniques for serological characterisation of *H. paragallinarum* field isolates are being investigated to assist diagnosis and hence minimise the economic burden this highly infectious bacterium places on the local poultry industry.

1.1 Classification of the infectious coryza causing agent

The first recorded isolation of a haemophilic bacillus from the nasal exudate of a naturally-infected bird occurred in 1931 by De Bliek (Nelson, 1933). He named this organism *Bacillus haemoglobinophilus coryza gallinarum.* In further studies on fowl coryza, Nelson (1938) noted that injection of uninfected experimental birds with exudate from the upper air passages of naturally-infected birds, produced a disease response which could be divided into three types, depending on disease onset and duration. A haemophilic bacterium, corresponding to that described by De Bliek, was regularly isolated from organisms infected with a type 1 coryza, characterised by rapid onset and short duration of the disease (Nelson, 1938). This bacterium was renamed *H. gallinarum* by Elliot and Lewis (1934), and was accepted as the causative agent of infectious coryza, requiring both haemin (X-factor) and nicotinamide adenine dinucleotide (NAD, V-factor) for growth (Schalm and Beach, 1936).

In several subsequent studies, it was repeatedly demonstrated that the causative agent of infectious coryza only required NAD for growth and not haemin (Page, 1962; Roberts *et al.*, 1964). Biberstein and White (1969) proposed that these X-factor-independent organisms, capable of synthesising porphyrin from δ-aminolaevulinic acid, should be renamed *H. paragallinarum.* Speculation as to whether *H. gallinarum* ever existed was resolved by Blackall and Yamamoto (1989b), when they concluded that the etiological agent of infectious coryza has always been an NAD-dependent, haemin-independent *H. paragallinarum.*

In February 1989, the emergence of an infectious bacterial disease resembling infectious coryza was seen in commercial chicken flocks in Kwazulu-Natal (Horner *et al.*, 1992). The biochemical, cultural, phenotypic and genetic (89% DNA homology with the ATCC 29545 reference strain) relatedness of this new organism to *H. paragallinarum* was shown by Mouahid *et al.* (1992) and Horner *et al.* (1995). It differed from typical *H. paragallinarum* in that it did not require NAD for growth, and is referred to as NAD-independent *H. paragallinarum* and designated Bisgaard Taxon 31 (Horner *et al.*, 1995). The NAD-independent *H. paragallinarum* strain rapidly became the predominant strain isolated from chickens showing signs of infectious coryza in South Africa. In a field study conducted at the Allerton Regional Veterinary Laboratory in Kwazulu-Natal, the ratio of NAD-independent to NAD-dependent outbreaks increased from 1.4:1 to 9.8:1 over a five year period (Figure 1.1; Horner *et al.*, 1995).

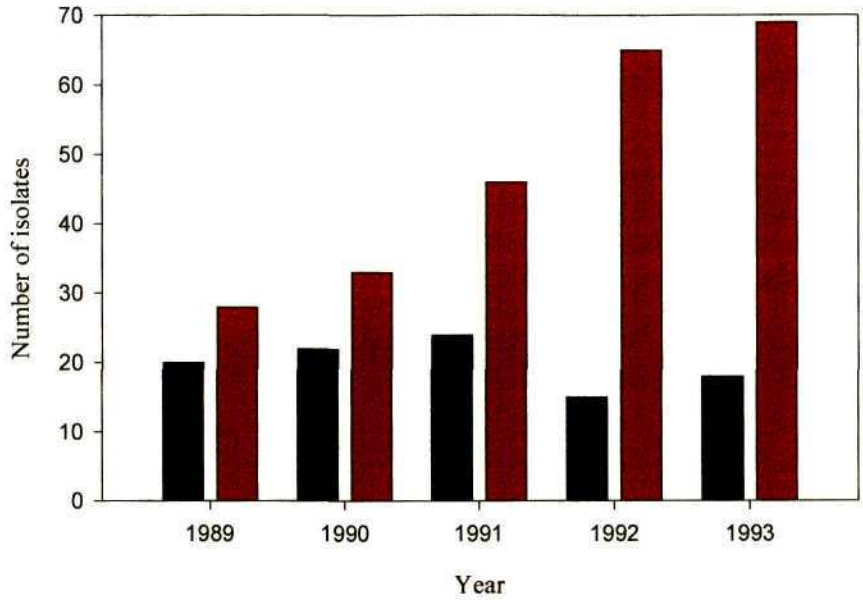


Figure 1.1 The relationship between outbreaks yielding NAD-dependent (■) or –independent (■) *H. paragallinarum* over the period 1989-1993 (Allerton Regional Veterinary Laboratory, South Africa).

Since 1993, infectious coryza has continued to cause problems in the local poultry industry, with the NAD-independent strain being the most prevalent and damaging (Allerton Regional Veterinary Laboratory Reports 1993-1996).

1.2 Clinical symptoms of infectious coryza and histopathological effects of *H. paragallinarum*

Infectious coryza is an acute respiratory disease, infecting both growing chickens and layers, which may take on a mild or chronic course of infection (Matsumoto, 1988). The disease is characterised by unilateral or bilateral nasal discharge of a watery or a viscid secretion, facial edema, conjunctivitis and sneezing (Figure 1.2). This is soon followed by clogging of the nostrils and sinuses with a putrid-smelling exudate which eventually causes extreme swelling of the face and closure of the eyes (Sarakbi, 1987). Infection of the lower respiratory tract may also occur causing rales, while diarrhoea and anorexia may develop as a result of decreased feed and water consumption (Fujiwara and Konno, 1965; Sarakbi, 1987). In most cases this disease is one of low mortality but high morbidity, with infected chickens recovering 7-10 days after exposure (Matsumoto, 1988). In some cases,

complicating agents, commonly *Mycoplasma galliseptica* or *Escherichia coli*, in addition to poor housing and inadequate nutrition, contribute to a more severe infection which often takes on a chronic course (Sarakbi, 1987; Yamamoto, 1991).

Figure 1.2 A broiler (left) and a layer (right) showing typical signs of infectious coryza (Allerton Regional Veterinary Laboratory)

Once the bacterium has entered the respiratory tract it firmly attaches itself to the ciliated epithelial cells of the mucous membrane in the nasal cavity, infraorbital sinuses and trachea. The principal pathological changes which occur include the disappearance of cilia, hyperplasia and desquamation of the colonised epithelial cells of the mucous membrane. The lamina propria of the mucous membrane demonstrates inflammatory oedema and infiltration of heterophils, and vacuolation in the mucous membrane of the infraorbital sinus is seen (Fujiwara and Konno, 1965; Matsumoto, 1988). In birds with lower respiratory tract infection, acute catarrhal bronchopneumonia is observed. The secondary and tertiary bronchi are filled with cell debris, and the epithelial cells of the air capillaries are swollen, hyperplasic and infiltrated with heterophils. The lamina propria of the mucous membrane of the nasal cavity becomes pronounced as a result of mast cell infiltration. The mast cell mediators, heterophil lysosomal enzymes and products of macrophages may be responsible for the vacuolar changes and cell damage that lead to infectious coryza (Sawata *et al.*, 1985a).

1.3 Phenotypic characteristics of *H. paragallinarum*

Numerous studies on the phenotypic properties of this avian *Haemophilus* have been conducted, including their morphology, growth requirements, and biochemical properties.

1.3.1 Morphology

All strains consist of Gram-negative, polar-staining, non-motile, non-spore-forming, pleomorphic, coccoid or rod-shaped cells 1-3 μm in length and 0.4-0.8 μm in width, with a tendency to filament formation (Schalm and Beach, 1936; Piechulla *et al.*, 1984; Yamamoto, 1991). In addition, virulent strains may possess a capsule. Colonies of NAD-dependent *H. paragallinarum* are grayish and typically tiny, reaching a diameter of up to 1 mm after 48 hours of growth in the presence of a feeder culture (Piechulla *et al.*, 1984; Blackall, 1989). In contrast, the NAD-independent isolates are clearer and more transparent and grow twice as fast (Horner *et al.*, 1992). Within 48-60 hours of growth, the organism undergoes degeneration showing fragments and indefinite forms.

1.3.2 Growth requirements

Co-factor requirements constitute the principal criterion for the inclusion of a Gram-negative bacterium in the genus *Haemophilus*, whereas species assignment is determined by the nature of these requirements (Biberstein and White, 1969). The NAD-dependent *H. paragallinarum* isolates require the oxidised form of the NAD co-factor at concentrations between 20-100 μg/ml of growth medium. Additional requirements for optimal growth include sodium chloride [1-1.5% (m/v)], chicken serum [1% (v/v)], and carbon dioxide (5-10%), at a pH and temperature range of 6.9-7.6, and 34-42°C respectively (Rimler *et al.*, 1976; 1977b). However, the NAD-independent *H. paragallinarum* field isolates display minimal co-factor requirements, and grow readily on blood agar plates, hereby casting doubt on the reason for inclusion of these bacteria in the genus *Haemophilus*.

1.3.3 Biochemical properties

The ability to reduce nitrate to nitrite, to ferment glucose without gas formation, to demonstrate oxidase activity, to possess the enzyme alkaline phosphatase, and the inability to produce indole or hydrolyse urea or gelatin, are uniform characteristics of avian haemophili (Piechulla *et al.*, 1984; Blackall, 1989). Detection of carbohydrate fermentation patterns plays a major role in the differentiation of *H. paragallinarum* from other avian haemophili. The failure of *H. paragallinarum* to

produce acid from trehalose and galactose, in addition to the lack of the enzymes catalase and α-glucosidase, and an inability to grow in air, uniquely distinguish *H. paragallinarum* from other avian haemophili, including *H. avium* (Table 1.1; Blackall and Reid, 1982; Blackall, 1983; Piechulla *et al.*, 1984). The biochemical properties of NAD-dependent and NAD-independent *H. paragallinarum* appear to be identical, with the exception of the ability of the NAD-independent strains to grow well in air (Horner *et al.*, 1992).

Table 1.1 Characteristics of an NAD-dependent *H. paragallinarum* strain compared to that of *H. avium* (Blackall and Reid, 1982; Blackall, 1983; Piechulla *et al.*, 1984).

Characteristic	***H. paragallinarum*** **Reference strain 0083**	***H. avium*** **ATCC 29546**
Effect on growth by:		
Haemin	ne	ne
NAD	↑	↑
CO_2	↑	ne
Chicken serum	↑	ne
Production of acid from:		
galactose	-[a]	+[b]
lactose	-	-
mannitol	+	-
sorbitol	+	-
trehalose	-	+
xylose	-	-
Catalase activity	-	+
α-glucosidase activity	-	+
Pathogenicity	yes	no

↑- improves growth, ne - no effect on growth

[a] positive result, [b] negative result

1.4 Serotypes and antigenic variation of *H. paragallinarum*

Prior to 1962, the study of immunity of chickens to infection with *H. gallinarum* had been hampered by the lack of serologic tests (Sato and Shifrine, 1964). A number of serologic tests have since been developed, with varying sensitivity and reproducibility. These include plate agglutination (Page, 1962), agar gel precipitation (Hinz, 1980), haemagglutination and haemagglutination inhibition (Kato *et al.*, 1965). More recently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in conjunction with a gel staining or immunochemical detection system, has been employed to study the whole cell and outer membrane protein profiles of NAD-dependent and NAD-independent *H. paragallinarum*, with limited success (Blackall and Yamamoto, 1989a; Blackall *et al.*, 1990d; Horner *et al.*, 1995). Currently, sequence comparisons of the evolutionary conserved 16S rRNAs by traditional ribotyping or restriction enzyme digestion of amplified 16S rDNA fragments, provide sensitive and reproducible diagnostic tools for allocating *H. paragallinarum* field isolates to previously established serotypes (Miflin *et al.*, 1995).

1.4.1 Plate agglutination

The first serological study of *H. paragallinarum* was conducted in 1962 in the United States of America by Page (1962). He employed a plate agglutination technique using *H. paragallinarum* whole cells from several field outbreaks as the antigen while antibodies, directed against these antigens, were raised in specific pathogen free white Leghorn chickens. In this test predetermined dilutions of antigen and antibody were mixed on a pre-warmed glass plate and agitated to allow interaction. The degree of agglutination was graded from one plus to four plus, while ambiguous results were regarded as negative. This technique revealed the existence of three serologic types, designated A, B and C, amongst pathogenic isolates studied. While Page's serotype A strain 0083 and B strain 0222 are still available, all of the serotype C strains were lost during the mid 1960s (Yamamoto, 1991; Blackall, 1989). In 1975, strain M was isolated from pullets in a Californian laboratory by Matsumoto and Yamamoto (1975), and was later typed by Rimler *et al.* (1977a) as serotype C strain Modesto. Subsequently Page's agglutination typing scheme classified Australian and South African isolates as serotype A and C (Blackall and Eaves, 1988).

In independent studies, Kato and Tsubahara identified three agglutinin serotypes, termed I, II and III, and their relationship with Page's types was initially unclear (Kume *et al.*, 1978). In an extension of the

work of Kato and Tsubahara, Sawata *et al.* (1978) indicated that serotype II and III were variants of serotype I, and they identified a further serotype, numerically designated 2. Kato and Tsubahara's serotype I (incorporating variants II and III), represented by strain 221, was renamed serotype 1, and was found to correspond serologically and biochemically to Page's serotype A strains 0083, W, Georgia, and Germany. An additional antigenic type, designated as serotype 2 and represented by strain H-18, was identified and found to correspond immunologically to Page's serotype C strain Modesto (Kume *et al.*, 1980b).

In 1973, Hinz published his findings regarding two antigens of *H. paragallinarum*, a heat-labile, serotype-specific antigen and a heat-stable common antigen shared by serotypes A and B. A close antigenic relationship was found to exist between serotypes of encapsulated *H. paragallinarum* organisms. Three different types of antigens were evident based on the agglutination test with immune rabbit serum, namely L antigen (heat-labile and trypsin-sensitive), HL antigen (heat-labile and trypsin-resistant), and HS antigen (heat-stable and trypsin-resistant; Figure 1.3; Sawata *et al.*, 1979). The L antigens are surface-located capsular substances which exist in three distinct forms, L1, L2 and L3. The L1 and L2 antigens are specific for serotypes 1 and 2 respectively, and confer protection to chickens against challenge exposure to homologous strains but not to heterologous strains (Kume *et al.*, 1980a).

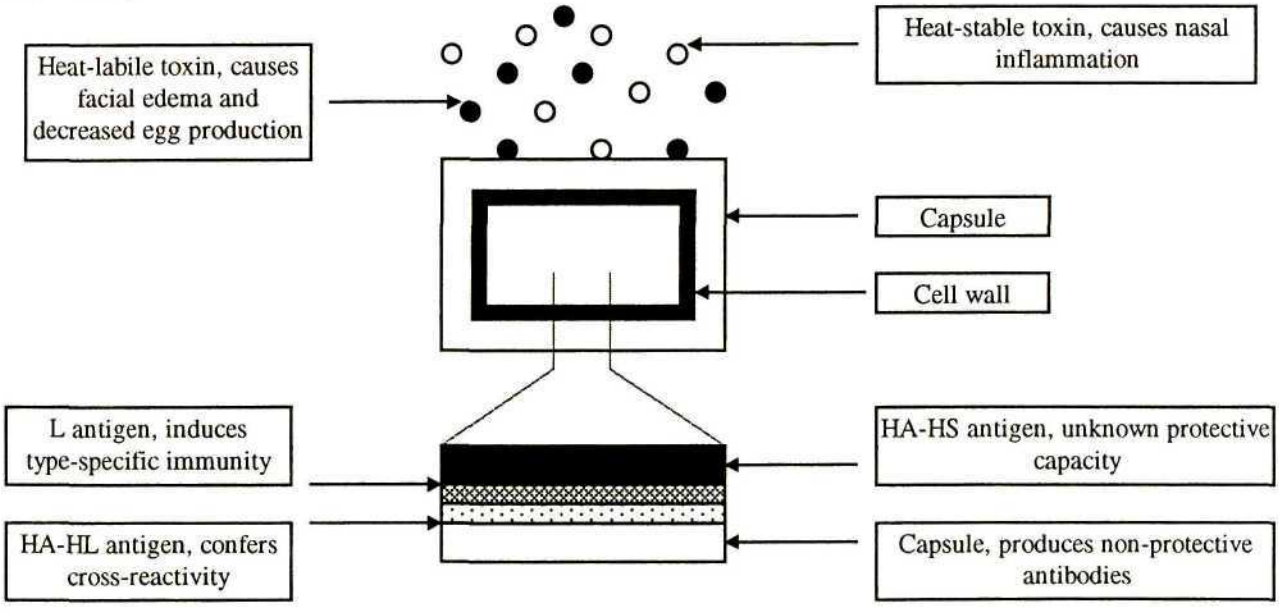


Figure 1.3 The structure, virulence factors and surface antigens of *H. paragallinarum* (adapted from Matsumoto, 1988).

A correlation between agglutination serotype specificity and immunotype-specific immunity became apparent. Serotype B strains could not be typed into either of Sawata's serotypes as they lacked a type-specific, heat-labile, trypsin-sensitive L antigen, however, these strains displayed L3 antigen, which was also common to serotypes 1 and 2 (Sawata *et al.*, 1980). Both HL and HS antigens are common between serotypes, the latter being somatic, and explain cross-reactivity reported by certain workers (Page, 1962; Thornton and Blackall, 1984). These antigens may be polysaccharide (Iritani *et al.*, 1981b), protein (Kume *et al.*, 1983b), or glycoprotein (Iritani *et al.*, 1980a) in nature.

Despite the success achieved in the late 1970s with characterisation of the antigenic variation amongst strains of *H. paragallinarum* employing the plate agglutination test, several limitations of this technique have been reported. Under certain cultural conditions *H. paragallinarum* cells were found to undergo spontaneous agglutination. Iritani *et al.* (1978) found that treatment of the bacterial cells with trypsin inhibited this agglutination while retaining the antibody-detecting ability of the antigen. However, the advantages of trypsin treatment became unclear when Sawata and Kume (1983) published evidence that the functional determinants of the bacterium were destroyed by trypsin. Several non-serotypable isolates have been reported, which have largely been attributed to the presence of hyaluronic acid, a hygroscopic capsule-forming substance thought to play a role in survival. Removal of the hyaluronic acid capsules using hyaluronidase render some of the non-typable isolates agglutinable (Rimler *et al.*, 1977a). Some isolates may possess material other than hyaluronic acid which masks the serospecific antigens rendering these isolates non-agglutinable and therefore non-serotypable (Rimler, 1979; Thornton and Blackall, 1984; Blackall and Eaves, 1988). Problems of non-serotypable or autoagglutinable isolates in the plate agglutination test gave rise to complications in the scheme for monitoring the serological distributions of populations of *H. paragallinarum* organisms, which in turn directly affects the efficacy of vaccines. Additional serotyping schemes have thus been developed.

1.4.2 Agar gel diffusion

Hinz (1980) developed a double immunodiffusion serotyping scheme to study the heat-stable antigens of *H. paragallinarum.* Six serotypes were identified on the basis of seven antigenic components. This technique was incapable of confirming a common heat-stable antigen between strains 221 (serotype 1) and H-18 (serotype 2). However, Hinz (1980) was able to establish that strains 0083 and 0222 of serotypes 1 and B respectively, carry distinct determinants as well as common antigenic factors. The identification of a unique serotype B antigenic determinant was in contradiction to reports by Sawata

et al. (1980), who were unable to class these strains into either of Sawata's serotypes. This technique is not widely used as the serotypes identified do not correlate with serotype-specific immunity (Thornton and Blackall, 1984).

1.4.3 Haemagglutination and haemagglutination inhibition

The first attempt to identify *H. paragallinarum* serotypes with different patterns of direct haemagglutination against red blood cells was described by Kato *et al.* (1965). Subsequent experiments revealed that both serotypes A and C possess antigenic determinants capable of haemagglutinating erythrocytes. They differ, however, in that untreated bacterial cell suspensions of serotype A will haemagglutinate fresh erythrocytes of various animals (Iritani *et al.*, 1981a; Kume *et al.*, 1980c; Sawata *et al.*, 1982), whereas both the antigen and the erythrocytes must be chemically treated before haemagglutination will occur with organisms of serotype C (Sawata *et al.*, 1982).

Two types of heat-labile haemagglutinins (HAs) were identified in cell suspensions of serotype A strain 221 (Yamaguchi and Iritani, 1980; Yamaguchi *et al.*, 1980). Trypsin-sensitive type 1 HA is biologically and immunologically similar to the agglutination serotype-specific L1 antigen, while trypsin-resistant type 2 HA mimicked the agglutinin serotype-common HL antigen.

In a separate study investigating haemagglutinins of genetically stable serotype 1 variants, Sawata *et al.* (1984a) indicated that at least three haemagglutinins were present on the outer membrane surfaces of the bacteria. Two types of heat-labile haemagglutinins, designated HA-L and HA-HL, and a heat-stable HA-HS haemagglutinin were identified (Sawata *et al.*, 1984a), and were classified due to their biological and immunological similarities with the agglutinin antigens L, HL and HS respectively. In addition, HA-L and HA-HL haemagglutinins were found to be similar to those of Yamaguchi and Iritani's type 1 HA and type 2 HA respectively. The three types of haemagglutinins may be differentiated on the basis of their biological and immunological properties (Table 1.2). Serotype specificity of haemagglutinins was correlated with HA-L haemagglutinin of serotype 1 (HA-L1 haemagglutinin) and serotype 2 (HA-L2 haemagglutinin; Kume *et al.*, 1983b), and is a potential candidate for serological typing of *H. paragallinarum* field isolates. In addition, Sawata and Kume (1983) postulated that the HA-L haemagglutinins are responsible for adherence or colonisation and protective activity (Takagi *et al.*, 1991a), but not for virulence. The relative sensitivity of HA-L haemagglutinins to trypsin revealed the removal of this haemagglutinin and the simultaneous

appearance of HA-HL and HA-HS haemagglutinins, indicating that both HA-HL and HA-HS are completely masked with HA-L haemagglutinin with HA-HS being situated on the inner part of the outer membrane (Sawata *et al.*, 1984a).

Table 1.2 The biological and immunological properties of the *H. paragallinarum* haemagglutinins (Kume *et al.*, 1983b; Sawata *et al.*, 1984a).

Property	**Haemagglutinin**			
	HA-L1	HA-L2	HA-HL	HA-HS
Sensitive (S) / Resistant (R) to:				
trypsin	S	S	R	R
formalin	S	S	R	R
hyaluronidase	R	R	R	R
Labile (L) / Stable (S) to:				
heat	L	L	L	S
Active (A) / Inactive (I) against:				
fresh RBCs	A	I	A	A
glutaraldehyde fixed RBCs	I	A	I	I

Kume *et al.* (1983a) proposed a serological classification scheme employing potassium thiocyanate-treated and sonicated bacterial cells, glutaraldehyde-fixed chicken erythrocytes, and rabbit anti-HA-L haemagglutinin sera in cross-haemagglutination inhibition tests. The results revealed three distinct serotypes, I, II and III, which were found to correspond to Page's serotype A, C, and B respectively. Further cross-absorption studies revealed differences within the three serotypes, and seven haemagglutinin sub-serotypes or serovars were recognised, corresponding to serotype A (HA-1, HA-2, HA-3), C (HA-4, HA-5, HA-6) and B (HA-7) (Table 1.3). In 1989, Eaves and his colleagues reported the existence of 15 Australian isolates which could not be classified as one of the present serovars in Kume's classification system. This new serovar was designated HA-8 and was allocated to serotype 1. Interestingly, a correlation was found between serovar-specific haemagglutinins and the geographical origin of the isolates. Furthermore, the common HA-L haemagglutinins shared by the serovars within the serotypes appear to be critical in inducing serotype-specific immunity. These facts lend support to the need for efficient monitoring systems for the determination of the serotype of the bacteria causing

infections in the field. A further serovar addition to the Kume haemagglutinin system was made by Blackall *et al.* (1990a) when serovar HA-9 was reported in Australian serotype II isolates (Table 1.3). These newly recognised serovars disrupted the assignment of serovars proposed by Kume *et al.* (1983a), and as the likelihood of further serovars being recognised was great, it was realised that the problem of illogical nomenclature would increase. An altered nomenclature for the Kume scheme was therefore suggested in which Kume's serotypes I, II and III would be changed to A, C and B respectively, while the serovars within each group would be renamed A-1 to A-4, C-1 to C-4 and B-1 (Table 1.3). The close linkage between the Page and Kume schemes was thus emphasised.

Table 1.3 Comparison of the new nomenclature for the Kume serotyping scheme for *H. paragallinarum* with the original nomenclature (Blackall *et al.*, 1990a).

Reference isolates	Original scheme		New scheme	
	Serotype	Serovar	Serotype	Serovar
221	I	HA-1	A	A-1
2403	I	HA-2	A	A-2
E-3C	I	HA-3	A	A-3
HP14	I	HA-8	A	A-4
H-18	II	HA-4	C	C-1
Modesto	II	HA-5	C	C-2
SA-3	II	HA-6	C	C-3
HP60	II	HA-9	C	C-4
2671	III	HA-7	B	B-1

The haemagglutinin serotyping system illustrated superior specificity and reproducibility, thereby successfully negating problems encountered by the agglutinin system, including reduction in the number of nontypable isolates. The efficacy of the haemagglutinin system is illustrated by the serological classification of 94 Australian isolates out of a total of 95, compared to the agglutinin system where only 60 of the 95 isolates were serotyped (Eaves *et al.*, 1989). Further success with the cross-haemagglutination inhibition test was reported by Yamaguchi *et al.* (1989) and Blackall *et al.* (1990b). Despite the merits of the haemagglutination inhibition test for serotyping A and C isolates, the characterisation of serotype B organisms remains controversial. Previously, Page's serotype B strains

(Spross and 0222) were found to be untypable (Sawata *et al.*, 1980) and were believed to be a variant of serotype A or C which had lost their serotype-specific antigens. Yamaguchi and co-workers (1990) carried out a study to clarify the existence of serotype-specific antigens in serotype B *H. paragallinarum* strains. Cross-absorption haemagglutination inhibition tests revealed six different kinds of haemagglutinin antigens in *H. paragallinarum* serotype B strains, of which one was found to be serotype-specific and was termed B-IV. Further work on serotype B strains is required, however the relatively low incidence of serotype B field isolates in South Africa (Blackall and Eaves, 1988) prioritises experimental studies on serotypes A and C.

1.4.4 SDS-PAGE: protein staining and immunochemical detection

Proteins perform a wide range of structural and metabolic functions in prokaryotes, eukaryotes and viruses. The diversity, including shape, charge, size, hydrophobicity and antigenicity, and the number of proteins produced - up to 2000 for some bacteria - provide ample scope for the development of typing and identification schemes based on comparative analysis of protein fingerprints. This approach has been applied successfully to bacteria for inter- and intra-species identification (Towner and Cockayne, 1993).

Generally, major protein profiles of strains within a species are very similar, but differ significantly from those of other species. Minor differences between strains within a species form the basis for *H. paragallinarum* strain allocation to Kume's established serotypes (A, B, and C) and their serovars (Blackall *et al.*, 1990b). Proteins may be typed on a whole-cell or semi-fractionated (e.g. outer membrane protein) level by non-denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and visualised with a protein staining or immunochemical detection system. Subsequently, a data base of protein profiles may be created against which the fingerprints generated from unknown field isolates may be compared (Towner and Cockayne, 1993).

1.4.4.1 Whole cell protein typing

Analysis of the protein profiles of *H. paragallinarum*, and generation of a serotype-specific fingerprint has not found wide application. Blackall and Yamamoto (1989a) reported on the whole-cell protein profiles of 15 NAD-dependent *H. paragallinarum* isolates. The patterns obtained were reproducible and contained 40-50 discrete bands. Two protein profile types were identified; type 1 was characterised by a prominent polypeptide at approximately 42 kDa, whereas type 2 lacked the 42 kDa protein but

expressed a major polypeptide at 40 kDa, which was indicative of type 2 organisms. In general, a species-specific fingerprint was found to be largely represented by proteins with molecular masses just greater than 68 kDa (Nicolet *et al.*, 1980). Further proteins in the region between 23 and 40 kDa as well as between 15 and 17 kDa are also important. However, no correlation between the serotype, and hence the serotype-specific antigens, and the whole-cell protein profile type of *H. paragallinarum* was noted, limiting the use of this technique for intra-species typing. Nevertheless, Nicolet *et al.* (1980) and Khan *et al.* (1987) recorded successful application of SDS-PAGE profiling for *H. parasuis* and *Mycoplasma gallisepticum* strains respectively, thereby discouraging abandonment of this technique.

A comparison of the whole cell protein profiles of NAD-independent *H. paragallinarum* isolates, from five different commercial poultry units, with representative NAD-dependent *H. paragallinarum* strains revealed marked differences in their protein banding patterns, suggesting differentially expressed proteins (Horner *et al.*, 1995). All of the NAD-independent *H. paragallinarum* isolates produced identical whole-cell profiles, prompting the idea that these newly emerged isolates may have originated from a single clone. Reducing SDS-PAGE revealed that the NAD-dependent *H. paragallinarum* isolates possess a unique 116 kDa protein, whereas only the NAD-independent *H. paragallinarum* isolates gave rise to a doublet at 60 kDa. Non-reducing SDS-PAGE also showed an additional protein band at 116 kDa, suggesting that this may be a single chain protein. Additional protein bands at 70 kDa and 25 kDa in NAD-independent isolates were also distinguished, while a common 68 kDa component appeared to have a slightly higher mobility in the NAD-dependent *H. paragallinarum* strains. Further characterisation of the antigenic differences between the isolates may form the basis of an effective subunit vaccine. As the serotype-specific antigens were previously demonstrated to be surface-located, the outer membrane proteins were identified as potential candidates for more specific identification of further antigenic variation between strains.

1.4.4.2 Outer membrane protein typing

The outer membrane of Gram-negative bacteria is a highly specialised structure that lies outside the cytoplasmic membrane and the peptidoglycan layer (cell wall), providing a physical and functional barrier between the bacterium and its surroundings. The specialised functions of the outer membrane, including regulation of solutes' passage through non-specific transmembrane channels, evasion of phagocytosis and complement action due to hydrophilic surface charges, and providing receptors for adhesion during colonisation, are reflected in its composition; namely cellular lipopolysaccharides,

phospholipids and a number of highly characteristic major proteins (Figure 1.4). At least three classes of major proteins have been characterised: matrix porins, OmpA proteins and murein lipoproteins. The matrix porins, Omp C, D and F, form transmembrane aqueous channels that allow passive, non-specific diffusion of solutes across the outer membrane. An additional function of the porin proteins is carried out by the outer membrane transport proteins, which are responsible for the uptake of large solute molecules present in the surrounding media at low concentrations eg. iron-carrier complex binding proteins. The OmpA proteins play a significant role in maintenance of cell morphology and outer membrane integrity, possibly through interactions with peptidoglycan. Finally, murein lipoproteins play a structural role in anchoring the outer membrane to the peptidoglycan layer via lysine (N-terminal) and cysteine (C-terminal) residues (Inouye, 1979; Osborn and Wu, 1980; Stanier *et al.*, 1986).

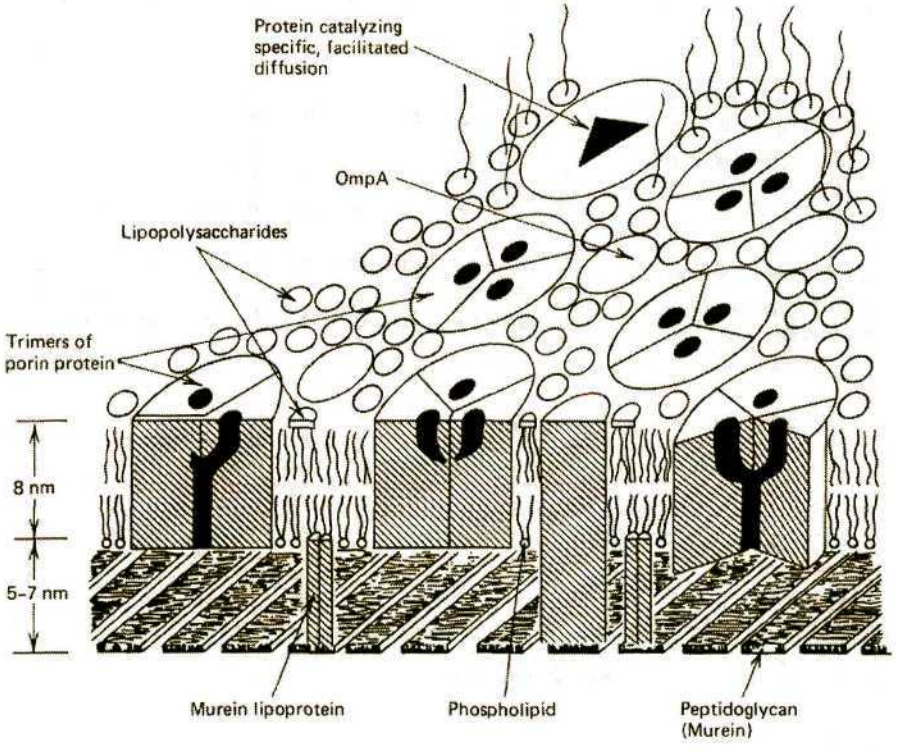


Figure 1.4 Schematic model of the outer membrane of *E. coli* showing the arrangement of the lipopolysaccharides, phospholipids and major proteins (Stanier *et al.*, 1986).

The sole study carried out on the outer membrane proteins of *H. paragallinarum* strains utilised NAD-dependent variants 0083, 0222, Modesto and HP31 (Blackall *et al.*, 1990d). The outer membrane proteins were isolated by a combination of sonic disruption, differential centrifugation and

selective solubilization in the non-ionic detergent Triton X-100 (Carlone *et al.*, 1986). This technique has been widely used for the preparation of outer membrane proteins of *Haemophilus* species, including *H. influenzae* (Carlone *et al.*, 1985; Saravani *et al.*, 1992) and *H. pleuropneumoniae* (Rapp *et al.*, 1986; Rapp and Ross, 1986). Reducing SDS-PAGE revealed a range of major and minor outer membrane proteins, shared by all the isolates studied, of which the most pronounced were designated OMP A through H (Table 1.4).

Table 1.4 Dominant outer membrane proteins found in NAD-dependent *H. paragallinarum* strains 0083, 0222, Modesto and HP31 (adapted from Blackall *et al.*, 1990d).

Outer membrane protein (OMP)	Estimated molecular mass of OMP (kDa)	Constant or variable molecular mass
OMP A	87.0	constant
OMP B	47.0	constant
	46.5	constant
OMP C	39.0	variable (38.0 kDa for 0222)
OMP D	36.5	constant
OMP E	32.5	constant
OMP F	31.0	constant
OMP G	26.5	constant
OMP H	31.5	constant (only present in Modesto and HP31)

The variability in OMP C forms the basis of recognition of the two whole cell profile types of *H. paragallinarum* previously noted (Blackall and Yamamoto, 1989a). Discrepancy in the molecular mass of this outer membrane protein, previously reported as 42 kDa for 0083, Modesto and HP31 and 40 kDa for 0222 (Blackall and Yamamoto, 1989), was observed with the latter estimation believed to be more accurate (Table 1.4; Blackall *et al.*, 1990d). The nature and function of OMPs A and C has been postulated. Heat-modifiability experiments characterised OMP C as a putative matrix-type protein (Blackall *et al.*, 1990d), forming diffusion pores for passive movement of hydrophilic substances, that require heat treatment (100°C) for dissociation from the peptidoglycan (Rosenbusch, 1974; Osborn and Wu, 1980). Immunoblot analysis of OMP C, employing chicken anti-*H. paragallinarum* HP31 serum, revealed OMP C to be non-immunogenic. In comparison OMPs A, B, E, F and G were recognised in

all four isolates by an immune serum directed against a serotype C vaccine strain, indicating considerable antigenic cross-reactivity amongst serotypes. In particular, OMP A was strongly recognised in all four strains by the immune system, a common situation in iron-regulated proteins. Subsequent experiments found that in response to iron limitation, *H. paragallinarum* organisms expressed four outer membrane iron-regulated proteins of 53, 62, 66 and 94 kDa (Ogunnariwo and Schryvers, 1992), with the 53 and 94 kDa proteins functioning as transferrin receptors. It is possible that this 94 kDa outer membrane protein and OMP A are considerably identical, both structurally and functionally. Transferrin binding outer membrane proteins of similar apparent molecular mass have been identified in pathogens such as *Pasteurella haemolytica* (Ogunnariwo and Schryvers, 1990) and *P. multocida* (Snipes *et al.*, 1988).

Despite the limited use of SDS-PAGE, combined with protein staining or immunochemical detection systems, for allocating *H. paragallinarum* isolates to Kume's established serotypes, much success has been achieved with closely related organisms, encouraging further application of this technique to the infectious coryza causing agent. Related *Haemophilus* species, including *H. influenzae* (Carlone *et al.*, 1985; Van Alphen *et al.*, 1983) and *H. pleuropneumoniae* (Rapp *et al.*, 1986), and *Mycoplasma synoviae* (Gurevich *et al.*, 1995), the etiological agent of a subclinical upper respiratory disease of chickens, are examples of bacteria characterised by SDS-PAGE, in conjunction with a silver protein stain, and immunoblotting. As the investigation carried out by Blackall *et al.* (1990d) did not extend to characterisation of the outer membrane proteins of NAD-dependent serovars, nor did it extend to the NAD-independent *H. paragallinarum* field isolates, there was much scope for further studies.

1.4.5 Restriction endonuclease analysis and ribotyping

Although the examination of proteins has been used successfully to type a variety of different microbes, this technique suffers from the limitation of phenotypic analysis rather than genotypic analysis. It is preferable to analyse the genotype which is not dependent on the expression of specific protein-encoding genes, and is therefore not subject to phenotypic variation. Methods for analysis of nucleic acids can be broadly divided into those which examine the size and structure of individual nucleic molecules (eg. restriction endonuclease analysis of chromosomal DNA), and those which examine the degree of relatedness between molecules by hybridisation procedures (eg. traditional ribotyping) or by restriction analysis of amplified 16S rDNA sequences.

Restriction endonuclease analysis (REA) of genomic DNA isolated from microorganisms has found wide use for identification of minor genomic differences between strains of related species. This method, given the acronym BRENDA (Bacterial Restriction Endonuclease DNA Analysis), involves extraction of chromosomal DNA from a homogenous population of organisms, digestion of the DNA with a restriction endonuclease, and electrophoresis of the DNA in an agarose gel. Restriction endonuclease recognition of specific base pairs in the double-stranded DNA and the subsequent cleavage thereof, generates a set of fragments that constitute a characteristic and reproducible fingerprint for any particular DNA (Towner and Cockayne, 1993).

Successful application of REA for serological typing of *Leptospira interrogan* serogroup Hebdomadis into serovars *hardjo* and *balcanica*, employing the enzyme *Eco*RI, was reported by Marshall *et al.* (1981). The potential for identifying genetically similar strains of *Campylobacter jejuni*, the causative agent of human enteritis, from two different sources (cattle and humans) by REA was demonstrated by Bradbury *et al.* (1984). Similarly, Marshall *et al.* (1985) showed different source isolations of gastroenteric *Escherichia coli* to originate from one epidemic strain. The success of BRENDA prompted Blackall *et al.* (1990c) to apply this technique to Australian isolates of *H. paragallinarum*. The restriction enzyme *Hin*dIII generated three REA profile types amongst the sixteen isolates studied, with reproducible differences in the fragments in the 5.1 to 21.2 kb size range. Although no correlation between serotype and REA profile type was noted, REA was demonstrated to be a useful tool in studying the epidemiology of infectious coryza outbreaks. Subsequent studies (Blackall *et al.*, 1991) established the suitability of REA for comparing the genetic relatedness of *H. paragallinarum* isolates. Four restriction enzymes (*Sma*I, *Bam*HI, *Eco*RI and *Hin*dIII) were examined using eight *H. paragallinarum* isolates from diverse sources. *Bam*HI, *Eco*RI and *Hin*dIII were found to be suitable for use with *H. paragallinarum*. Comparative analysis revealed *Hin*dIII to generate the largest number of DNA fragments, thus allowing ease of observation of genetic variation, and thereby confirming the results previously reported by Blackall *et al.* (1990c). However, these *Hin*dIII REA patterns exhibited little genetic variation between *H. paragallinarum* isolates, suggesting that all Australian isolates prior to 1991 evolved from a few original strains.

Although REA provides the basis of a good typing method, in practice it is often difficult to distinguish individual bands in chromosomal DNA fingerprints, due to their number and close proximity. A combination of REA of chromosomal DNA with nucleic acid hybridisation techniques has since found

extensive application for typing a wide range of microorganisms (Towner and Cockayne, 1993). Nucleic acid hybridisation techniques employ specific, single-stranded, labeled DNA probes which will anneal with any complementary sequence generated following restriction endonuclease treatment of chromosomal DNA of the microorganism of interest. Visualisation of the labeled probe-bound chromosomal DNA fragments is facilitated by one of several commercially available detection kits. The choice of probe is crucial, with the most powerful approach to current probe design exploiting the large data base of known 16S rRNA sequences (Grimont and Grimont, 1991; Towner and Cockayne, 1993).

Ribosomes are multimolecular aggregates of protein and RNA, which are composed of two non-covalently associated subunits, a 30S particle and a 50S particle. The heavy 50S subunit contains two specific ribosomal RNAs (rRNAs)- a 23S rRNA and a 5S rRNA, while the light 30S subunit contains a single 16S rRNA (Wittmann, 1983). These rRNAs are encoded for by the rRNA operon (rrn; Figure 1.5), and are associated with single copies of several different ribosomal proteins. Analysis of the sequence of a 16S rRNA revealed many regions of self-complementarity which are capable of forming double-helical segments. Comparison of 16S rRNA sequences of similar and distantly related microbes revealed the double-stranded regions to be highly conserved. 'Universal' probes specific for these sequences will recognise and hybridise to similar sequences of a wide range of bacteria. As some bacterial species are genetically homogenous, all strains within that species will produce a single rRNA gene restriction pattern. On the other hand, some species show genetic heterogeneity such that different strains within the same species may give different rRNA gene restriction patterns. Comparison of these fingerprints facilitates inter- and intra-species characterisation in techniques known as ribotyping or 16S rDNA restriction analysis (Wittmann, 1983; Grimont and Grimont, 1991; Towner and Cockayne, 1993; Jordens and Leaves, 1997).

The application of ribotyping to a few NAD-dependent and NAD-independent *H. paragallinarum* isolates was conducted by Miflin and co-workers (1995). Briefly, the typing technique involved restriction endonuclease digestion of *H. paragallinarum* chromosomal DNA, employing one of three restriction enzymes *Hin*dIII, *Hpa*II and *Ssp*I, separation of the resulting fragments by agarose gel electrophoresis and subsequent capillary transfer to a nylon membrane. By employing the 16S rRNA universal primers, 27f and 1525r, for polymerase chain reaction (PCR) amplification of the 16S rRNA gene sequence of *H. paragallinarum* strain ATCC 29545, a double-stranded 16S rDNA probe was generated. Following hybridisation of a digoxigenin-labeled 16S rDNA probe to immobilised

complementary DNA sequences, the reaction was visualised using a digoxigenin-detection kit and the resulting DNA fingerprints were analysed for typing.

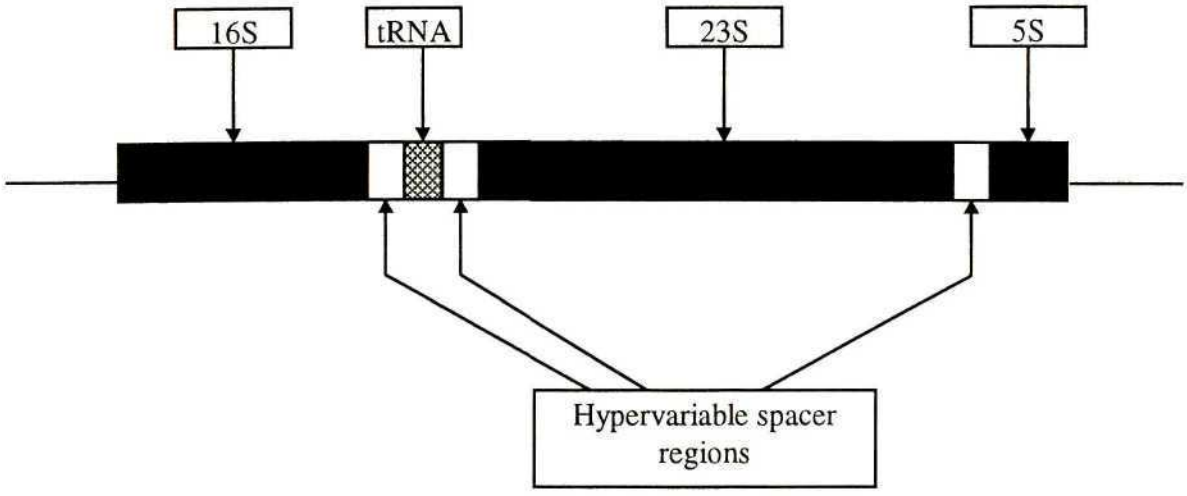


Figure 1.5 Schematic representation of the *rrn* operon, showing the 16S, 23S and 5S rRNA coding genes (adapted from Jordens and Leaves, 1997).

Digestion of *H. paragallinarum* genomic DNA with *Hin*dIII, *Hpa*II and *Ssp*I, resulted in several fragments that contained rRNA gene sequences and were able to bind the 16S rDNA probe. Analysis of the results obtained revealed a correlation between the serotype of the isolate and the ribotyping fingerprint. Some slight banding differences within a serotype were observed and these may enable the subsequent subdivision of isolates into their serovars. In addition, application of ribotyping to the NAD-independent *H. paragallinarum* field isolates was demonstrated, providing evidence of the versatility of this technique and the genetic relatedness of the *H. paragallinarum* strains of varying NAD-dependency.

Further evidence confirming ribotyping as one of the most powerful and highly discriminating molecular-based methods for species identification and epidemiological typing of intra-species bacterial strains was documented. Ribotyping has been successfully employed for the identification of *Campylobacter* species (Moureau *et al.*, 1989), mycoplasmas (Yogev *et al.*, 1988), and *Candida* species (Magee *et al.*, 1987). However, the most valuable application of ribotyping is its ability to type strains within the same species. Isolates of *Pasteurella multocida*, the etiologic agent of a chronic respiratory infection in domestic and wild fowl, were ribotyped in an attempt to elucidate transmission patterns and identify the reservoir(s) of the causative bacterium. Four ribotyping profiles were

identified which demonstrated the presence of three strains of *P. multocida* in a single outbreak, the recurrence of a single strain over an eleven-month period, and the presence of common strains (Snipes *et al.*, 1989). Further studies on isolates of *P. multocida* distinguished the serotype 3,4 live fowl cholera vaccines from serotype 3,4 field strains on the basis of 8 different *Eco*RI- and *Pst*I-generated ribotyping fingerprints (Snipes *et al.*, 1990). These results support the use of ribotyping with a 16S rDNA probe for taxonomic and epidemiologic investigations on avian bacterial infections. This method has also been used for intra-species characterisation of *Campylobacter jejuni* and *C. coli* (Russell *et al.*, 1994) and *Streptococcus iniae* strains (Eldar *et al.*, 1997).

The use of nucleic acid hybridisation analysis as a specific and sensitive tool for typing and epidemiological studies has been irresputably demonstrated. The major draw-back of this method is that it is labour-intensive, time-consuming and relatively complex. PCR-based methods of strain characterisation that specifically target *rrn* sequences have been applied to the inter- and intra-species characterisation of bacteria (Jordens and Leaves, 1997). Amplified 16S rDNA restriction analysis (16S ARDRA) has been shown to differentiate species of, amongst others, streptococci (Jayarao *et al.*, 1992; Eldar *et al.*, 1997). The application of this technique for differentiating NAD-dependent and -independent *H. paragallinarum* strains has not been reported.

1.5 Pathogenicity of *H. paragallinarum*

Little is known about the factors influencing virulence of *H. paragallinarum*, even though several surface-located components have been proposed as virulence determinants with potential as targets for vaccine design. At least three virulence-associated antigens have been described for *H. paragallinarum*, including the hyaluronic acid-containing capsule (Sawata and Kume, 1983), a lipopolysaccharide resembling the heat-stable antigen (Iritani *et al.*, 1980b), and a protective polysaccharide, that is toxic to the host chicken (Iritani *et al.*, 1981b).

Highly encapsulated organisms of *H. paragallinarum* were found to induce gross lesions in chickens, whereas nonencapsulated organisms lacked pathogenicity, thereby correlating the degree of virulence with the amount of capsule (Sawata *et al.*, 1978; Kume *et al.*, 1980a). Hyaluronidase treatment of highly encapsulated variants of serotype 1 destroyed the virulence determinant, rendering the organism non-pathogenic (Kume and Sawata, 1984). This hyaluronic acid-containing capsule was demonstrated

to play a role in the pathogenicity of *H. paragallinarum* as the primary structure associated with attachment to the cilia of the host cells, and the subsequent development of lesions typical of infectious coryza. In addition, the capsule functions as a natural defence structure against the bactericidal action of complement, activated in the absence of antibodies (Sawata *et al.,* 1984b). Colonisation by the nonencapsulated variants is facilitated by the surface-located L and HA-L antigens (Sawata *et al.*, 1985b). Capsule-associated components, especially those of the outer membrane, including lipopolysaccharide, polysaccharide, immunogenic factors and antigens responsible for serological classification, play a crucial role in the survival and multiplication of the organism on the surface of the mucosal cells and are responsible for induction of local inflammation (Matsumoto, 1988). A lipopolysaccharide (a soluble endotoxin) was isolated from the supernatant fluids of cultured serotype A and C strains (Iritani *et al.,* 1980b). This antigen was found to resemble the common heat-stable (HS) agglutinin and was toxic to chickens inducing nasal exudation and inflammation (Matsumoto, 1988). A polysaccharide was isolated from serotype A and C strains which causes hydropericardium in chickens, and may be similar to the L3 agglutinin or it may originate from a capsular substance (Iritani *et al.*, 1981b). The HA-L and L antigens provide protective immunity to all variants of *H. paragallinarum* (Kume *et al.*, 1984).

Clearly, the virulence factors governing *H. paragallinarum* infection are not well characterised. Further studies on these pathogenicity-inducing determinants are clearly essential to identify potential targets for vaccine desig. Such vaccines will curb the number of outbreaks and release the stranglehold this infection places on local poultry industries.

1.6 Control of *H. paragallinarum*: efficacy of current vaccines

The use of vaccines to protect flocks against infectious coryza was first demonstrated by Nelson (1938). He showed that chickens which had recovered from infection displayed a temporary resistance to reinfection with cultures of *H. gallinarum.* Infectious coryza vaccines have been used in South Africa since 1975, but in the 1980s it became apparent that the vaccines were becoming less effective in controlling the disease such that infectious coryza re-emerged in the 1990s as one of the most serious bacterial diseases affecting the local poultry industry (Bragg, 1997).

The severity of infectious coryza has been found to be alleviated by various sulfonamides (e.g. sulfachloropyrazine-sulfadimine and chlortetracycline-trimethoprim) and antibiotics (e.g. erythromycin and oxytetracycline). However, none of these therapeutic agents have been found to be bactericidal and drug resistance does develop (Reece and Coloe, 1985). Greater success has been documented employing a preventative control method and thus the efficacy of current infectious coryza vaccines will be evaluated.

Commercial bacterins, containing broth-propagated, thiomersal-inactivated whole cells of *H. paragallinarum*, adsorbed to an aluminium hydroxide gel adjuvant, provides a safe and effective vaccine, offering protection against infection when injected subcutaneously or intramuscularly (Matsumoto and Yamamoto, 1971; 1975; Davis *et al.*, 1976). Infectious coryza has been found to be one of the few diseases of poultry that can be prevented by implementing efficient vaccination schemes; in which bacterins of sufficient quality are capable of preventing:

i) clinical signs of coryza, including nasal exudates, sinus swelling and facial edema with conjunctivitis;

ii) persistence of the organism in the respiratory tract and;

iii) egg production and weight gain loss (Matsumoto, 1988).

Extensive research has been undertaken with regard to the selection of strains for inclusion in an infectious coryza vaccine, especially since several workers have established that immunity induced by bacterins is serotype-specific (Rimler *et al.*, 1977a; Kume *et al.*, 1980c; Blackall and Reid, 1987). In an extensive cross-protection study carried out by Rimler *et al.* (1977a), vaccines against serotype A organisms provided protection against a homologous challenge, but not against a heterologous challenge (i.e. serotype C). These observations were confirmed by Kume *et al.* (1980c), who found that there was no cross-protection between serotype 1 and serotype 2, each serotype characterised by serotype specific antigen L1 and L2 respectively. A subsequent study carried out on serotype 1 organisms provided further evidence that serotype 1 *H. paragallinarum* isolates provide protection against challenge exposure with homologous isolates (Kume and Sawata, 1984). The correlation between serotype and protective immunity was also established in Australia where protection against *H. paragallinarum* serotypes A and C was afforded by aluminium hydroxide-adsorbed, thiomersal-inactivated vaccines in a serotype-specific manner (Blackall and Reid, 1987). The introduction of bivalent and trivalent vaccines was necessitated to provide adequate protection against the different serotypes found in the field. Bacterins containing inactivated *H. paragallinarum* whole

cells of serotypes A (0083), B (Spross), and C (H-18) provide broader protection fifty-five weeks post-vaccination (Jacobs *et al.*, 1992). However, protection against serotype B and C strains was not equivalent to that found for serotype A and unexpectedly, the trivalent vaccines did not provide protection against challenge by serotype C organisms six weeks post-vaccination.

Protective immunity is thus related to the serotype of the organism included in the vaccine and the serotype of the challenging organism. These findings lead to the conclusion that the serotype-specific antigens may play a protective role. It has since been established that chickens which have L agglutinins (Kume *et al.*, 1980a; c) and haemagglutination-inhibition antibodies against HA-L antigen (Sawata *et al.*, 1982) are protected against bacterial challenge. Kume *et al.* (1980a; b) established that flocks inoculated with serotype 1 strains produced both serotype 1-specific agglutinin and haemagglutination-inhibition antibody, whereas those injected with serotype 2 produced serotype 2-specific agglutinin and were protected against homologous challenge exposure. Following treatment of the sonicated serotype 2 strains with potassium thiocyanate, detection of the haemagglutination-inhibition antibody was possible (Sawata *et al.*, 1982). Haemagglutination inhibition tests revealed serotype 2 haemagglutinin to be immunologically distinct from serotype 1 haemagglutinin, and that the HI antibody titre correlated with protective activity against challenge exposure with serotype 2 (C). A significant correlation was observed between the haemagglutination-inhibition antibody titre of the serum of chickens vaccinated with inactivated *H. paragallinarum* vaccines and the rate of clearance of these organisms from the nasal passages of the upper respiratory tract (Kume *et al.*, 1984). The greater the HI titre against HA-L haemagglutinin, the more rapid the rate of nasal clearance. Chickens with a serum titre of 1:40 or higher completely cleared a challenge exposure within 12 hours, whereas chickens with titres of between 1:5 and 1:20 cleared the administered bacteria within 48 hours post-challenge. Birds with HI titres lower that 1:5 did not clear *H. paragallinarum* organisms from the nasal passages which were thus able to establish themselves in the host and elicit clinical signs of infectious coryza in 2 days (Kume *et al.*, 1984). The identification of the antigenic determinants providing protective activity is crucial in vaccine design.

Of interest is the degree of cross-protection amongst the serovars within a serotype. Using the Kume scheme, Australian isolates of *H. paragallinarum* employed in vaccine production were classified as serovars C-2, C-4, and A-4 (Eaves *et al.*, 1989; Blackall *et al.*, 1990a). Cross-protection was found between serovars C-2 and C-4 when challenged by serovars C-4 (90% and 100% respectively) and C-2

(50% and 70% respectively). In contrast, poor cross-protection was noted when a vaccine based on a serovar A-4 strains was challenged with either serovar C-2 or C-4 (Blackall, 1991). These observations stress the need for efficient monitoring systems for serological determination of the *H. paragallinarum* field isolates to facilitate their inclusion in the local vaccines.

Over the last thirty years *H. paragallinarum* isolates of serovar A-1, B-1, C-2, and C-3 (Figure 1.6) have been serotyped (Bragg, 1997). The distributions of isolates among these serovars indicate that the number of serotype A isolates had decreased significantly, due to a high level of cross-protection between the different serovars of serotype A, whereas the number of C-3 isolates had increased suggesting very limited cross-protection between C-3 and C-1 or C-2. These changes in the relative abundance of the different serovars may be explained by considering the composition of infectious coryza vaccines employed in South Africa. The first vaccine produced in this country in 1975 consisted of strain 0083 (serovar A-1). In 1982, the valency of this vaccine was increased with the emergence of serotype B (0222) strains, and by 1995 had increased again to include a serovar C-2 field isolate (Bragg *et al.*, 1996). These results indicate that provided the current vaccination protocols contain *H. paragallinarum* isolates representing the serovars predominating in the field, vaccines provide an effective preventative method for controlling infectious coryza. Implementation of efficient monitoring systems for serotyping the organisms causing infection in the field will enable the implementation of effective and efficient vaccination schemes.

The recent emergence of NAD-independent isolates of serotype A and C-3, potentially capable of evading the immune response of the host, provides concern that current vaccination protocols may become ineffective against these NAD-independent isolates (Bragg *et al.*, 1997). Further research on the serotypes of the NAD-independent isolates, and hence the serotype-specific antigens that confer protection, is required to assess the efficacy of current vaccination protocols on the NAD-independent isolates.

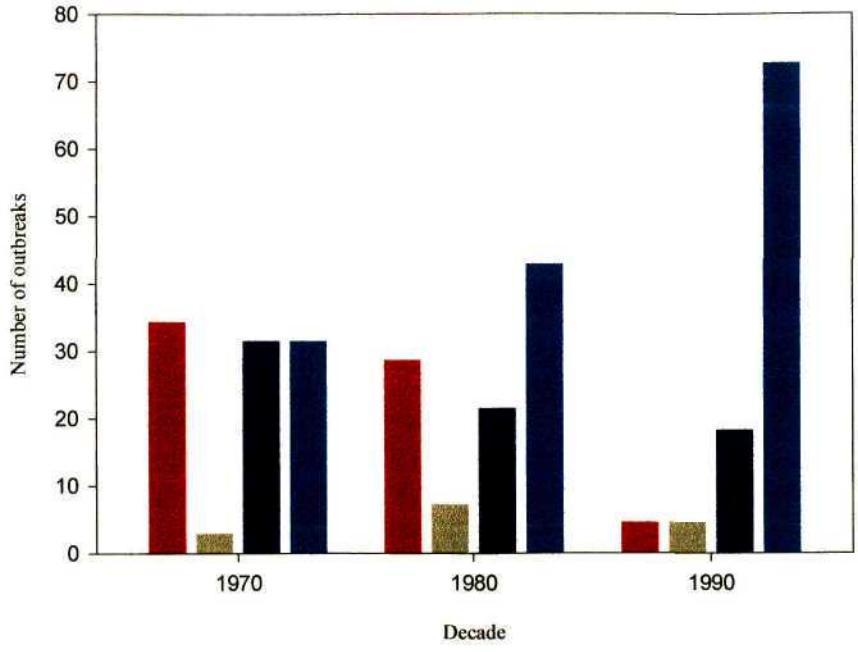


Figure 1.6 Changes in the relative abundance of serotypes A (■), B (■), and C (■), and serovar C-3 (■) of NAD-dependent *H. paragallinarum* isolates over the past three decades (Bragg *et al.*, 1996).

1.7 Objectives of the current study

Immunity against re-infection displays a dependency on the serotype, and to an extent the corresponding serovar, of the infecting NAD-dependent or NAD-independent *H. paragallinarum* field isolate. Serological determination of the *H. paragallinarum* field isolates, employing a variety of agglutination tests, has been met with variable success. Consequently, the chief aim of the current study was to identify a suitable, unambiguous sero-determining technique offering the advantages of rapidity and convenience. Following a PCR-based diagnosis of the isolated organisms as *H. paragallinarum* (Chapter 3), characterisation of the NAD-dependent and the highly virulent NAD-independent *H. paragallinarum* strains was carried out. Two different approaches were explored: one was based on the physical traits of the organism, while the other utilised conserved ribosomal RNA sequences. The phenotypic-based study was facilitated by comparison of the whole cell and outer membrane proteins by Tris-tricine SDS-PAGE, in conjunction with a gel staining or an immunochemical detection system (Chapter 4). In addition, unique protein determinants were identified for inclusion in the current vaccine as a target for a conditioned immune response. Furthermore, as a result of the successful application of genotypic typing techniques to strains of *H. paragallinarum*, in which the conserved 16S rRNA gene sequences are exploited, a similar procedure was undertaken for type analysis of the locally isolated NAD-independent *H. paragallinarum* bacteria (Chapter 5).

CHAPTER 2

General Materials and Methods

A variety of fundamental biochemical techniques were employed throughout this study and are described here for convenience. More specific experimental details are described later in their appropriate sections.

2.1 Materials

Most of the commonly used chemicals used in this study were from BDH (Poole, England), Merck (Darmstadt, Germany) or Boehringer Mannheim (Mannheim, Germany), and were of the highest purity available. Protein molecular mass standards were obtained from Pharmacia LKB Biotechnology (Lund, Sweden). The restriction endonucleases *Hin*dIII, *Bam*HI, *Stu*I and *Apa*I, lambda DNA, a set of deoxynucleotide triphosphates (dNTPs), a digoxigenin DNA labeling and detection kit, bovine serum albumin (BSA) and 2,2'-azino-di-(3-ethyl)-benzthiozoline sulphonic acid (ABTS), were from Boehringer Mannheim (Mannheim, Germany). The restriction endonucleases *Hpa*II, *Ssp*I, and *Sma*I were purchased from New England BioLabs (Hertfordshire, UK), 0.2 ml natural thin wall PCR tubes were from Quality Science Plastics (Petaluma, USA), and the Qiaex II agarose gel extraction kit was from Qiagen (Hilden, Germany). Biotin hydrazide, nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), ovalbumin, oleic acid-albumin, nicotinamide adenine dinucleotide (NAD), RNAse A, thiamine-HCl, extravidin-alkaline phosphatase conjugate, and rabbit anti-chicken IgY- and sheep anti-rabbit IgG-alkaline phosphatase conjugate were obtained from Sigma (St Louis, USA). Nunc Immuno Maxisorp F96 multiwell plates were from Nunc Intermed (Roskilde, Denmark). Polystyrene cell culture flasks and Petri dishes, disposable sterile 0.45 µm pre-filters and disposable sterile polypropylene cryogenic vials were from Corning/Costar (Cambridge, USA). Chicken serum, casein hydrolysate and primers for polymerase chain reaction amplification were obtained from Gibco BRL Life Technologies (Paisley, England). Columbia blood agar was purchased from Unipath (Hampshire, England) while *Taq* DNA polymerase was obtained from Bioline (London, UK). Microbank® beads were from Pro-Lab diagnostics (Austin, USA). Magnagraph transfer nylon membrane was from Micron separations (Westborough, USA) whereas Hybond™C hybridisation transfer membrane was obtained from Amersham International

(Buckinghamshire, UK). Distilled water (dist. H_2O) was obtained with a Milli-RO® 15 Water Purification System (Millipore, Marlboro, USA). Deionised water was obtained with a Milli-Q Plus Ultra-Pure Water System (Millipore, Marlboro, USA), and had a minimum resistivity of 18 MΩ.cm.

2.2 Protein assays

Studies involving the characterisation of proteins require methods for specific, reproducible, and preferably rapid quantitation of small amounts of sample. The micro-Bradford dye binding assay (Bradford, 1976), as modified by Read and Northcote (1981), was employed in this study for protein quantitation of all samples where the detergent Triton X-100 was absent. Any solutions containing Triton X-100 were quantified using the disodium salt bicinchoninic acid (BCA-Na_2) (Smith *et al.*, 1985).

2.2.1. Bradford Dye-binding assay

The Bradford dye-binding assay was routinely used for protein quantitation because it provides a simple, rapid, sensitive, specific and inexpensive technique for protein quantitation (Bradford, 1976; Read and Northcote, 1981). The assay is based on a change in absorbance maximum from 465 nm (cationic red form) to 595 nm (anionic blue form) when the Serva Blue G dye binds to the protein. At 595 nm, the extinction coefficient of the blue dye-protein complex is far greater than that of the unbound dye, and allows for higher sensitivity in protein quantification (Bradford, 1976). The dye binds primarily to basic amino acids, especially arginine and to a lesser extent to histidine, lysine, tyrosine, tryptophan and phenylalanine via van der Waals forces and hydrophobic interactions (Compton and Jones, 1985).

A major advantage of the technique is the relative lack of interference by most commonly used chemicals. The effects of small amounts (less than 1%) of Tris, acetic acid, sucrose, 2-mercaptoethanol, glycerol, ethylene diamine tetraacetic acid (EDTA), Triton X-100 and sodium dodecyl sulfate (SDS) can be eliminated using an appropriate blank. However, at concentrations of 1% or greater, the anomalies caused by the interfering compound cannot be overcome and the compound (most commonly detergents) must be removed (Bradford, 1976).

The major disadvantage of this assay is the wide variation in dye-binding of different individual proteins, necessitating use of a suitable protein standard for each particular application. To minimise this variation, Read and Northcote (1981) modified the acid/alcohol ratios and increased the dye concentration, thereby increasing sensitivity but retaining the simplicity and rapidity of the original method described by Bradford (1976). This modified assay is linear up to 25 μg of protein (Splittgerber and Sohl, 1989).

Three protein assays have been established: a macro-assay with a working range of 5-25 μg of protein, a micro-assay for the determination of 1-5 μg of protein and a microtitre plate assay which is accurate in the range 0.2-1 μg of protein. The micro-assay was employed in the present study.

2.2.1.1 Materials

Dye Reagent. Serva Blue G dye (50 mg) was dissolved in a mixture of 88% (v/v) phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). The solution was made up to 500 ml with dist. H_2O and stirred for 30 min at room temperature on a magnetic stirrer. The resulting solution was filtered through Whatman No. 1 filter paper and stored in an amber coloured bottle for up to 6 months. Visual checks were made for precipitation prior to use, and if precipitation was observed, the reagent was filtered and re-calibrated.

Standard protein solution. Ovalbumin (1 mg) was dissolved in dist. H_2O (1 ml). This solution was diluted in dist. H_2O to 100 μg/ml for the assay.

2.2.1.2 Procedure

Assays for the standard curve were carried out in quintuplicate at 5 concentrations of ovalbumin. Ovalbumin standard (10-50 μl of a 100 μg/ml working solution i.e. 1-5 μg protein) was diluted to a final volume of 50 μl with dist. H_2O in polyethylene microfuge tubes. Dye reagent (950 μl) was added and the solution was immediately mixed by inversion of the tube. The colour was allowed to develop for 2 min after mixing, and the A_{595} was read in 1 ml plastic microcuvettes. The colour was stable for up to 1 hour, so accurate timing was not necessary (Bradford, 1976). Concentrated samples were diluted in buffer, and dye reagent was added as before. Buffer alone, added to dye reagent, served as the blank. The cuvettes were cleaned after use with 25% (v/v)

sodium hypochlorite. Linear regression analysis of the data yielded the following equation allowing protein concentration of the samples to be calculated:

$$\text{Protein concentration } (\mu g/50\ \mu l) = \frac{A_{595} - 5.714 \times 10^{-4}}{0.1503}$$

2.2.2 Bicinchoninic Acid (BCA) Assay

BCA, in the form of its water-soluble sodium salt, provides a sensitive, stable and highly specific complexing reagent for the detection of copper. In this assay the Folin-Ciocalteau reagent, used in the Lowry assay (Lowry *et al.*, 1951), is replaced with BCA thereby overcoming some of the limitations of the Lowry procedure. In alkaline solution, a coloured complex will form between the protein's peptide bonds and the copper atoms (Cu^{2+}), thereby reducing the copper. BCA can then form a 2:1 complex with Cu^{+}, resulting in a stable, highly coloured chromophore with an absorbance maximum at 562 nm (Smith *et al.*, 1985). The total colour yield is a sum of contributions from two different sources: readily oxidisable protein components (e.g. tyrosine, cysteine and tryptophan), and the reaction between the peptide bonds and divalent copper.

The sensitivity and intensity of colour development is directly related to the temperature of incubation. Therefore a range of incubation protocols, varying the temperature and time of incubation, were investigated which generated several colour response curves indicating the optimal incubation protocol to be employed for protein concentration determination within a specific range. The following incubation protocols yielded satisfactory results:

i) room temperature for 2 hours, with a sensitivity for 20-120 µg of protein;

ii) 37°C for 30 min, with a sensitivity for 20-120 µg of protein and;

iii) 60°C for 30 min, with a sensitivity for 5-25 µg of protein (Smith *et al.*, 1985).

An advantage of the BCA assay is its relative resistance to interference by detergents and denaturing agents. However, reducing sugars, copper-chelating reagents, especially EDTA, and buffers which will change the pH of the reaction solution, will interfere considerably with the reaction.

This method was employed in the present study as a comparison to the results obtained from the Bradford assay (Section 2.2.1), as the samples employed often contained more that 1% (v/v) Triton X-100.

2.2.2.1 Materials

BCA stock reagents.

Reagent A: Bicinchoninic acid (BCA-Na_2; 5 g), $Na_2CO_3.H_2O$ (10 g), Na_2 tartrate (0.8 g), NaOH (2 g) and $NaHCO_3$ (4.75 g) were dissolved in 480 ml of dist. H_2O. The pH was adjusted to 11.25 using 50% (v/v) NaOH or solid $NaHCO_3$, and the solution was made up to 500 ml with dist. H_2O.

Reagent B: $CuSO_4.5H_2O$ (8 g) was dissolved in dist. H_2O (200 ml).

BCA working reagent. Reagent A (100 vol) was mixed with Reagent B (2 vol). The resulting solution was an apple-green colour and was prepared weekly.

Standard protein solution. Ovalbumin (1 mg) was dissolved in dist. H_2O (1 ml). This solution was diluted in dist. H_2O to 100 µg/ml for the assay.

2.2.2.2 Procedure

Assays for the standard curve were carried out in quintuplicate at 5 concentrations of ovalbumin. Ovalbumin standard (20-100 µl of a 100 µg/ml working solution i.e. 2-10 µg protein) was diluted to a final volume of 100 µl with dist. H_2O in polyethylene microfuge tubes. BCA working solution (2 ml) was added to the protein solution with immediate colouring. The solutions were incubated at 37°C for 30 min, cooled to room temperature and their absorbances were measured at 562 nm. Test samples were diluted in buffer, and dye reagent was added as before. Buffer alone added to BCA working reagent served as the blank. Linear regression analysis of the data yielded the following equation allowing protein concentration of the samples to be calculated:

$$\text{Protein concentration } (\mu g/100\ \mu l) = \frac{A_{562} - 7.0 \times 10^{-4}}{0.0177}$$

2.3 Tris-tricine SDS-PAGE

Electrophoresis describes the separation of charged particles through a molecular sieving gel towards an electrode of opposite charge, under the influence of an externally applied electric field. Native PAGE cannot distinguish between the effects of size, shape and charge on electrophoretic mobility. SDS-PAGE was developed to overcome these limitations, and is regularly employed as an analytical technique for the evaluation of the purity of a protein sample (Laemmli, 1970). SDS is an anionic detergent which binds tightly to most proteins at about 1.4 mg of SDS per mg of protein. Globular native amphoteric proteins are thereby converted into highly negatively charged rodlike complexes, the length of which is dependent on the molecular mass of the protein. In this way, the charge to mass ratio of all proteins in the sample are rendered equal and proteins are subsequently separated on the basis of size alone. A truer estimation of molecular mass may be obtained by treatment of the sample with a reducing agent, such as 2-mercaptoethanol, which effectively opens up the protein molecule facilitating unhindered binding of the SDS to disulfide bonded regions. As an inverse relationship exists between the logarithm of molecular mass of a protein and the distance migrated by the protein in the gel, a standard curve may be constructed by running standard proteins of known molecular mass alongside the protein sample of interest.

For bands to be well resolved, the starting band of sample must be as narrow as possible, and the diffusion of the protein bands in the gel must be minimised. With this in mind Ornstein (1964) and Davis (1964) developed a discontinuous system, which makes use of two different buffer systems and two different gel porosities. The sample and stacking gel contain a Tris-HCl buffer, pH 6.8, whereas the pHs of the Tris-HCl running gel buffer and the Tris-glycine electrode buffer are 8.8 and 8.3 respectively. At pH 6.8 the glycine ions are poorly dissociated and have a small negative charge and hence a low mobility. The proteins are stacked into a sharp interface between the highly mobile chloride ions and the glycine ions. At the separating gel interface, the increased pH causes the glycine ion to dissociate further, thereby increasing their mobility. The glycine ions overtake the proteins, which are left to destack in a uniform voltage gradient at constant pH. In the separating gel the protein stack is subjected to molecular sieving effects which resolve the proteins on the basis of their size and shape.

In a modification of the system described by Laemmli (1970), the upper cathodic chamber contains a Tris-tricine buffer (pH 8.9), where the more mobile tricine ion has replaced the

traditional glycine ion (Schägger and von Jagow, 1987). In addition, the separating and stacking gels, as well as the sample and the lower anodic chamber contain a Tris-HCl buffer with pH-values in the range 8.25 to 8.45. When an electrical potential is applied, proteins will become focused in the stacking gel between the tricine (trailing) and chloride (leading) ions. The use of the tricine trailing ion, as well as the increased pH employed in this system, facilitates increased resolution of the low M_r proteins from the SDS micelles, especially in the 5-20 kDa range.

2.3.1 Materials

Monomer solution [49.5% (m/v) acrylamide, 3% (m/v) *N,N'*-methylenebisacrylamide]. Acrylamide (48 g) and *N, N'*-methylenebisacrylamide (3 g) were dissolved in 100 ml of deionised H_2O. The solution was filtered through Whatman No. 1 filter paper and stored at room temperature in an amber-coloured bottle.

Gel buffer [3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris (72.7 g) and SDS (0.6 g) were dissolved in 150 ml deionised H_2O, the pH was adjusted to 8.45 with HCl and the buffer was made up to 200 ml with deionised H_2O. The buffer was filtered through Whatman No.1 filter paper and stored at 4°C.

Anode buffer [0.2 M Tris-HCl, pH 8.9]. Tris (24.22 g) was dissolved in 950 ml of dist. H_2O, the pH was adjusted to 8.9 with HCl and the buffer was made up to 1 litre with dist. H_2O.

Cathode buffer [0.1 M Tris-HCl, 0.1 M tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (12.2 g), tricine (17.9 g) and 10% (m/v) SDS (10 ml) were thoroughly mixed in 1 litre of dist. H_2O and the pH was adjusted to 8.25 with HCl if necessary.

10% (m/v) Ammonium persulfate. Ammonium persulfate (0.1 g) was dissolved in dist. H_2O (1 ml) just before use.

Non-reducing treatment buffer [3 M Tris-HCl, 4% (m/v) SDS, 20% (m/v) glycerol, pH 8.45]. Gel buffer (2.5 ml), 10% (m/v) SDS (4 ml) and glycerol (2 ml) were made up to 10 ml with dist. H_2O. Aliquots (1 ml) were stored frozen for up to 3 months.

Molecular mass markers. Standards for molecular mass determination were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Lyophilised markers were reconstituted in non-reducing treatment buffer (100 µl) and heated at 100°C for 5 min for Coomassie staining or immunoblot analysis. For silver staining, the reconstituted markers were diluted 1:50 in non-reducing treatment buffer, and stored at 4°C.

2.3.2 Procedure

For SDS-PAGE, the Bio-Rad Mini-PROTEAN II® electrophoresis cell was assembled as described in the manufacturer's manual. Before use, the glass plates, spacers, combs and casting stand gaskets were washed with soap water and cleaned with alcohol. The two glass plates (inner plate 7.3×10.2 cm, outer plate 8.3×10.2 cm) were positioned in the clamp assembly, separated by 1.5 mm polyethylene spacers. Removable silicone gaskets ensure that the acrylamide solution does not leak from the sandwich assembly.

Table 2.1 Reagents for two Tris-tricine (Schägger and von Jagow, 1987) gels for the Bio-Rad Mini-PROTEAN II® electrophoresis cell.

Reagent	Separating Gel (10%)	Stacking Gel (4%)
Gel Buffer (ml)	5	1.5
Monomer (ml)	3	0.5
Ammonium persulfate (µl)	50	30
TEMED (µl)	5	12
Dist. H_20 (ml)	7	4

The separating gel solution was run into the space between the two glass plates, to a depth of 3 cm from the top of the outer glass plate, and was overlayered with dist. H_2O to exclude atmospheric oxygen which inhibits polymerisation. Polymerisation involves production of an acrylamide monomer chain which is cross-linked by the bifunctional compound *N*, *N*'-methylenebisacrylamide. *N*, *N*, *N*', *N*'-tetramethylethylenediamine (TEMED) catalyses the formation of free radicals from ammonium persulfate, which in turn initiate polymerisation (Hames and Rickwood, 1981). Once the gel had set (approx. 1 h, evidenced by the re-appearance of an interface between the gel solution and the overlayered dist. H_2O), the water was removed by inversion of the casting stand. Stacking gel solution was poured into the sandwich to the top of the outer glass plate, and a 10-well comb inserted to form the sample application wells. Once the gel had set, the comb was removed and the wells were washed with dist. H_2O. The gel sandwiches were then transferred from the casting stand to the U-shaped inner cooling core gaskets, creating an upper and a lower chamber in the electrophoresis unit.

Cathode buffer (containing SDS) was poured into the upper chamber. Sample (at least 100-300 ng protein per band for silver staining) was mixed with an equal volume of non-reducing treatment buffer. The samples were incubated in a boiling water bath for 90 s to facilitate more uniform binding of the SDS. Bromophenol blue marker dye (1-3 µl), which migrates with the buffer front, was added to each sample prior to loading to monitor the progress of electrophoresis. Anode buffer was introduced into the lower chamber and the apparatus was connected to a power pack. The voltage was set at 80 V (maximum current) until the bromophenol blue front had migrated through the stacking gel. The voltage was then increased to 100 V for the remainder of the electrophoretic run. Electrophoresis was continued until the bromophenol blue marker dye was 0.5 cm from the bottom of the separating gel. The gels were removed and placed in an appropriate staining solution for visualisation or prepared for immunoblot analysis.

2.4 Silver staining of proteins in polyacrylamide gels

This method provides an attractive alternative to the traditionally used Coomassie blue G-250 protein stain, in that proteins may be detected in the nanogram range. The sensitivity of this technique, which is based on the reduction of silver ions to metallic silver, is only surpassed by radioactive labelling. However, several practical problems including reproducibility, ease of

handling and background staining were previously not sufficiently solved. Blum *et al.* (1987) modified the silver staining procedure to avoid non-specific background staining without the loss of sensitivity or contrast. They observed that the addition of sodium thiosulfate, which chemically dissolves silver salts by complexation, facilitated optimal contrast between the visualised protein on a colourless, transparent background and pre-treatment with this salt increased the sensitivity of this technique. In this way the practical problems previously described were overcome. Increased sensitivity demanded that extra care was taken in cleaning electrophoretic plates and staining containers by soaking them in nitric acid.

2.4.1 Materials

30% (v/v) Nitric acid. Nitric acid [545 ml of a 55% (v/v) solution] was diluted to 1 litre with deionised H_2O.

Fix solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) formaldehyde]. Methanol (100 ml), acetic acid (24 ml) and formaldehyde [100 µl of a 37% (v/v) solution] were diluted to 200 ml with deionised H_2O.

Wash solution 1 [50% (v/v) ethanol]. Ethanol (100 ml) was diluted to 200 ml with deionised H_2O.

Pretreat solution [0.02% (m/v) sodium thiosulfate]. $Na_2S_2O_3.5H_2O$ (40 mg) was dissolved in deionised H_2O (200 ml).

Impregnate solution [0.2% (m/v) silver nitrate, 0.075% (v/v) formaldehyde]. $AgNO_3$ (400 mg) was dissolved in deionised H_2O (199.85 ml) and formaldehyde [150 µl of a 37% (v/v) solution] was added just before use.

Develop solution [6% (m/v) sodium carbonate, 0.0004% (m/v) sodium thiosulfate, 0.05% (v/v) formaldehyde]. Na_2CO_3 (12 g) and $Na_2S_2O_3.5H_2O$ (4 ml of pretreat solution above) were thoroughly mixed in 195 ml of deionised H_2O. Formaldehyde [100 µl of a 37% (v/v) solution] was added just before use.

Stop solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (100 ml) and acetic acid (24 ml) were diluted to 200 ml with deionised H_2O.

Wash solution 2 [50% (v/v) methanol]. Methanol (100 ml) was diluted to 200 ml with deionised H_2O.

2.4.2 Procedure

All steps were carried out on an orbital shaker at room temperature and in scrupulously clean glass containers. Gloves were worn to prevent contamination of the gels. After electrophoresis, the gels were soaked in fix solution for more than 1 h (preferably overnight) and washed in wash solution 1 (3 × 20 min) to remove any acetic acid. Gels were placed in pretreat solution (1 min), washed in deionised H_2O (3 × 20 s) and placed in the impregnate solution (25 min). After washing (2 × 20 s in deionised H_2O), the developer was added. As soon as bands were visible, the gels were washed in deionised H_2O until the bands were fully developed. The stop solution was added (> 20 min), the gels washed in wash solution 2 (10 min), and stored in a sealed plastic bag, in the dark, until photographed.

2.5 Immunochemical techniques

2.5.1 Generation and isolation of antibodies

Antibodies were raised in chickens and rabbits following inoculation with antigen emulsified with an adjuvant. The adjuvant enhances an immune response by providing a depot from where the antigen is slowly released. It also stimulates cellular responses such as those provided by macrophages. The presentation of the antigen on the surface of the macrophages increases the efficiency of their presentation to the lymphocytes and hence facilitates prolonged and improved antigen exposure to the immune system of the experimental animal (Roitt, 1991). Generally, the adjuvant used in the initial inoculation is Freund's complete adjuvant, which contains killed *Mycobacterium tuberculosis* cells and mineral oil, and is prepared by trituration of the immunogen and the adjuvant. Subsequent inoculations contain the antigen of interest emulsified with Freund's incomplete adjuvant, which consists of mineral oil, to further stimulate the selected B-cell clone, which produces antibodies specific to the inoculated antigen (Roitt, 1991).

Some bacteria and their isolated antigens are extremely immunogenic, possessing the ability to stimulate and maintain an immune response without the addition of adjuvants (Thorpe, 1994). In the present study an aluminium hydroxide gel (commercially available as 'Alhydrogel'), to which *H. paragallinarum* whole cell bacteria were adsorbed, was employed during antibody production (Reid and Blackall, 1987). The antibody-stimulating ability of these microorganisms was exploited during booster injections, in which the aluminium hydroxide adjuvant was omitted from intravenously inoculated normal saline suspensions of live *H. paragallinarum* bacteria.

The water-soluble polymer, polyethylene glycol (PEG), has been successfully employed for the simple and convenient precipitation of IgG and IgY (Polson *et al.*, 1964; 1985). PEG is a non-denaturing precipitating agent which operates on a steric exclusion mechanism whereby proteins are concentrated in the extrapolymer space, eventually exceeding their solubility limit (Ingham, 1990). The concentration of the polymer required to precipitate a protein is a function of the nett charge on the molecule, which is determined by the pH of the surrounding medium (Polson *et al.*, 1964).

The protocol used to purify IgG from rabbit serum was that described by Polson *et al.* (1964), while a similar procedure (Polson *et al.*, 1980; 1985) was followed for the isolation of IgY from chicken egg yolks.

2.5.1.1 Materials

Borate-buffered saline, pH 8.6. Boric acid (2.16 g), NaCl (2.19 g), NaOH (0.7 g) and 37% (v/v) HCl (0.62 ml) were dissolved in 950 ml of dist. H_2O, the pH was adjusted to 8.6 with NaOH, and the buffer was made up to 1 litre with dist. H_2O.

100 mM Na-phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. $NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist. H_2O, the pH was adjusted to pH 7.6 using NaOH, and the solution was made up to 1 litre with dist. H_2O.

2.5.1.2 Procedure for the isolation of IgG from rabbit serum

Blood was obtained from rabbits by cardiac puncture and allowed to clot overnight at 4°C. The supernatant serum was separated by centrifugation (1000 x g, 10 min, 4°C), and mixed with

2 volumes of borate-buffered saline. Solid PEG (6 kDa) was added to the diluted serum to a concentration of 14% (m/v), dissolved with constant, gentle stirring, and the resulting solution centrifuged (12 000 x g, 10 min, RT). The pellet was dissolved in the original serum volume, using 100 mM Na-phosphate buffer, pH 7.6. PEG was again added to a final concentration of 14% (m/v), dissolved by stirring, and the solution was centrifuged (12 000 x g, 10 min, RT). The pellet was re-dissolved in half of the original serum volume, using 100 mM Na-phosphate buffer, pH 7.6, containing 60% (v/v) glycerol. IgG fractions were stored at -20°C.

To determine the concentration of IgG, the absorbance of a 1/40 dilution of a purified IgG fraction in 100 mM Na-phosphate buffer, pH 7.6, was read at 280 nm in 1 cm quartz curvettes against a Na-phosphate blank. The protein concentration was calculated using the equation:

$A = Elc$

where A = Absorbance at 280 nm,

E = Extinction coefficient of IgG = 1.43 mg/ml/cm (Hudson and Hay, 1980) and,

c = IgG concentration.

2.5.1.3 Procedure for the isolation of IgY from chicken egg yolks

Egg yolks were separated from the egg white and carefully washed under running water to remove all traces of albumin. The yolk sac was punctured and the yolk volume determined in a measuring cylinder. Two volumes of 100 mM Na-phosphate buffer, pH 7.6, was added and mixed in thoroughly. Solid PEG (6 kDa) was added to a final concentration of 3.5% (m/v) and dissolved by gentle stirring. The precipitated vitellin fraction was removed by centrifugation (4 420 x g, 30 min, RT), and the supernatant was filtered through absorbent cotton wool to remove the lipid fraction. The PEG concentration was increased to 12% [i.e. 8.5% (m/v) was added], the solution was mixed thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant was discarded and the pellet was dissolved in 100 mM Na-phosphate buffer, pH 7.6, in a volume equal to that obtained after filtration. PEG was then added to a final concentration of 12% (m/v), the solution was mixed thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant was discarded and the final antibody pellet was dissolved in 1/6 of the original egg yolk volume, using 100 mM Na-phosphate buffer, pH 7.6, and stored at 4°C.

The concentration of IgY was determined in the same manner as that for IgG, using an extinction coefficient ($E_{280}^{1\ mg/ml}$) of 1.25 (Coetzer, 1985).

2.5.2 Enzyme-linked immunosorbent assay (ELISA)

Immunoassays exploit the specific, high-affinity interaction of an antibody and its complementary antigen to provide information about the concentrations of the antibody or antigen in samples of unknown concentration. In principle, the chemical conjugation of an enzyme to either an antibody or an antigen allows the detection of immune complexes immobilised on a solid phase. This forms the basis of the enzyme-linked immunosorbent assay (ELISA), first introduced by Engvall and Perlmann (1971). This quantitative technique complements western blotting, which provides qualitative information regarding the specificity of the antibody.

An indirect, non-competitive, solid phase ELISA is commonly employed to assess the specificity and titre of an antibody. Briefly, antigen is coated onto the walls of the wells of a polystyrene microtitre plate by non-specific hydrophobic interactions, and the primary antibody to be quantified is allowed to form immune complexes with the immobilised antigen. The degree of reactivity, and hence the amount of primary antibody, is detected using an enzyme conjugated to a secondary antibody, which will recognise the primary antibody-immobilised antigen complex. The detection system employed in the present study utilised sheep anti-rabbit IgG, or rabbit anti-chicken IgY antibodies coupled to horse-radish peroxidase (HRPO) by the method of Hudson and Hay (1980). The enzyme (HRPO) has a high specificity for the hydrogen acceptor H_2O_2, which together catalyse the oxidation of ABTS (2,2′-azino-di-(3-ethyl)-benzthiozoline sulphonic acid), a hydrogen donor, to yield a soluble blue-green-coloured complex which may be measured spectrophotometrically at 405 nm.

In the present study, ELISA was used to monitor the progress of immunisation during inoculation protocols and hence to determine the titre of the rabbit anti-*H. paragallinarum* whole cell (WC) IgG and chicken anti-*H. paragallinarum* WC IgY antibodies. Plots of A_{405} versus log antibody concentration were constructed. The titre of the primary antibodies was taken as the highest dilution at which the primary antibody response was greater than that of a pre-immune antibody control sample.

2.5.2.1 Materials

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $NaHPO_4.2H_20$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dist. H_2O (1 litre).

Fix [70% (v/v) methanol]. Methanol (70 ml) was diluted to 100 ml with dist. H_2O.

0.5% (m/v) Bovine serum albumin-PBS (BSA-PBS). BSA (0.5 g) was dissolved in PBS (100 ml).

0.1% (v/v) PBS-Tween. Tween-20 (1ml) was diluted to 1 litre with PBS.

0.15 M Citrate-phosphate buffer, pH 5.0. A solution of citric acid.H_2O (21 g/l) was titrated with a solution of $NaHPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

Substrate solution [0.05% (m/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]. ABTS (7.5 mg) and H_2O_2 (7.5 µl) were dissolved in citrate-phosphate buffer, pH 5.0 (15 ml) for each ELISA plate.

Stopping buffer (citrate-phosphate-0.1% (m/v) NaN_3). NaN_3 (0.1 g) was dissolved in citrate-phosphate buffer (100 ml).

2.5.2.2 Procedure

Wells of microtitre plates (Nunc Immuno Maxisorp F96 plates) were coated with antigen (150 µl, overnight at 4°C) at a predetermined concentration (0.1 µg/ml) in PBS. The antigen was fixed with 70% (v/v) methanol for 10 min at room temperature. Non-specific binding of antibody was prevented by blocking the wells with 0.5% (m/v) BSA-PBS (200 µl, 1 h at 37°C), followed by washing of wells three times with 0.1% (v/v) PBS-Tween. The same washing step was repeated after each subsequent incubation step with antibodies. Serial doubling dilutions of the primary antibody in 0.5% BSA-PBS (1 mg/ml) was added to individual wells (100 µl) and incubated (2 h at 37°C). The secondary antibody-HRPO conjugate (120 µl) was incubated (1h at 37°C) at a suitable dilution (1:400 for sheep anti-rabbit IgG and 1:300 for rabbit anti-chicken IgY). Finally ABTS/H_2O_2 substrate (150 µl) was added and incubated in the dark for optimal colour

development. The enzyme reaction was terminated after 25 min by the addition of NaN_3-citrate-phosphate buffer (50 µl), and the absorbance was read at 405 nm in a Bio-Tek EL 307 ELISA plate reader. Titration curves were constructed from the spectrophotometric data.

2.5.3 Western blotting

In this technique, proteins separated by Tris-tricine SDS-PAGE (Section 2.3) and immobilised on nitrocellulose, a matrix with a high binding affinity for proteins (approximately 80-100 µg/cm^2; De Maio, 1994), were identified and characterised using specific antibodies as probes. Alternatively, western blotting may be used for the qualitative analysis of antibodies prepared against purified proteins.

The protocol followed was according to that described by Towbin *et al.* (1979), with the following modification. Methanol was included in the transfer buffer to enhance the hydrophobic binding of the protein-SDS complexes to the nitrocellulose (Goodenham, 1984). Following transfer of the proteins of interest, the unoccupied binding sites on the nitrocellulose membrane are blocked with non-fat milk. Detection of the proteins then proceeds immunochemically. Briefly, the antibody of interest is allowed to react with the immobilised antigen. This reaction is detected by an enzyme-linked secondary antibody (e.g. a sheep anti-rabbit IgG-alkaline phosphatase conjugate), which catalyses a reaction with a precipitating substrate (eg. BCIP/NBT) to yield a coloured band on the nitrocellulose membrane.

In the present study western blotting was employed to identify different epitopes on the surfaces of NAD-dependent and -independent *H. paragallinarum* field isolates. Analysis of the whole cell and outer membrane protein profiles generated when probed with each of anti-*H. paragallinarum* WC strain 0083, 0222, Modesto and 95/03938 antibodies, may aid in the serological classification of these field isolates (see Chapter 4).

2.5.3.1 Materials

Blotting buffer [25 mM Tris-HCl, 192 mM glycine, pH 8.3]. Tris (9.08 g) and glycine (43.2 g) were dissolved in 1.4 litres of dist. H_2O and methanol (600 ml) was added. Prior to use, 10% (m/v) SDS (2 ml) was added.

0.1% (m/v) Ponceau S. Ponceau S (0.1 g) was dissolved in 1% (v/v) glacial acetic acid (100 ml).

Tris buffered saline [TBS; 20 mM Tris-HCl, 200 mM NaCl, pH 7.4]. Tris (2.42 g) and NaCl (11.69 g) were dissolved in 950 ml of dist. H_2O, the pH was adjusted to 7.4 with HCl and the buffer made up to 1 litre with dist. H_2O.

Blocking solution [5% (m/v) low fat milk powder]. Low fat milk powder (5 g) was dissolved in TBS (100 ml).

Detection buffer [50 mM Tris-HCl, 5 mM $MgCl_2$, pH 9.5)]. Tris (0.61 g) and $MgCl_2$ (0.05 g) were dissolved in 90 ml of dist. H_2O, the pH was adjusted to 9.5 with HCl and the buffer was made up to 100 ml with dist. H_2O.

Substrate solution [3% (m/v) 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), 1.5% (m/v) nitro blue tetrazolium (NBT) in detection buffer]. BCIP (30 mg) and NBT (15 mg) were dissolved in detection buffer (100ml).

2.5.3.2 Procedure

For western blotting, the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell® was set up as described in the manufacturer's manual. The nitrocellulose membrane was cut to a suitable size and, to avoid the entrapment of air, carefully floated onto blotting buffer at a 45° angle, before being totally immersed for 15-30 min. The immersed nitrocellulose membrane was then sandwiched, with the gel lying squarely on top of it, between 3 pieces of Whatman No. 4 filter paper and two fiber pads, also completely wetted in blotting buffer. This sandwich was then positioned in a locking gel cassette clamping system and placed in the buffer tank, with the nitrocellulose membrane towards the anodal side of the chamber, along with a magnetic stirrer bar, to ensure even distribution of cooling, and the Bio-Ice cooling unit. The voltage was set at 30 V (maximum current) and blotting was allowed to proceed overnight.

Following transfer, the nitrocellulose membrane was removed and stained with Ponceau S (1 min) to assess the efficiency of transfer and to mark the positions of the molecular mass markers on the membrane. The excess stain was removed by rinsing in dist. H_2O, the positions of the markers

were carefully marked with a pencil, and the membrane was destained by the addition of a drop of 500 mM NaOH to the dist. H_2O. Unoccupied sites on the nitrocellulose membrane were blocked with blocking solution for 1 h, washed in TBS (3 × 5 min) and incubated for 2 h with primary antibody diluted in 0.5% (m/v) BSA-TBS. Following washing in TBS (3 × 5 min), the membrane was incubated in alkaline phosphatase-linked secondary antibody diluted in 0.5% (m/v) BSA-TBS for 1h. The membrane was washed in TBS (3 x 5 min), immersed in substrate solution (BCIP/NBT) and reacted in the dark until purple-coloured bands were clearly evident against a lightly stained background. The membrane was finally removed from the substrate solution, washed in dist. H_2O and dried between two sheets of filter paper to ensure good preservation of bands.

2.6 Agarose gel electrophoresis

Agarose is a linear galactan hydrocolloid which is isolated from agar or directly recovered from agar-bearing marine algae. It consists of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro α-L-galactopyranose residues which form a porous supporting medium for the resolution of large proteins and nucleic acids (Garfin, 1990; Allen and Budowle, 1994).

In the present investigation, agarose gel electrophoresis was applied to the separation of chromosomal DNA molecules for concentration determination, the examination of polymerase chain reaction (PCR) products for amplification confirmation, and to the resolution of restriction enzyme digested DNA for restriction fragment length polymorphism (RFLP) analysis. On application of an electric field, these negatively charged DNA molecules migrate towards the positively charged anode, at a rate which is determined by several parameters, including the molecular size and conformation of the DNA, the agarose concentration, and the composition of the electrophoresis buffer (Sambrook, 1989).

Molecules of linear double-stranded DNA become orientated in an end-on position under the influence of an electric field (Fisher and Dingman, 1971; Aaij and Borst, 1972) and migrate through the gel matrix at a rate which is inversely proportional to the logarithm of the number of base pairs (Helling *et al.*, 1974). Furthermore, DNAs of the same molecular size, but which have assumed a superhelical circular, nicked circular, or linear conformation will migrate at varied rates (Thorne, 1966; 1967). The relative mobilities of the three DNA forms depend primarily on the

agarose concentration, but are also influenced by, amongst other criteria, the ionic strength of the electrophoresis buffer (Johnson and Grossman, 1977).

A linear relationship was observed between the logarithm of the electrophoretic mobility of DNA molecules and the gel concentration, such that an increase in the percentage agarose from 0.3-2.0% (m/v) was accompanied by an increased efficiency in the range of separation of linear DNA molecules from 5-60 kb to 0.1-2 kb respectively (Sambrook, 1989).

The composition and ionic strength of the electrophoresis buffer directly affects the electrophoretic mobility of DNA. In the absence of ions, electrical conductance is minimal and therefore the DNA migrates very slowly. In contrast, electrical conductance is extremely efficient and significant amounts of heat are generated when buffers of high ionic strength are used. The most commonly used electrophoresis buffer contains both Tris-acetate and EDTA (TAE) which, despite its relatively low buffering capacity, is favoured over Tris-borate (TBE) and Tris-phosphate (TPE) buffers as it is relatively inexpensive and has a comparative resolving power with a slightly faster rate of migration (Sambrook, 1989).

The most common and convenient method to visualise the electrophoretically-separated DNA fragments is staining with the fluorescent dye ethidium bromide (Sharp *et al.*, 1973). This dye contains a planar group which intercalates between the stacked bases of DNA, displaying increased fluorescent yield compared to that of free dye in solution. The DNA absorbs ultraviolet radiation at 254 nm and transmits it to the dye, while the dye itself is capable of absorbing radiation at 302 and 366 nm. This energy is re-emitted at 590 nm in the red-orange region of the visible spectrum and facilitates direct visualisation of the DNA on a ultraviolet transilluminator (Sambrook, 1989).

The size of the DNA fragments or amplicons of interest, or the concentration of chromosomal DNA may be determined by visual comparison with fragments generated by *Hin*dIII restriction enzyme digestion of lambda DNA. Lambda DNA, in its circular form, consists of 48 502 bp containing six *Hin*dIII recognition sites. Digestion of this DNA with *Hin*dIII generates seven fragments 23130, 9416, 6682, 4361, 2322, 2027, and 564 bp in length (Boehringer Mannheim Technical Bulletin). From a known concentration of uncut lambda DNA, the concentration of

these restriction fragments may be derived and hence used as a comparison for determining the concentration of DNA samples.

2.6.1 Materials

50 X Tris-acetate stock solution [TAE buffer; 40 mM Tris-acetate, 1 mM EDTA, pH 8.0]. Tris (242 g) was dissolved in 800 ml dist. H_2O, glacial acetic acid (57.1 ml) was added and the buffer made up to 900 ml with dist. H_2O. EDTA (18.612 g) was dissolved in 90 ml dist. H_2O, the pH was adjusted to 8.0 with glacial acetic acid and the solution was made up to 100 ml with dist. H_2O. Finally, the Tris buffer and the EDTA solution were combined and autoclaved (121°C, 15 min).

TAE working solution. TAE buffer (20 ml) was diluted to 1 litre with dist. H_2O.

1% (m/v) agarose. Agarose (0.4 g) was dissolved in TAE working solution (40 ml) in an Erlenmeyer flask with the neck loosely plugged with cotton wool. The solution was heated in a microwave oven with intermittent mixing to ensure that all the grains of agarose were dissolved in the minimum time required.

1.5% (m/v) agarose. Agarose (0.6 g) was dissolved with heating in TAE working solution (40 ml) as described above.

Gel-loading buffer [1 mM EDTA, 0.5% (m/v) bromophenol blue, 50% (v/v) glycerol]. EDTA (0.007 g) and bromophenol blue (0.1 g) were dissolved in dist. H_2O (10 ml), and glycerol (10 ml) was added and thoroughly mixed in.

1% (m/v) Ethidium bromide. Ethidium bromide (0.2 g) was dissolved in dist. H_2O (20 ml) and stored in an aluminium foil-wrapped bottle.

Tris-EDTA buffer [TE buffer; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0]. Tris (0.121 g) and EDTA (0.037 g) were dissolved in 95 ml dist. H_2O, the pH was adjusted to 8.0 with HCl and the buffer was made up to 100 ml with dist. H_2O.

*Hin*dIII-cut lambda DNA standard markers. Lambda DNA (160 µl of a 0.25 µg/ml stock solution) was digested overnight at 37°C with *Hin*dIII (10 µl of a 10 U/µl stock solution) in a mixture containing reaction buffer B (Section 5.3.1.1; 20 µl) and made up to a final volume of 200 µl with TE buffer.

2.6.2 Procedure

The open ends of a clean, dry perspex casting tray (100 x 66 mm) were sealed with masking tape to form a mould for polymerisation of the agarose on a horizontal surface. The dissolved agarose solution was cooled to 60°C and ethidium bromide (1 µl) was added and mixed in thoroughly. A perspex 12-well comb was positioned in the casting tray and the warm agarose solution was carefully poured into the mould, ensuring that no air bubbles were formed. After the gel had set completely (15-20 min at room temperature), the masking tape was removed and the casting tray was positioned in the electrophoresis tank. TAE working solution was added to cover the gel to a depth of approximately 1 mm, and ethidium bromide (5 µl) was added and mixed in to ensure even distribution within the buffer. Gel-loading buffer (1 µl) was added to the DNA samples prior to loading to increase the density of the DNA sample and to monitor the progress of electrophoresis. The apparatus was connected to a power pack and the voltage was set at 100 V (maximum current) for 1.0-1.2 hours. On completion of the electrophoretic run, the casting tray was removed from the electrophoresis tank and the gel was examined on an ultraviolet transilluminator, prior to photographing through a red filter employing Polaroid type 667 black and white instant film. The concentration of the DNA was determined by visual comparison with *Hin*dIII generated lambda DNA fragments of known size and concentration.

CHAPTER 3

Growth, morphology and diagnosis of *H. paragallinarum*

In this chapter the conditions for growth of both the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates to confirm their species classification and to further facilitate their serotypical characterisation will be detailed. Scanning electron microscopic analysis of *H. paragallinarum* enabled morphological comparison of the strains of varying NAD-dependency. In addition, a polymerase chain reaction-based diagnostic assay to verify the classification of isolated strains as the causative agent of infectious coryza will be discussed.

3.1 Growth of *H. paragallinarum* reference strains and field isolates

The causative agent of infectious coryza has been classified according to its growth requirements. All avian Haemophili display a minimal requirement for NAD (Page, 1962). However, the NAD-dependent *H. paragallinarum* strains show improved growth in the presence of sodium chloride (Rimler *et al.*, 1977b), chicken serum (Blackall and Reid, 1982), oleic acid-albumin, thiamine-HCl (Blackall and Yamamoto, 1990), and carbon dioxide (Rimler *et al.*, 1976). In contrast, the highly virulent and fast-spreading strain dominating infectious coryza outbreaks in Kwazulu-Natal, exhibits minimal co-factor requirements, growing readily on blood agar plates (Horner *et al.*, 1992). A dense growth of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates is essential for phenotypic and genotypic characterisation studies. As sufficient material cannot be obtained from clinical samples, broth and agar culture media have been successfully employed as a bulking-up strategy.

3.1.1 NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates

The reference strains and field isolates employed in the present study, their corresponding serovar and the bacterial source are listed in Table 3.1.

Table 3.1 Reference strains and field isolates of NAD-dependent and -independent *H. paragallinarum*.

Strain	NAD requirement	Serotype (-serovar)	Reference / Field strain	Source
0083	+[a]	A-1	R	Blackall[d]
221	+	A-1	R	Blackall
2403	+	A-2	R	Blackall
ATCC 29545	+	A	R	Blackall
0222	+	B-1	R	Blackall
Modesto	+	C-2	R	Blackall
32	+	A	F	Bragg[e]
39	+	A	F	Bragg
41	+	A	F	Bragg
37	+	C-2	F	Bragg
43	+	C-2	F	Bragg
46	+	C-3	F	Bragg
1130	-[b]	A	F	Bragg
1742	-	A	F	Bragg
541	-	C-3	F	Bragg
95/03938	-	ud[c]	F	Horner[f]
93/09472	-	ud	F	Horner
93/00449	-	ud	F	Horner
96/11756	-	ud	F	Horner
96/12954	-	ud	F	Horner
RH 2390	-	ud	F	Horner

[a] NAD-dependent, [b] NAD-independent

[c] serotype is undetermined

[d] P.J. Blackall, Animal Research Institute, Queensland, Australia

[e] R.R. Bragg, University of Pretoria, Gauteng, South Africa

[f] R.F. Horner, Allerton Regional Veterinary Laboratory, Kwazulu-Natal, South Africa

3.1.2 Related avian bacterial strains

Four different strains of bacteria belonging to families closely related to *H. paragallinarum* were selected to test the specificity of a PCR-based diagnostic test (Section 3.3). Isolates previously characterised as *Pasteurella multocida*, *P. gallinarum*, *P. haemolytica* and *Actinobacillus pleuropneumoniae* were chosen.

3.1.3 Culture media

Columbia blood agar [3.9% (m/v) columbia blood agar, 5% (v/v) sheep blood]. Columbia blood agar (39 g) was dissolved in 1 litre of dist. H_2O with heating. The solution was autoclaved (121°C, 15 min), cooled to 50°C in a water bath and sterile citrated sheep blood (50 ml) was added asceptically. The media was aliquoted (25 ml) into sterile Petri dishes, and stored at 4°C.

Chocolate agar. Columbia blood agar base was prepared as described above with the exception that, following the addition of the sheep blood, the medium was heated to 75°C in a water bath. The medium was agitated at short intervals without producing bubbles. When the blood had 'browned', the media was cooled to 50°C and aliquoted into sterile Petri dishes.

Brain heart infusion broth [3.7% (m/v) brain heart infusion broth, 0.1% (m/v) agar]. Brain heart infusion broth (37 g) was dissolved in 1 litre of dist. H_2O. On dissolution, agar (1 g) was added and dissolved with heating. The medium was dispensed into sterile glass tubes (7 ml), autoclaved (121°C, 15 min) and stored in the dark at 4°C.

Test Medium agar [TM; 1% (m/v) casein hydrolysate, 1% (m/v) NaCl, 0.1% (m/v) yeast extract, 0.05% (m/v) glucose, 1.5% (m/v) agar, pH 7.5]. Casein hydrolysate (10 g), NaCl (10 g), yeast extract (1 g), glucose (0.5 g) and agar (15 g) were dissolved, with heating, in 950 ml dist. H_2O, the pH was adjusted to 7.5 with HCl, and the solution made up to 1 litre. The medium was autoclaved (121°C, 15 min) and cooled to 50°C in a water bath.

Test Medium agar containing chicken Serum and NAD [**TM/SN**, 1% (m/v) NAD, 0.05% (m/v) thiamine-HCl, 0.5% (v/v) chicken serum, 0.0016% (v/v) oleic acid-albumin]. The basic test medium agar was supplemented with the necessary co-factors, previously sterilised through 0.45 μm filters. NAD (1.25 ml), thiamine-HCl (5 ml), heat-inactivated chicken serum (5 ml) and

oleic acid-albumin (16 µl) were added per litre of media. The media was mixed thoroughly and dispensed (25 ml) into sterile Petri dishes and stored at 4°C.

TMB. This media is a broth version of TM/SN and is made according to the same protocol except the agar is omitted. The broth was dispensed (50 ml) into polystyrene cell culture flasks and stored at 4°C.

3.1.4 Isolation, growth and harvesting of *H. paragallinarum*

3.1.4.1 *H. paragallinarum* reference strains

Reference strains of *H. paragallinarum* (Table 3.1) were obtained from P.J. Blackall (Animal Research Institute, Australia). Freeze-dried samples were reconstituted in sterile dist. H_2O (1 ml), inoculated into TMB (Section 3.1.3; 15 ml) and incubated at 37°C for 48 hrs. The purity of the sample was assessed by plating a small volume of broth onto blood agar plates with a *Staphylococcus hyicus* feeder culture, which supplies the co-factor NAD. Dense growth of the reference strains was obtained by growth in TMB (Section 3.1.3; 250 ml) for genotypic analysis and on TM/SN (Section 3.1.3) plates for phenotypic analysis.

3.1.4.2 *H. paragallinarum* field isolates

H. paragallinarum was isolated from the sinuses of chickens showing clinical signs of infectious coryza. The heads of the chickens were removed in the post mortem room at Allerton Regional Veterinary Laboratory and disinfected with heating before an incision was made into the left and right sinuses of the bird with a sterile scalpel blade. Samples were collected from the sinuses with a sterile swab, inoculated onto blood agar plates, with a *S. hyicus* feeder culture, and incubated at 37°C for 48 hrs under 10% CO_2 tension. Following incubation, the plates were examined for purity and satellitism, in which the NAD-dependent *H. paragallinarum* colonies were found to be largest closest to the feeder streak and smaller when further removed from the feeder streak (Figure 3.1A). Those colonies which displayed uniform growth across the plate, growing independent of the *S. hyicius* streak, are NAD-independent *H. paragallinarum* colonies (Figure 3.2A).

The relative colony size of the NAD-dependent and -independent *H. paragallinarum* field isolates may be observed when strains are inoculated onto TM/SN (Section 3.1.3) plates in a three-way

streak. After a 48 hr incubation, the NAD-independent *H. paragallinarum* field isolates (Figure 3.2B) had grown more rapidly and to a larger colony size than the NAD-dependent isolates (Figure 3.1B). The NAD-dependent and -independent *H. paragallinarum* colonies isolated were stored either by freeze-drying or attached to porous beads in the Microbank® system (Section 3.1.5), until required for further studies. Freeze-dried *H. paragallinarum* field isolates (Table 3.1) obtained from R.R. Bragg (University of Pretoria, South Africa) were reconstituted as described in Section 3.1.4.1.

Dense populations of the NAD-dependent and -independent *H. paragallinarum* field isolates were obtained by growth in TMB (Section 3.1.3; 250 ml) for genotypic analyses and on TM/SN plates (Section 3.1.3) for comparative phenotypic analyses. Broth colonies were harvested by centrifugation (10 000 x g, 30 min, 10°C), whereas cultures on agar plates were carefully scraped off using sterile swabs, and resuspended in PBS (Section 2.5.2.1; 10 ml).

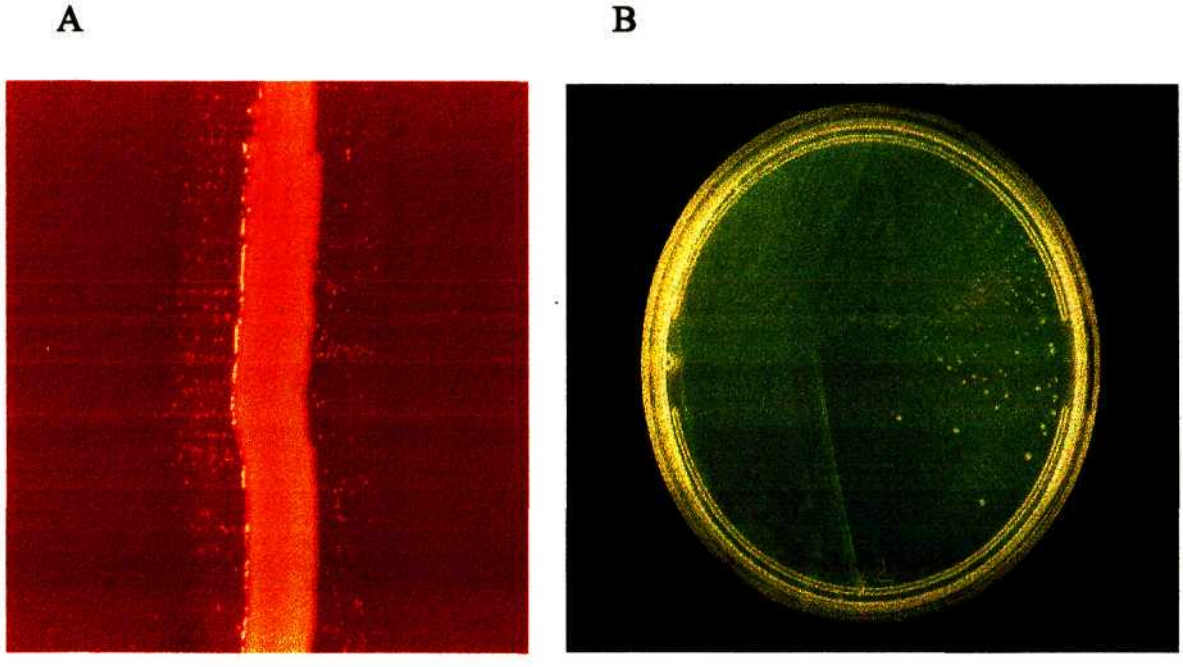


Figure 3.1 NAD-dependent *H. paragallinarum* field isolate and strain Modesto. **(A)** Blood agar plate containing a *S. hyicus* feeder culture inoculated with an NAD-dependent *H. paragallinarum* field isolate (Allerton Regional Veterinary Laboratory, South Africa). **(B)** TM/SN plate inoculated with NAD-dependent *H. paragallinarum* strain Modesto.

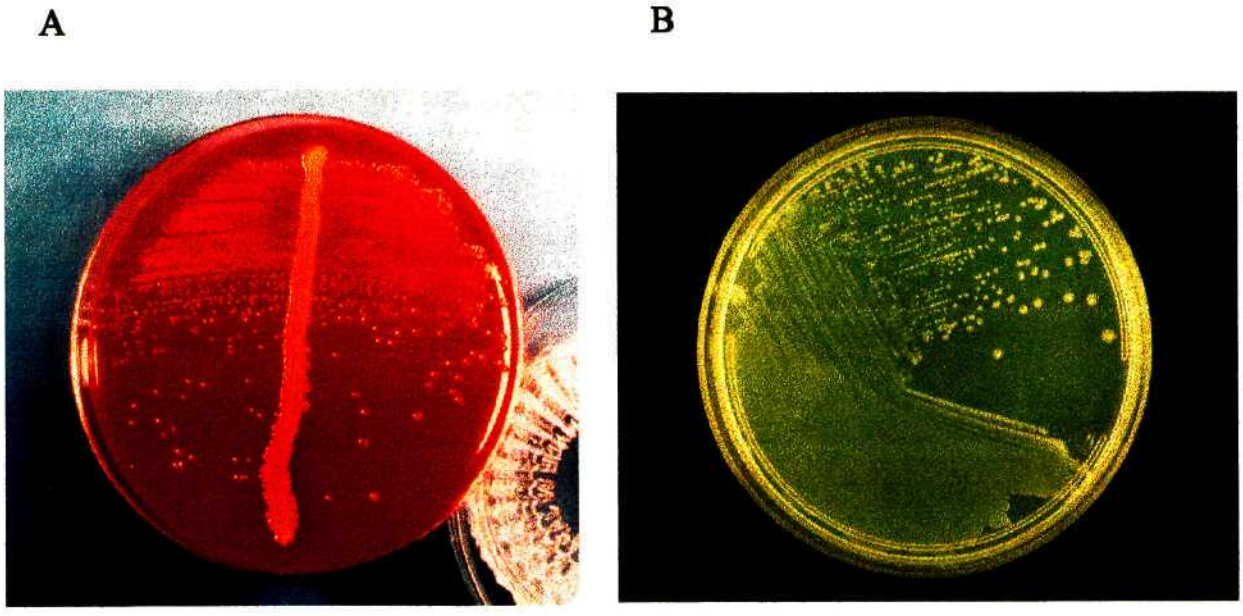


Figure 3.2 NAD-independent *H. paragallinarum* field isolate and strain 93/00449. **(A)** Blood agar plate containing a *S. hyicus* feeder culture inoculated with an NAD-independent *H. paragallinarum* field isolate (Allerton Regional Veterinary Laboratory, South Africa). **(B)** TM/SN plate inoculated with NAD-independent *H. paragallinarum* strain 93/00449.

3.1.5 Storage of *H. paragallinarum*

Two methods were applied for the storage of NAD-dependent and -independent *H. paragallinarum* field isolates: freeze-drying or attachment to porous beads in the Microbank® system.

Freeze-drying involves the removal of water from a microbial culture by sublimation under reduced pressure (Isaac and Jennings, 1995). NAD-independent *H. paragallinarum* field isolates were grown in brain heart infusion broth (Section 3.1.3), dispensed into vials and freeze-dried overnight. The NAD-dependent *H. paragallinarum* reference strains and field isolates were freeze-dried following growth in specific pathogen free (SPF) chicken eggs. The organism to be freeze-dried was grown on chocolate agar plates (Section 3.1.3) at 37°C under 10% CO_2 tension for 48 hrs. A few bacterial colonies were removed from the plate, resuspended in saline solution (0.1 ml) and inoculated, in triplicate, into the yolk of six-day old SPF chicken eggs. The eggs were sealed with heated wax and placed in a heated incubator (37°C) for 48 hrs. The viability of

the embryo was checked after 24 hrs, and after 48 hrs the yolk was asceptically removed and dispensed into sterile vials for freeze-drying overnight. The viability and purity of the sample was assessed by plating a small volume of yolk onto a blood agar plate (Section 3.1.3) with a *S. hyicus* feeder culture. All freeze-dried cultures were stored at -20°C.

The Microbank® system is a commercially available storage system which consists of approximately 25 acid-washed porous beads in a cryovial containing cryopreservant. This storage system may be used equally efficiently for NAD-dependent and -independent *H. paragallinarum* strains. The bacterial organism to be stored was grown on chocolate agar (Section 3.1.3) at 37°C, under 10% CO_2 tension for 48 hrs. A few colonies were carefully picked from a pure culture using a sterile wire loop and added to the cryopreservant. Following gentle inversion (4-5 times), to emulsify the organism and facilitate binding to the porous beads, excess cryopreservant was removed and the vials were stored at -80°C until required. For reconstitution, the vial was warmed to room temperature and 1-2 beads were asceptically removed into TMB (Section 3.1.3; 10 ml), and incubated at 37°C under 10% CO_2 tension to facilitate growth.

3.2 Morphology of *H. paragallinarum* reference strains and field isolates

3.2.1 Scanning electron microscopy

All *H. paragallinarum* strains were reported to be Gram-negative, polar-staining, non-motile, non-spore-forming, pleomorphic, coccoid or rod-shaped cells, 1-3 μm in length and 0.4-0.8 μm in width, with a tendency to filament formation (Schalm and Beach, 1936; Piechulla *et al.*, 1984; Yamamoto, 1991). In the present investigation scanning electron microscopy (SEM) was employed to study the morphology of an NAD-dependent *H. paragallinarum* reference strain and an NAD-independent *H. paragallinarum* field isolate.

The scanning electron microscope utilises a focused beam of high energy electrons that systematically scans across the surface of the specimen. A variety of signals are produced by the interaction of the beam with the specimen, including secondary electrons which are drawn to a positively biased detector system. Conversion of this electron signal to an electronic signal portrays an image of the specimen on a cathode ray tube. Resolution in the region of 3-5 nm has been reported (Flegler *et al.*, 1993; Postek *et al.*, 1980).

The most critical step in SEM is adequate preparation of the specimen; free of preparation artefacts and capable of rendering clear life-like images in the 'hostile' environment of the electron microscope. Specimen preparation involves fixation of the biological tissue and subsequent dehydration using a method exploiting the critical point of a solvent. The dried specimen is then mounted onto a metal stub, sputter coated to render the sample surface conductive, and viewed in the electron microscope.

Fixation is the most critical operation during sample preparation, required to preserve the fine structure of the cells and to make them resistant to aleration and distortion during subsequent preparatory treatments. Chemical methods, employing non-coagulative fixatives such as glutaraldehyde which form intra- and inter-molecular cross-links, thereby conferring structural stability, or mechanical methods, in which the cell is plunge-frozen at -180°C in liquid nitrogen to preserve the tissue in a form which closely resemble the living state.

The removal of water from the fixed specimen may be achieved by air drying, freeze drying or the most commonly used method of critical point drying (CPD). Following chemical dehydration by passage through a graded organic solvent series, this dehydrating solvent is replaced with liquid carbon dioxide. The ability of the latter solvent to change from a liquid to a gaseous phase, without the latent heat of vaporisation, is exploited to yield a critical point dried sample which has not been subjected to the damaging forces of surface tension (Humphreys *et al.*, 1978).

Finally the dried specimen is attached to a mounting stub and coated with a very thin layer of an electrically conductive metal enabling visualisation of satisfactory images in the SEM. Sputter coating is a routine procedure in which large inert argon gas molecules are ionized to bombard target molecules such as gold or gold:palladium (60:40), which become dislodged and are available for deposition on the specimen surface (Flegler *et al.*, 1993; Postek *et al.*, 1980).

3.2.2 Sample preparation

3.2.2.1 Materials

Washing buffer [0.05 M cacodylate buffer, pH 7.10]. Cacodylic acid (dimethylarsinic acid-sodium salt, 8 g) was dissolved in 950 ml dist. H_2O, the pH was adjusted to 7.1 with NaOH and the buffer was made up to 1 litre with dist. H_2O.

Buffered fixative [3% (v/v) glutaraldehyde]. Glutaraldehyde [10.7 ml of a 28% (v/v) solution] was diluted to 100 ml with washing buffer.

2% (m/v) osmium tetroxide. Osmium tetroxide (2 g) was dissolved in washing buffer (100 ml).

30, 70 and 90% (v/v) ethanol series solutions. Ethanol (30, 70, and 90 ml respectively) was diluted to 100 ml with dist. H_2O.

3.2.2.2 Procedure

NAD-dependent *H. paragallinarum* reference strain Modesto and NAD-independent *H. paragallinarum* field isolate 93/00449 were grown on TM/SN plates (Section 3.1.3) at 37°C under 10% CO_2 for 48 hrs. Using protective gloves, the agar was cut into sections no larger that $3mm^3$, containing between 1-3 colonies, and incubated in buffered fixative for 45 min. The sections were washed (2 x 5 min) in washing buffer and post-fixed in 2% (m/v) osmium tetroxide for 30 min. Following washing (2 x 5 min) in washing buffer, the agar sections were dehydrated by passage through an ethanol series. This involved successive incubations (5 min each) in solutions of increasing ethanol percentage [30, 70 and 90% (v/v)], followed by incubation in 100% (v/v) ethanol (3 x 5 min). The sections were transferred to wire mesh baskets in 100% alcohol and the baskets were positioned inside the critical point drying (CPD) apparatus. During the CPD process the liquid CO_2 gradually replaces the organic solvent in the sample, with the efficiency of the process being increased by flushing the chamber several times. The chamber was then heated until the critical point of CO_2 (31.3°C at 75.5 kg/cm^2 pressure) was reached and the sample was dried in this dense gaseous environment. The CPD process was completed after approximately 2 hrs. The dried samples were removed from the wire mesh baskets and securely mounted onto the polished upper surfaces of an SEM specimen stub using double-sided tape. The mounted specimens were coated with gold employing a sputter coater and viewed under the scanning electron microscope.

3.2.3 Results and discussion

SEM revealed the NAD-independent *H. paragallinarum* field isolate colonies (Figure 3.4A) to grow to a greater density than the NAD-dependent *H. paragallinarum* reference strain colonies (Figure 3.3A). This may be attributed to the minimal co-factor requirements for growth exhibited

by the NAD-independent *H. paragallinarum* strains. In addition, the NAD-dependent bacterial cells assumed a predominantly coccoid morphology (Figure 3.3B) with some tendency to filament formation (Figure 3.3C), while the NAD-independent bacterial cells were largely rod-shaped (Figure 3.4B). It may be postulated that the availability of essential growth factors decreased over a 48 hr period, thereby stunting the growth of the NAD-dependent strains and promoting coccoid-shaped cells.

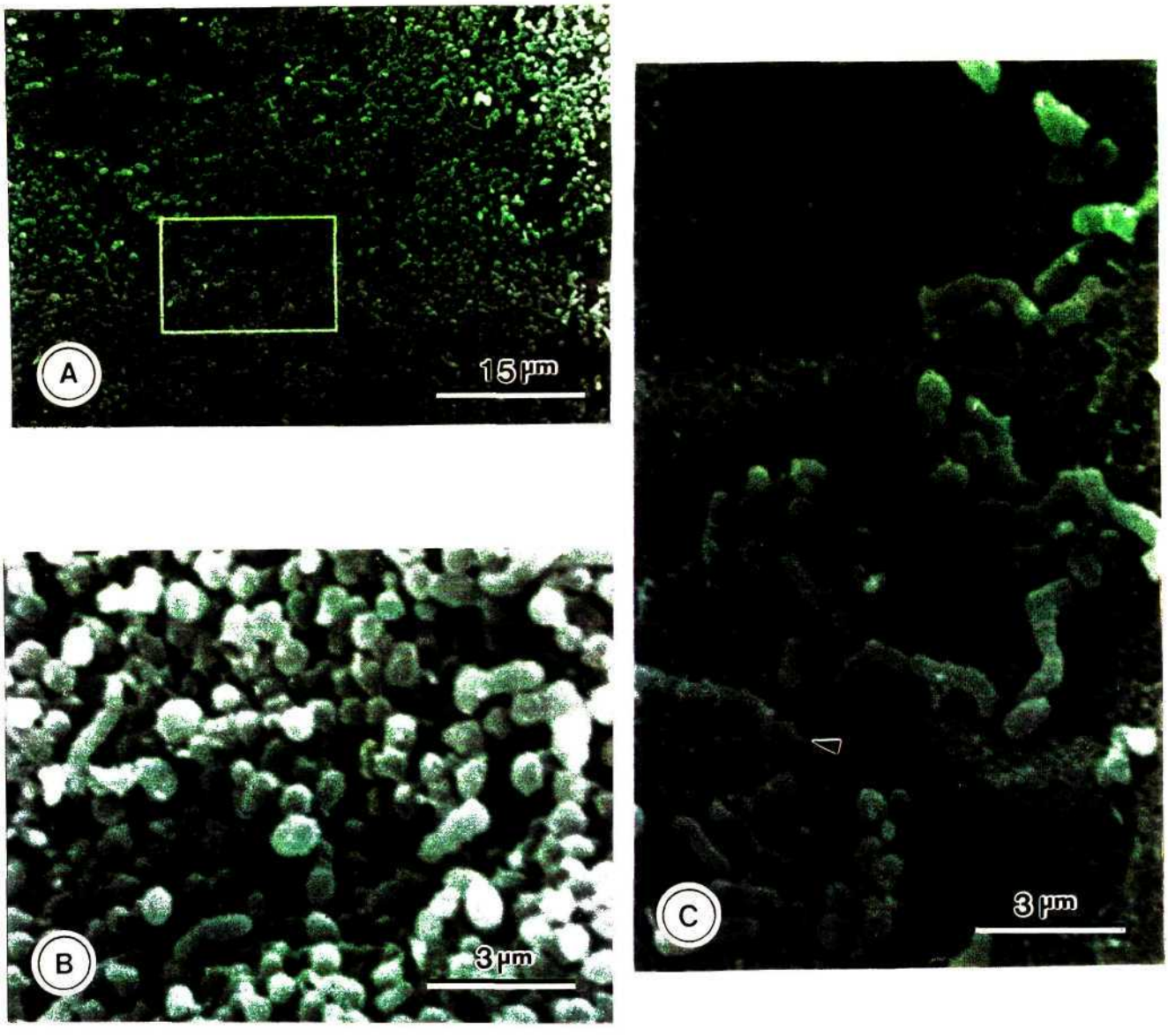


Figure 3.3 Scanning electron micrograph of an NAD-dependent *H. paragallinarum* reference strain Modesto colony. (A) Illustrates the density of the bacterial cells. **(B)** The same tissue at increased magnification showing coccoid-shaped cells. **(C)** The same tissue demonstrating the tendency to filament formation (arrow).

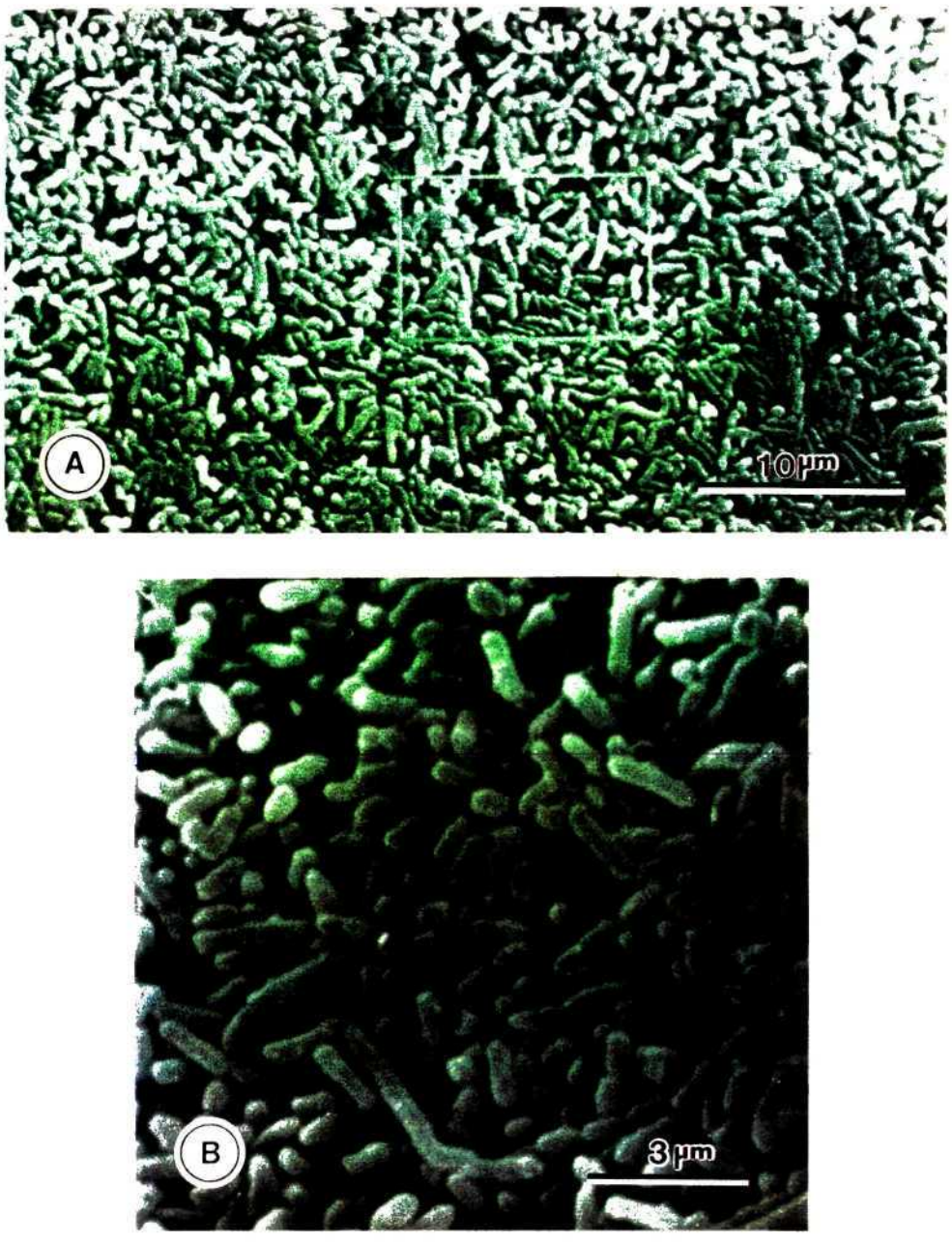


Figure 3.4 Scanning electron micrograph of an NAD-independent *H. paragallinarum* field isolate 93/00449 colony. **(A)** Illustrates the density of the bacterial cells. **(B)** The same tissue at higher magnification showing predominantly rod-shaped cells.

3.3 Diagnosis of *H. paragallinarum* reference strains and field isolates

The primary approach to diagnosis of infectious coryza is the isolation and identification of *H. paragallinarum* (Blackall and Yamamoto, 1990). However, several complications have been reported with this approach. Firstly, NAD-dependent *H. paragallinarum* is a difficult organism to grow as a pure culture *in vitro*, requiring complex media containing several growth additives (Blackall and Yamamoto, 1990). In addition, NAD-dependent *H. paragallinarum* is a relatively slow-growing organism, thus other organisms can overgrow and mask its presence. The third complicating factor is that *H. paragallinarum* is not the only avian haemophilic organism, with *Pasteurella avium* (formerly *H. avium*), *P. volantium* and *Pasteurella* species A also infecting flocks of birds (Mutters *et al.*, 1985).

The recent development of nucleic acid techniques for the detection of pathogens was applied as an alternative approach to cultural diagnosis of *H. paragallinarum*, and involves a species-specific PCR-based method (Chen *et al.*, 1996). Development of this diagnostic assay involved the identification of four clones specific for *H. paragallinarum*, following screening of a genomic DNA library of *H. paragallinarum* strain Modesto. The probes based on these clones were approximately 1.8, 2.3, 3.5, and 5.5 kb in size. The smallest probe, termed P601, was partially sequenced using the chain termination method, and primers for two PCR tests were designed from the sequence data. Both PCR tests, termed HPG-1 and HPG-2, were demonstrated to be specific for *H. paragallinarum* and sensitive to 1 pg DNA. The application of HPG-2 PCR test directly to colonies and clinical samples led to this test being favoured over HPG-1 (Chen *et al.*, 1996), with *H. paragallinarum* diagnosis affirmed by the amplification of a 0.5 kb DNA band.

3.3.1 Polymerase chain reaction

The polymerase chain reaction (PCR), devised and named by Mullis and colleagues (1987), is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA (Taylor, 1991). PCR may be broken down into three steps, which are repeated in cycles. Firstly, the duplex sample DNA is denatured by heating, producing a single-stranded template. Secondly, the two primers are annealed to complementary regions at opposite ends of the single-stranded DNA template, conferring the specificity to the PCR reaction. The temperature and length of time required for primer annealing is dependent on

the base composition, length, and concentration of the amplification primers (Innis and Gelfand, 1990). Finally, the primers are extended by *Thermus aquaticus* (*Taq*) DNA polymerase catalysed nucleotide additions in the *de novo* synthesis of the region of DNA flanked by the two primers. The extension time is dependent upon the length and concentration of the target sequence and upon the temperature, which is typically 72°C (Innis and Gelfand, 1990). This process is repetitive with the number of copies of product increasing exponentially.

The requirements of the PCR reaction are simple: deoxyribonucleotide triphosphates to provide both the activation energy and nucleosides for the synthesis of DNA, thermostable *Taq* polymerase, amplification primers, template DNA, and buffer containing magnesium to improve the fidelity of the enzyme (Eckert and Kunkel, 1991; Taylor, 1991). The advantages of PCR include simplicity, speed and an inherent sensitivity to small amounts of DNA (Giovannoni, 1991).

In the present study, PCR was employed for the diagnosis of the infectious coryza-causing agent, *H. paragallinarum.* Evidence for confirmed identification was provided by the specific amplification of a 500 bp band, when the HPG-2 PCR test (Chen *et al.*, 1996) was applied directly to *H. paragallinarum*-like clinical samples, to plated whole cell colonies and to extracted chromosomal DNA.

3.3.2 DNA sample preparation

3.3.2.1 Materials

All solutions were filtered through 0.45 µm filters or autoclaved (121°C, 15 min) before use.

2.5% (m/v) Proteinase K. Proteinase K (25 mg) was dissolved in dist. H_2O (1 ml) and stored at -20°C.

Saline-EDTA [0.85% (m/v) sodium chloride, 0.05 M EDTA, pH 7.6]. Sodium chloride (0.17 g) and EDTA (0.372 g) were dissolved in 18 ml dist. H_2O, the pH was adjusted to 7.6 with HCl and the solution was made up to 20 ml with dist. H_2O.

2% (m/v) Lysozyme. Lysozyme (0.4 g) was dissolved in dist. H_2O (20 ml) and stored at 4°C.

25% (m/v) SDS. SDS (5 g) was dissolved in dist. H_2O (20 ml).

1% (m/v) RNAse A. RNAse A (0.01 g) was dissolved in dist. H_2O (1 ml) and stored at -20°C. Prior to use aliquots of the solution (10.5 µl per chromosomal DNA extraction) were boiled at 100°C for 10 min.

3 M sodium acetate, pH 5.2. Sodium acetate (40.81 g) was dissolved in 95 ml dist. H_2O, the pH was adjusted to 5.2 with acetic acid, and the solution was made up to 100 ml with dist. H_2O.

3.3.2.2 Procedure

Clinical samples. Sinus swabs were taken from five commercial chickens exhibiting clinical symptoms resembling infectious coryza, for direct diagnosis of *H. paragallinarum.* The swab was soaked in PBS (Section 2.5.2.1; 1 ml) in a 1.5 ml microfuge tube and centrifuged (264 x g, 5 min, RT) to pellet the blood. The supernatant was transferred to a new tube and centrifuged (11 148 x g, 15 min, RT). The supernatant was discarded while the pellet was resuspended in 20 µl (final volume) reaction buffer (Section 3.3.3.1) containing proteinase K (8 µl) and incubated at 56°C for 1 hr. The sample was heated at 98°C for 10 min to inactivate the proteinase K and then held on ice for 10 min before being subjected to the HPG-2 PCR test (Section 3.3.3).

Plated whole cell colonies. From an agar plate which had been incubated overnight at 37°C under 10% CO_2 tension, one or two *H. paragallinarum*-like colonies were removed and resuspended in 10-20 µl of deionised H_2O in a 0.6 ml natural thin wall PCR tube. The sample was heated at 98°C for 10 min, held on ice for 10 min and the HPG-2 PCR test (Section 3.3.3) was performed on the whole cell sample.

Extraction of chromosomal DNA. Chromosomal DNA was extracted in a scaled-down version of standard DNA extraction methods (Blackall *et al.*, 1995). NAD-dependent and -independent *H. paragallinarum* reference strain and field isolate bacterial cells were grown in 100 ml of TMB (Section 3.1.3; 16 hr, 37°C, 10% CO_2), collected by centrifugation (10 000 x g, 30 min, 10°C) and washed three times in PBS (Section 2.5.2.1) with centrifugation (10 000 x g, 5 min, 10°C). The cells were resuspended in saline-EDTA (1 ml) to which lysozyme (1 ml) was added and the

suspension was incubated at 37°C for 1 hr. Thereafter SDS (10 µl) and proteinase K (80 µl) were added, and the solution was incubated at 60°C for 1 hr. The mixture was reincubated at 37°C for 30 min following the addition of RNAse A (10.5 µl). The resulting suspension was subjected to repeated phenol-chloroform extractions for protein removal. The final aqueous phase was removed and supplemented with 0.25 volumes of 3 M sodium acetate and 2.5 volumes of ice-cold ethanol, held at -70°C for 1 hr and centrifuged (14 000 x g, 20 min, 10°C). The resulting DNA pellet was washed in 70% (v/v) ethanol, air-dried, and resuspended in deionised H_2O (50-100 µl). The concentration of the chromosomal DNA was determined, following 1% (m/v) agarose gel electrophoresis (Section 2.6). The DNA samples were stored at -20°C.

3.3.3 HPG-2 PCR reaction conditions

A 500 bp gene sequence was amplified with primers N1/R1 (Figure 3.5), designed from sequences on the P601 probe, identified from the genomic DNA library generated from *H. paragallinarum* strain Modesto (Chen *et al.*, 1986).

Forward Primer (N1):

5' TGA GGG TAG TCT TGC ACG CGA AT **3'**

Reverse Primer (R1):

5' CAA GGT ATC GAT CGT CTC TCT ACT **3'**

Figure 3.5 Nucleotide sequences of the HPG-2 PCR primers used to amplify gene sequences from *H. paragallinarum* strain Modesto probe P601 (Chen *et al.*, 1996).

3.3.3.1 Materials

10 X PCR reaction buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% (v/v) Tween-20]. Tris (0.8 g) was dissolved in deionised H_2O (9.5 ml) and the pH was adjusted to 8.8 with HCl. $(NH_4)_2SO_4$ (0.2 g) was dissolved in this solution, Tween-20 (10 µl) was added and the solution was made up to 10 ml with deionised H_2O. The resulting mixture was aliquoted into 1 ml fractions and stored at -20°C.

50 mM $MgCl_2$. $MgCl_2$ (0.048 g) was dissolved in deionised H_2O (10 ml). This solution was aliquoted into 1 ml fractions and stored at -20°C.

dNTP stock solutions [100 mM of each dATP, dCTP, dGTP, dTTP]. dATP (4.912 mg), dCTP (4.672 mg), dGTP (5.072 mg), and dTTP (5.071 mg) were individually dissolved in a 100 mM lithium chloride solution, pH 7.0 (100 µl).

dNTP working solution [200 µM of each dATP, dCTP, dGTP, and dTTP]. dATP, dCTP, dGTP and dTTP (20 µl of each stock solution) were combined and diluted to 1 ml with deionised H_2O.

Stock N1 primer solution. Freeze-dried N1 primer (407 µg) was reconstituted in TE buffer (Section 2.6.1; 543 µl).

Stock R1 primer solution. Freeze-dried R1 primer (477 µg) was reconstituted in TE buffer (Section 2.6.1; 622 µl).

Working N1 primer solution. Stock N1 primer solution (20 µl) was diluted to 100 µl with deionised H_2O.

Working R1 primer solution. Stock R1 primer solution (20 µl) was diluted to 100 µl with deionised H_2O.

3.3.3.2 Procedure

The PCR mixture contained PCR reaction buffer (5 µl), $MgCl_2$ (2 µl), dNTP working solution (5 µl), primers N1 and R1 (1 µl of each), and template DNA (10-200 ng of chromosomal DNA, 1-2 colonies of plated whole cell colonies or a single clinical swab) diluted to a final volume of 50 µl with deionised H_2O. The mixture was heated at 98°C for 2.5 min in a Perkin-Elmer 2400 thermocycler, before 1.25 U *Taq* DNA polymerase was added. The samples were amplified for 25 cycles. Each cycle consisted of a denaturation step at 94°C for 1 min, followed by annealing at 55°C for 1 min and extension at 72°C for 2 min. The final extension step was at 72°C for 10 min to allow complete extension of all DNA products. Verification of the correct size of the amplicon

was established by running each completed PCR reaction product (5 µl) through a 1% (m/v) agarose gel. The specificity of the HPG-2 PCR test for strains of *H. paragallinarum* was assessed by the incorporation of negative controls, namely the related bacterial isolates of *P. multocida*, *P. gallinarum*, *P. haemolytica*, and *A. pleuropneumoniae*. In addition, internal controls in which one of three essential reaction mixture components, namely chromosomal DNA, PCR reaction primers or *Taq* DNA polymerase, were omitted were included in the PCR cycle to test for 'false positive' results.

3.3.4 Results and discussion

Optimisation of the HPG-2 PCR test for application to *H. paragallinarum* reference strains and field isolates was necessary as no amplification was obtained under the conditions described by Chen *et al.* (1986). Several PCR tests were carried out in which the concentration of the template DNA and the ratio of template DNA to primer concentration were varied, with no success. However, reduction of the sensitivity of the PCR reaction by decreasing the annealing temperature from 65°C to 55°C, was accompanied by amplification of a 0.5 kb band.

The optimised HPG-2 PCR test was used directly on sinus swabs obtained from five commercially-infected chickens. These samples adequately provided a template for the amplification of a 500 bp DNA fragment (results not shown), thereby confirming the presence of *H. paragallinarum* and hence the diagnosis of infectious coryza. The application of the HPG-2 PCR test to a range of plated NAD-dependent and -independent *H. paragallinarum* whole cell colonies exhibited the amplification of a 500 bp DNA fragment (results not known). These results confirmed the successful application of PCR to both clinical swabs and plated colonies, previously reported by Chen *et al.* (1996) and provide further evidence for the implementation of this technique as a diagnostic tool in the field.

The HPG-2 PCR test was used to confirm the identification of all the NAD-dependent and -independent *H. paragallinarum* (Table 3.1) strains employed in this study. This test, employing chromosomal DNA as the template, was found to be equally effective for confirming the classification of both the NAD-dependent reference strains (Figure 3.6) and field isolates (results not shown) as well as the NAD-independent field isolates (Figure 3.7). The specificity of HPG-2 PCR was illustrated by the incorporation of several related avian Haemophili in the test.

A negative result was obtained for *P. gallinarum*, *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae* (Figure 3.6, lanes 8-11). In addition, three internal controls were included that contained all of the components of the PCR with the exception of the template DNA, primers N1/R1, and *Taq* DNA polymerase. These controls produced negative results, indicating that there was no contamination or mispriming in the test samples, which could give rise to a false positive result. Confirmation of *H. paragallinarum* classification of the bacterial cells under observation was pivotal to safeguard subsequent phenotypic and genotypic comparisons amongst the strains with varying NAD dependency.

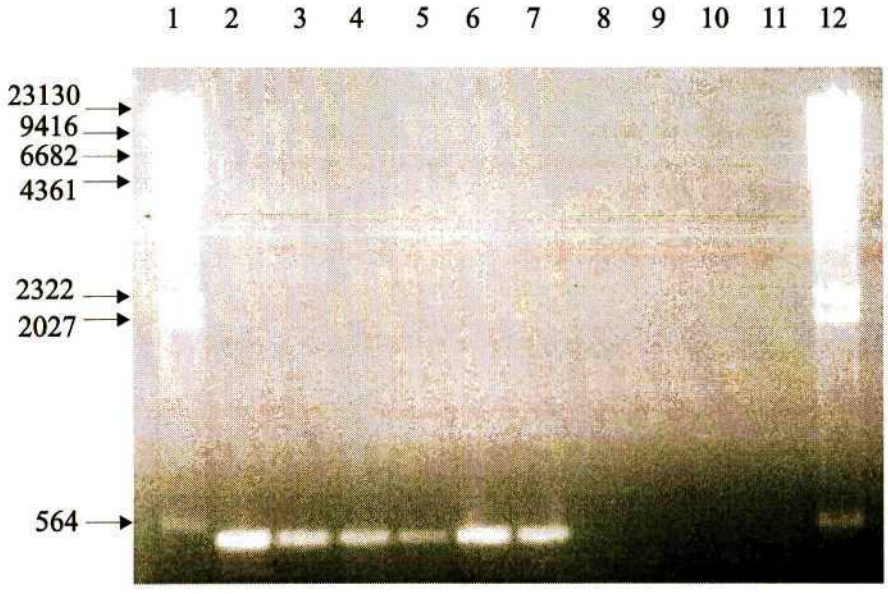


Figure 3.6 HPG-2 PCR analyses of the chromosomal DNA of NAD-dependent *H. paragallinarum* reference strains and related avian Haemophili strains. Lanes 1 and 12, *Hin*dIII-cut lambda DNA molecular weight ladder, **lanes 2-7,** *H. paragallinarum* strains **[lane 2,** 0083; **lane 3,** 221; **lane 4,** 2403; **lane 5,** ATCC 29545; **lane 6,** 0222; **lane 7,** Modesto]; **lane 8,** *P.gallinarum*; **lane 9,** *P. haemolytica*; **lane 10,** *P. multocida*; **lane 11,** *A. pleuropneumoniae*.

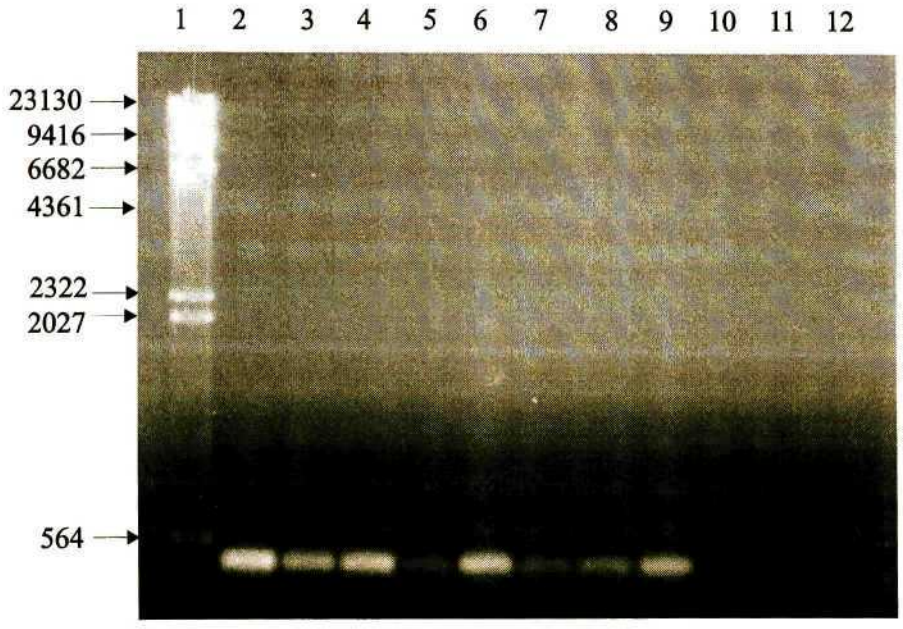


Figure 3.7 HPG-2 PCR analyses of the chromosomal DNA of NAD-independent *H. paragallinarum* field isolates. Lane 1, *Hin*dIII-cut lambda DNA molecular weight ladder; **lanes 2-9,** *H. paragallinarum* strains [**lane 2,** 95/03938; **lane 3,** 93/09472; **lane 4,** 93/00449; **lane 5,** 96/11756; **lane 6,** 96/12954; **lane 7,** RH 2390; **lane 8,** 1130; **lane 9,** 541]; **lane 10,** No DNA control; **lane 11,** No primer control; **lane 12,** No *Taq* polymerase control.

1.4 Conclusions

In this part of the study the fastidious growth requirements of the NAD-dependent *H. paragallinarum* reference strains and field isolates, as well as the comparatively basic growth conditions required by the NAD-independent *H. paragallinarum* field isolates were detailed. Morphological analysis of these strains of varying NAD-dependency revealed the NAD-independent colonies to grow to a greater density than those exhibiting NAD-dependency, and to assume a predominantly rod-shaped conformation. In contrast, the NAD-dependent bacterial cells were largely coccoid. Furthermore, the classification of the isolated strains as the causative agent of infectious coryza was confirmed using a species-specific PCR-based test.

Consequently, the 'bulked up' NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates were subjected to phenotypic (Chapter 4) and genotypic (Chapter 5) analyses, for allocation to one of the previously established serotypes and/or serovars.

CHAPTER 4

Analysis of *H. paragallinarum* protein profiles for serotype allocation and characterisation of major outer membrane proteins

4.1 Introduction

The immense diversity and number of microbial proteins facilitate the implementation of identification and typing schemes. Comparative analysis of the protein fingerprint of the bacterial isolate with established reference templates forms the basis of these schemes (Towner and Cockayne, 1993). The importance of intra-species typing of the divergent *H. paragallinarum* field isolates is two-fold. Firstly, protection against reinfection has been demonstrated to be serotype-specific. Flocks of chickens were shown to be protected against challenge with a strain of homologous serotype but not against isolates of heterologous serotype (Kume *et al.*, 1980a; b). In addition, a limited degree of cross-protection was found to exist amongst the serovars within a serotype (Blackall, 1991). Secondly, serotype-specificity is conferred by the serotype-specific protective antigens located on the outer membrane surfaces of the bacterium (Sawata *et al.*, 1984a).

Methods for allocation of the *H. paragallinarum* field isolates to Kume's established serotypes (A, B and C) and their serovars (Blackall *et al.*, 1990a) were essential to enable the inclusion of the predominant field isolate type in the current vaccine. To this end the bacterial proteins were typed on a whole cell or semi-fractionated [e.g. outer membrane protein (OMP)] level by non-denaturing SDS-PAGE. Visualisation of the protein fingerprints was achieved by the sensitive silver protein stain or by an immunochemical detection system in which polyclonal anti-whole cell antibodies were raised against a classical reference strain representing each serotype. The success achieved in subtyping other *Haemophilus* species, including *H. influenzae* (Barenkamp *et al.*, 1981a; b; Murphy *et al.*, 1983; Saravani *et al.*, 1992) and the related etiologic agent, *M. synoviae*, causing subclinical upper respiratory tract infections in chickens (Gurevich *et al.*, 1995), suggested application of these subtyping methods to *H. paragallinarum.*

The biochemical (Van Alphen *et al.*, 1983; Coulton and Wan, 1983) and immunological (Lam *et al.*, 1980; Murphy *et al.*, 1986; DeMaria *et al.*, 1996) properties of several of the major OMPs of *H. influenzae* have been characterised. Structural and functional analysis (recognition of heat-modifiable and surface-exposed OMPs, identification of outer membrane lipopolysaccharides, and N-terminal sequencing of unique major OMPs) of individual virulence-associated molecules comprising the outer membrane of *H. paragallinarum* will enable identification of potential targets for improved vaccine design, coupled with improved efficacy of the current vaccines and more efficient control of infections in the field.

The whole cell protein profiles of several NAD-dependent *H. paragallinarum* reference strains and field isolates (Blackall and Yamamoto, 1989a), as well as a small number of NAD-independent *H. paragallinarum* field isolates (Horner *et al.*, 1995) have been examined. However, application of OMP profiling to the NAD-dependent *H. paragallinarum* reference strains is limited (Blackall *et al.*, 1990d) and has not been applied to typing of the NAD-dependent and-independent field isolates prior to the present study. This Chapter describes attempts to allocate local *H. paragallinarum* field isolates to previously established serotypes and, to a certain extent, to their corresponding serovars by whole cell protein (WCP) and OMP profiling. To this end non-reducing SDS-PAGE, coupled with protein staining and immunochemical detection systems were used. In addition, novel efforts to characterise individual OMPs of *H. paragallinarum* strains will be described.

4.2 Growth of *H. paragallinarum*

The protein content of a bacterial cell may be influenced by environmental factors to the extent that the expression of the virulence-associated determinants are affected by the culture conditions (Davies *et al.*, 1992; Bragg *et al.*, 1995). To allow generation of reproducible comparative protein profiles, the growth conditions were standardised. Special attention was given to the formulation of culture media (Section 3.1.3), preparation of the bacterial inoculum (Section 3.1.5), and the incubation conditions, including temperature, gaseous environment and conditions for cell harvesting (Section 3.1.4).

4.3 Preparation of whole cell proteins

Standardisation of protein sample preparation is critical, yet difficult, in that bacterial cells may exhibit a varied susceptibility to the method of cell-breakage (Jackson, 1985). Bacterial cells may be broken by chemical methods, including heat-treatment in the anionic detergent SDS or by enzymatic lysis with lysozyme, and physical methods such as sonication or shaking with glass beads (Coakley, 1977). In the present study it was both effective and convenient to disrupt the NAD-dependent and -independent *H. paragallinarum* bacterial cells by chemical treatment in SDS. This was followed by boiling to allow denaturation of the proteins and efficient binding of SDS to the protein molecules.

4.3.1 Procedure

The protein concentration of harvested NAD-dependent and -independent *H. paragallinarum* bacterial cells suspended in PBS (Section 2.5.2.1) was determined using the method of Bradford (1976; Section 2.2.1). Protein samples (100-300 ng of protein per band expected) were aliquoted into 1.5 ml microfuge tubes and mixed with an equal volume of non-reducing treatment buffer (Section 2.3.1). The mixture was heated at 100°C for 5 min for complete denaturation of the whole cell proteins and subsequent uniform binding of the negatively charged SDS molecules. The denatured samples were analysed under non-denaturing SDS-PAGE conditions (Section 2.3) coupled with a silver protein staining (Section 2.4) or immunochemical (Section 2.5.3) detection system.

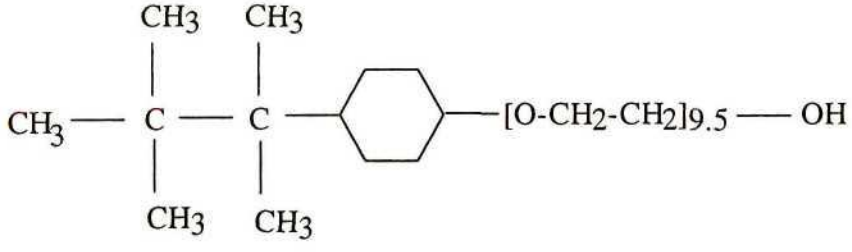
4.4 Isolation of OMPs

The extraction of OMPs from the membranes of Gram-negative bacteria has been successfully achieved employing a combination of sonication, centrifugation and selective solubilisation with one of a wide range of detergents (Schnaitman, 1971; Filip *et al.*, 1973; Ganong and Delmore, 1991). Detergents are amphiphilic molecules with hydrophobic moieties that coalesce into thermodynamically stable aggregates called micelles, and hydrophilic moieties which interact with the aqueous environment rendering the detergent molecules soluble. Solubilisation of membranes proceeds by detergent binding to the hydrophobic regions on the component proteins and the interaction of the detergent's hydrophilic regions with the surrounding aqueous phase, such that

the membrane partitions into a bilayer until it is no longer stable and disintegrates into soluble lipid and protein components (Findlay, 1990; Neugebauer, 1990; Von Jagow *et al.*, 1994).

The hydrophilic structures in detergents can be nonionic, anionic, cationic or zwitterionic. As nonionic detergents, most notably Triton X-100 (Blall *et al.*, 1990d) and Triton X-114 (present study), have been successfully applied to the preparation of the OMPs from *H. paragallinarum* strains, the structure, parameters for solubilisation and mechanism of action of only these detergents will be discussed. Detergents of the Triton X series are polyoxyethylene derivatives containing a polymeric $(O\text{-}CH_2\text{-}CH_2)_n\text{-}OH$ chain of variable length (Figure 4.1). The most important parameters with regard to solubilisation include the critical micelle concentration [CMC, which specifies the minimum detergent concentration (0.015% (m/v) for Triton X-100 and 0.011% (m/v) for Triton X-114) at which micelles begin to form and is thus sufficient to solubilise the membrane proteins], the critical micellar temperature [CMT, above which micelle formation occurs], and the cloud point [a characteristic temperature above the CMT at which detergents undergo phase separation (64°C for Triton X-100 and 22°C for Triton X-114)] (Neugebauer, 1990; Von Jagow *et al.*, 1994).

Triton X-100



Triton X-114

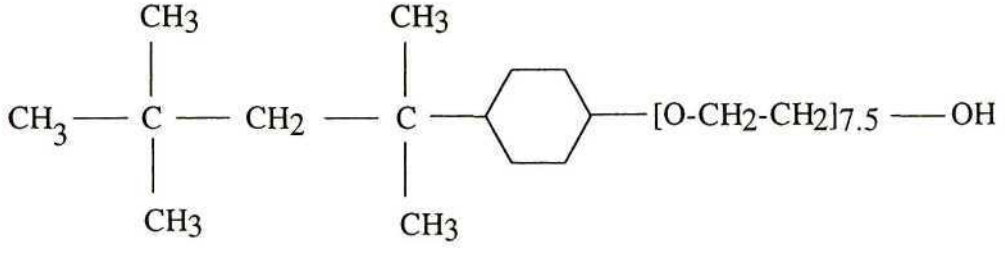


Figure 4.1 Chemical structure of the detergents Triton X-100 (Findlay, 1990) and Triton X-114 (Brusca and Radolf, 1994).

Triton X-100 exercises a selective solubilizing effect on the cytoplasmic membrane of Gram-negative bacteria thereby separating it from the detergent-insoluble outer membrane proteins (Schnaitman, 1971; Filip *et al.*, 1973; Carlone *et al.*, 1986; Blackall *et al.*, 1990d). The mechanism of action of Triton X-100 is not understood but resistance to solubilisation is believed to be dependent, at least in part, on the action of the divalent cation Mg^{2+} in stabilisation of the outer membrane. In contrast, partitioning of the membrane proteins by phase separation is well documented. Phase separation results from dehydration of the oxyethylene chains at high temperatures decreasing the detergent's aqueous solubility such that the suspension partitions into a detergent-rich phase and a detergent-depleted phase (Ganong and Delmore, 1991; Brusca and Radolf, 1994). Bordier (1981) recognised that the low cloud point of Triton X-114 could be exploited for separating hydrophilic peripheral proteins and hydrophobic integral membrane proteins. The distribution of proteins depends on their relative hydrophobicity: amphiphilic integral membrane proteins partition into the lower detergent-rich phase, and the hydrophilic, globular proteins partition into the upper aqueous phase.

In the present study a comparison of methods for selective solubilisation, using Triton X-100, and phase separation, using Triton X-114, of the OMPs of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates was carried out for selection of the most effective protocol for subsequent investigations.

4.4.1 Selective solubilisation using Triton X-100

4.4.1.1 Materials

HEPES buffer [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4]. HEPES (2.383 g) was dissolved in 950 ml dist. H_2O, the pH was adjusted to 7.4 with HCl and the buffer was made up to 1 litre with dist. H_2O.

2% (v/v) Triton X-100. Triton X-100 (2 ml) was diluted to 100 ml with HEPES buffer.

4.4.1.2 Procedure

The detergent-insoluble OMPs were isolated using a rapid microprocedure (Carlone *et al.*, 1986), modified as described by Blackall *et al.* (1990d). The optical density of the harvested bacterial cells was adjusted to between 0.5 and 0.6 at 600 nm by dilution with PBS (Section 2.5.2.1).

Samples of the adjusted bacterial suspensions were aliquoted into 1.5 ml microfuge tubes and centrifuged (5 000 x g, 10 min, 4°C). The supernatant fluid was discarded and the pellets were resuspended in 1.5 ml of cold HEPES buffer and washed by centrifugation (15 600 x g, 2 min, 4°C). Following removal of the supernatant, the pellets were suspended in 1 ml of HEPES buffer and, while kept on ice, sonicated (8 bursts, 10 s each) with an Ultrasonicator. The unbroken cells and debris were removed by centrifugation (15 600 x g, 2 min, 4°C). The supernatant was transferred to a clean 1.5 ml microfuge tube, and the cell membranes were sedimented by centrifugation (15 600 x g, 45 min, 4°C). The gel-like cell membrane pellets were completely suspended in HEPES buffer (0.2 ml) by repeated pipetting and the cytoplasmic membranes were selectively solubilized by addition of an equal volume of 2% (v/v) Triton X-100. The resulting suspension was incubated at room temperature for 30 min with intermittent mixing and centrifuged (15 600 x g, 45 min, 4°C) to pellet the outer membranes. The supernatant was decanted and the membranes were washed without resuspension in 0.5 ml of HEPES buffer, and then resuspended in 50 to 100 µl HEPES buffer. The concentration of the isolated OMPs was determined using the BCA assay (Section 2.2.2). Protein samples (100-300 ng of protein per expected band) were aliquoted into 1.5 ml microfuge tubes and mixed with an equal volume of non-reducing treatment buffer (Section 2.3.1). The suspension was heated to 100°C for 90 s for complete denaturation of the proteins and to allow uniform binding of the SDS molecules. The denatured samples were analysed under non-denaturing SDS-PAGE conditions (Section 2.3) coupled with a silver protein staining (Section 2.4) or immunochemical (Section 2.5.3) detection system.

4.4.2 Phase separation using Triton X-114

4.4.2.1 Materials

2% (v/v) Triton X-114. Triton X-114 (2 ml) was diluted to 100 ml with PBS (Section 2.5.2.1).

4.4.2.2 Procedure

The concentration of the bacterial suspension was adjusted to 200 µg/ml with PBS (Section 2.5.2.1) in a final volume of 200 µl. Following the addition of an equal volume of 2% (v/v) Triton X-114, selective extraction of the outer membranes proceeded at 4°C for a minimum of 1 h, but preferably overnight. Prior to phase separation any detergent-insoluble material was

removed by centrifugation (13 000 x g, 10 min, 4°C). The supernatant was retained and phase separation was induced by incubation at 37°C for 10 min, followed by centrifugation (13 000 x g, 10 min, RT). The aqueous phase was transferred to a new microfuge tube and Triton X-114 was added to a final concentration of 2% (v/v). The phases were separated and centrifuged (13 000 x g, 10 min, RT), and the aqueous phase was collected for analysis. The detergent phase (approx. 50 µl) was thoroughly mixed with 1 ml PBS (Section 2.5.2.1) at 0°C, the suspension was warmed to 37°C to induce phase separation and centrifuged as before. The aqueous phase was discarded and the proteins of the detergent-rich phase were precipitated (1 h at -20°C) by addition of 10 volumes of acetone to remove the detergent which has been found to cause smearing during SDS-PAGE analysis (Brusca and Radolf, 1994). The resulting precipitate was pelleted by centrifugation (13 000 x g, 10 min, 4°C) and washed three times with PBS (Section 2.5.2.1) to remove any residual detergent. The protein concentration of both the detergent and aqueous phases was determined using the BCA assay (Section 2.2.2). Protein samples (100-300 ng of protein per expected band) were aliquoted into 1.5 ml microfuge tubes and mixed with an equal volume of non-reducing treatment buffer (Section 2.3.1). The suspension was heated to 100°C for 90 s for complete denaturation of the proteins and to allow uniform binding of the SDS molecules. The denatured samples were analysed under non-denaturing SDS-PAGE conditions (Section 2.3) coupled with a silver protein staining detection system (Section 2.4).

4.5 Identification of heat-modifiable OMPs

Many Gram-negative bacteria have one or more OMPs which exhibit heat-modifiable behaviour (Beher *et al.*, 1980; Van Alphen *et al.*, 1983; Coulton and Wan, 1983; Rapp *et al.*, 1986). Two mechanisms for these heat-dependent changes have been proposed. OMPs are unusually, yet characteristically, rich in β-structured polypeptides, which are stable in an SDS environment but undergo a conformational change to α-helices upon heating (Nakamura and Mizushima, 1976; Hancock and Carey, 1979). The β-structured conformation is maintained by hydrophobic interactions, however, upon heating to 100°C the resulting molecular agitation weakens these interactions such that an α-helical conformation is assumed (Mizushima, 1974). At temperatures below 85°C the β-folded protein has a greater mobility on SDS-PAGE and therefore an apparent lower molecular mass than the fully unfolded protein. In this case, heat modification reflects a complete unfolding of the protein when heated (Chen *et al.*, 1980) and implies that the protein is

held together in its unheated native state by strong non-covalent forces, typically hydrogen bonds (Darby and Creighton, 1993). In addition, heat modification may be observed if insufficient heat is supplied, such that proteins may remain tightly bound to the peptidoglycan layer and are therefore unable to enter the electrophoretic gel (Rosenbusch, 1974). These properties of tight non-covalent association of the OMP with the peptidoglycan layer and resistance to denaturation at temperatures below 85-100°C are characteristic of the matrix-type proteins (Osborn and Wu, 1980).

In the present investigation, efforts to identify possible heat-modifiable OMPs were carried out as the basis for a broad preliminary characterisation of some of the virulence-associated antigens into those which are peptidoglycan-associated and those which are non-covalently ordered into β-structures in the native state.

4.5.1 Procedure

Isolation of OMPs was carried out as described in Section 4.4.1. Protein samples (100-300 ng of protein per expected band) were aliquoted into 1.5 ml microfuge tubes and mixed with an equal volume of non-reducing treatment buffer (Section 2.3.1). Prior to electrophoresis (Section 2.3), the samples were heated at various temperatures (25, 37 and 100°C) and incubation times (10 and 30 min).

4.6 Identification of the surface-exposed OMPs

Proteins of the outer membrane are anchored to the peptidoglycan layer via lysine (N-terminal) and cysteine (C-terminal) residues (Stanier *et al.*, 1986). Pandher and Murphy (1996) deduced that proteolytic cleavage of these anchoring residues would release the attached polypeptide and enable the identification of those OMPs that are surface-exposed. The serine proteases trypsin and chymotrypsin were chosen due to their specificity for basic (especially lysine and arginine) and hydrophobic (e.g. phenylalanine and leucine) residues respectively (Kraut, 1977).

The identification of the OMPs which are exposed to the environment of the host was essential in this study. Further characterisation of the OMPs, performing possible protective or adhesive roles, was the ultimate goal for the identification of potential targets for vaccine design.

4.6.1 Materials

Trypsin buffer [0.041 M Tris-HCl, 0.012 M calcium chloride, pH 8.0]. Tris (0.497 g) and $CaCl_2$ (0.133 g) were dissolved in 95 ml dist. H_20, the pH was adjusted to 8.0 with HCl and the buffer was made up to 100 ml with dist. H_2O.

Chymotrypsin buffer [0.08 M Tris-HCl, 0.1 M calcium chloride, pH 8.0]. Tris (0.968 g) and $CaCl_2$ (1.110 g) were dissolved in 95 ml dist. H_2O, the pH was adjusted to 8.0 with HCl and the buffer was made up to 100 ml with dist. H_2O.

1% (m/v) Trypsin. Trypsin (10 mg) was dissolved in 1 ml of trypsin buffer.

1% (m/v) Chymotrypsin. Chymotrypsin (10 mg) was dissolved in 1 ml of chymotrypsin buffer.

4.6.2 Procedure

The optical density of harvested bacterial cells was adjusted to between 0.5 and 0.6 by dilution with PBS (Section 2.5.2.1). The cells were collected by centrifugation in a microfuge (10 000 rpm, 1 min, RT) and washed in an appropriate enzyme buffer (1 ml). Following centrifugation (10 000 rpm, 1 min, RT), the pellets were resuspended in trypsin or chymotrypsin buffer (250 µl) to which the enzymes trypsin and chymotrypsin (5 µl) were added respectively. Negative controls were included in which bacteria were suspended in enzyme buffer lacking the corresponding enzyme. The bacterial cells were incubated on a rotary shaker for 1 h at 37°C for proteolytic digestion. Following centrifugation (10 000 rpm, 1 min, RT) the cells were washed in PBS (Section 2.5.2.1) and resuspended in a combination of PBS (Section 2.5.2.1; 50 µl) and non-reducing treatment buffer (Section 2.3.1; 50 µl) for electrophoretic analysis (Section 2.3). Visualisation of the surface-exposed OMPs was facilitated using either a silver stain (Section 2.4) or an immunochemical detection system (Section 2.5.3).

4.7 Detection of lipopolysaccharides

Bacterial lipopolysaccharides, or endotoxins, are components of the outer membrane of Gram-negative bacteria and have been implicated as virulence determinants in many pathogenic microorganisms including strains of *H. paragallinarum* (Iritani *et al.*, 1980b).

Lipopolysaccharides (LPSs) are complex, amphipathic molecules consisting of two covalently-linked components: a hydrophobic glycolipid called lipid A, and a hydrophilic polysaccharide. Within this basic conserved structure, a significant level of structural and antigenic variability exists (Towner and Cockayne, 1993). Lipid A is responsible for the toxic activity of endotoxin and has a largely conserved structure. The polysaccharide component is structurally divided into three regions: an inner and outer core region of monosaccharides, and an O-polysaccharide-rich region (Kabir *et al.*, 1978). The latter region, in comparison to the core constituents, displays a vast degree of variation. However, this O-polysaccharide is not found in all strains. Consequently, the absence of the O-polysaccharide in some variants, due to a genetic mutation or an elementary lack of expression thereof, led to the introduction of the term rough LPS (Towner and Cockayne, 1993). On the other hand, LPS containing the O-polysaccharide is called smooth LPS. In strains of *Haemophilus*, including *H. influenzae*, LPS is naturally rough (Inzana, 1983) and is referred to as lipooligosaccharide. The structural components of LPS in strains of *H. paragallinarum* have, as yet, not been detailed.

Traditionally, LPSs were extracted from bacterial cells employing various combinations of aqueous phenol, chloroform, and petroleum ether (Westphal and Jann, 1965; Galanos *et al.*, 1969). However, the large quantity of bacteria required and the potential safety hazard imposed by the phenol, led to Hitchcock and Brown (1983) developing a non-solvent-based method. The relative simplicity of this technique and the degree of reproducibility obtained in comparison with the profiles of pure LPS, favoured the use of this new method over previously described techniques. Hitchcock and Brown (1983) exploited the action of proteinase K to digest the bacterial proteins of temperature-lysed bacterial cells, leaving the protease-resistant LPSs in solution.

A highly sensitive periodic acid-silver stain has been described for the identification of electrophoretically-separated lipopolysaccharides (Dubray and Bezard, 1982; Tsai and Frasch, 1982). Detection of polysaccharides containing 1,2-diols forms the basis of this staining technique, which may thus be extended to the identification of glycoproteins which, amongst others, function as receptors for binding. Periodic acid is responsible for oxidising the 1,2-diols to produce aldehyde functions which are subsequently detected with a protein silver stain. The

sensitivity of this silver stain is reportedly comparable to autoradiography (Tsai and Frasch, 1982).

4.7.1 Preparation of lipopolysaccharides

4.7.1.1 Materials

Lysing buffer [1 M Tris-HCl, pH 6.8, 2% (m/v) SDS, 4% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol]. Tris (12.113 g) was dissolved in 80 ml deionised H_2O and the pH was adjusted to 6.8 with HCl. SDS (2 g), 2-mercaptoethanol (4 ml) and glycerol (10 ml) were added and mixed in thoroughly, and the buffer was made up to 100 ml with deionised H_2O.

25% (m/v) Proteinase K. Proteinase K (25 mg) was dissolved in lysing buffer (1 ml).

4.7.1.2 Procedure

The optical density of the bacterial cells was adjusted to between 0.5 and 0.6 by dilution with PBS (Section 2.5.2.1). The cells were collected by centrifugation (13 000 x g, 10 min, RT), resuspended in lysing buffer (50 µl) and heated at 100°C for 10 min. Following lysis, proteins were digested with proteinase K (10 µl) at 60 °C for 1 h. Negative controls were included in which bacterial cells were suspended and heated in lysing buffer, lacking the enzyme proteinase K. Following incubation, the LPS preparations and their corresponding controls, were subjected to non-reducing SDS-PAGE (Section 2.3) with a modification in that the percentage of the separating gel was increased from 10 to 15%, and visualised with a periodic acid-silver stain (Section 4.7.2).

4.7.2 Periodic acid-silver staining

4.7.2.1 Materials

Fix solution [25% (v/v) isopropyl alcohol, 10% (v/v) acetic acid]. Isopropyl alcohol (250 ml) and acetic acid (100 ml) were mixed together and made up to 1 litre with deionised H_2O.

0.2% (v/v) periodic acid. Periodic acid (0.2 ml) was diluted to 100 ml with deionised H_2O.

Impregnate solution [20% (m/v) $AgNO_3$, 0.1 M NaOH]. $AgNO_3$ (20 g) was dissolved in 100 ml of deionised H_2O. Of this solution, 4 ml was slowly added to a mixture of fresh NH_4OH (2.8 ml)

and 0.1 M NaOH (21 ml) with vigorous agitation to redissolve the brown precipitate which forms upon mixing. The resulting solution was made up to 100 ml with deionised H_2O.

Developing solution [0.05% (m/v) citric acid, 10% (v/v) methanol, 0.019% (v/v) formaldehyde]. Citric acid (0.05 g) was dissolved in 85 ml of deionised H_2O, methanol (10 ml) and formaldehyde (51 µl of a 37% (v/v) solution) were added and the solution was made up to 100 ml with deionised H_2O.

4.7.2.2 Procedure

All steps were carried out on an orbital shaker at room temperature unless otherwise stated. On completion of the electrophoretic run, the gels were soaked in fix solution overnight and washed in 7.5% (v/v) acetic acid (30 min). The gels were incubated in 0.2% (v/v) periodic acid (1 h, 4°C) for oxidation of the 1,2-diols and washed in several changes of deionised H_2O over a period of 3 h. The water was removed and the gels were placed in impregnate solution (10 min), washed in deionised H_2O (3 x 10 min) and the developing solution was added. After about 2-5 min, the reaction was terminated with Ilford Pan F photographic fixer and the gels were stored in a sealed plastic container, in the dark, until photographed.

4.8 N-terminal sequence analysis

Identification of the N-terminus of proteins enables characterisation of its structural and functional domains, and is determined using repeated cycles of the Edman degradation reaction (Matsudaira, 1990). Each degradation cycle consists of three steps: coupling, cleavage, and conversion. In the coupling step, the α-amino group of the N-terminus of the protein is modified with the Edman reagent, phenyl isothiocyanate, under basic conditions to generate a phenylthiocarbamoyl (PTC) polypeptide. The second step involves cleavage of the PTC-N-terminal residue from the protein by either liquid or gaseous trifluoroacetic acid to liberate an anilinothiazoline (ATZ)-amino acid derivative of the original N-terminal residue, and the shortened polypeptide. Finally, the unstable ATZ-amino acid is converted by acid treatment to the more stable phenylthiohydantoin (PTH)-amino acid. Identification of these derivatives is facilitated through a combination of ultraviolet (UV) absorbance and high-performance liquid chromatography (HPLC), with a sensitivity of 1-10 pmol (Matsudaira, 1989; Serwe and Meyer, 1994). The new N-terminal

residue is now available for another Edman degradation cycle. This procedure is commonly performed by automated gas-phase sequenators for the simple and efficient analysis of purified proteins, which have been resolved by SDS-PAGE and then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Matsudaira, 1987). The resulting N-terminal amino acid sequence may be compared with existing sequences held in protein sequence databases, permitting the identification and classification of the protein of interest (Lottspeich, 1994).

A number of post-translational modifications of protein molecules may confound the sequencing process, most notably, the N-terminal blocking of a peptide or protein. Approximately 50% of all naturally occurring proteins are N-terminally modified by an acetyl, a formyl, or a pyroglutamic acid group (Lottspeich *et al.*, 1994). These blocked proteins cannot be sequenced by conventional Edman degradation steps, requiring prior enzymatic or chemical cleavage. In addition to N-terminal blockage as a native property of a protein, blockage may be introduced during sample preparation or purification. To this end, the Tris-tricine gels were polymerised four days in advance to ensure that all the reactive peroxide radicals were depleted. In addition, the scavenger thioglycollic acid was included in the electrotransfer buffer to reduce the possibility of N-terminal blockage (Legendre *et al.*, 1993).

In the present study, N-terminal sequence analysis was applied to an approximately 30 kDa OMP of a representative NAD-independent *H. paragallinarum* field isolate, namely 96/11756. This investigation was conducted due to an apparent similarity of this protein with the unique, serotype-determining OMP of NAD-dependent *H. paragallinarum* field isolate 46. As this isolate belongs to serotype C-3, the predominant type infecting local flocks of birds, it was thus of interest to ascertain the structural and hence functional properties of this protein, for evaluation of its potential as a target for vaccine design.

4.8.1 Materials

Electrotransfer buffer [10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), 10% (v/v) methanol, 0.1 mM thioglycollic acid, pH 11.0]. CAPS (2.213 g) was dissolved in 850 ml dist. H_2O, and methanol (100 ml) and thioglycollic acid (ρ = 1.33 g/ml; 7 µl) were added. The pH was adjusted to 11.0 with NaOH, and the buffer was made up to 1 litre with dist. H_2O.

Destain solution [30% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (30 ml) and acetic acid (10 ml) were diluted to 100 ml with dist. H_2O.

Stain solution [0.1% (m/v) amido black in destain solution]. Amido black (0.1 g) was dissolved in destain solution (100 ml).

4.8.2 Procedure

The OMPs of NAD-independent *H. paragallinarum* field isolate 96/11756 were prepared as described in Section 4.4.1. Protein sample (approximately 25 µg) was aliquoted into a 1.5 ml microfuge tube, mixed with an equal volume of non-reducing treatment buffer (Section 2.3.1) and heated at 100 °C for 2 min. Tris-tricine separating gels were prepared as described in Section 2.3 three days in advance, while the stacking gels were prepared one day in advance. The protein sample was loaded into the sample application wells and electrophoresed at 80 V (maximum current) until the bromophenol blue front had migrated through the stacking gel. The voltage was then increased to 100 V for the remainder of the electrophoretic run. Following electrophoretic separation, proteins were electrotransferred (as per Section 2.5.3) to PVDF membranes pretreated in 100% (v/v) methanol (15 s), deionised H_2O (5 min) and then stored in electrotransfer buffer. On completion of electrotransfer (0.4 A, 45 min), the PVDF membrane was washed in deionised H_2O (5 min) and immersed in stain solution (2 min). The protein of interest (approx. 30 kDa) was excised with a clean, sharp scalpel, and destained (10 min). The membrane strip was then rinsed in deionised H_2O (2 x 5 min), air dried and transferred to a 1.5 ml microfuge tube for storage at - 20°C. N-terminal sequencing was undertaken on an automated Perkin Elmer Applied Biosystems Procise 491 Instrument at the Molecular Biology Unit, University of Natal (Pietermaritzburg), South Africa.

4.9 Production of chicken and rabbit anti-*H. paragallinarum* whole cell antibodies

For completeness sake, the protocol for immunisation is described here. However, these procedures were carried out at Allerton Regional Veterinary Laboratory (Kwazulu-Natal, South Africa) to generate serotype-specific antibodies for use in a serotype-determining diagnostic assay, namely haemagglutination inhibition.

4.9.2 Procedure

Antibodies against NAD-dependent *H. paragallinarum* whole cells from reference strains 0083, 0222 and Modesto, representing serotypes A, B, and C respectively, and against NAD-independent *H. paragallinarum* whole cells from field isolate 95/03938 were raised in chickens and rabbits. Two chickens and two rabbits were used for each strain, as different species may respond differently to the same immunogen (Harlow and Lane, 1988). The animals were immunised intramuscularly with a total of 1 mg of each of the four strains, which had been triturated with an equal volume of thiomersal-inactivated aluminium hydroxide adjuvant into a thick emulsion. Further inoculations of live bacteria of each strain, suspended in PBS (Section 2.5.2.1), were administered intravenously every 4-6 days. Chicken eggs were collected weekly from the start of the immunisation protocol over a 16-week interval, while blood was only collected from rabbits 6-weeks post-immunisation by cardiac puncture. Anti-*H. paragallinarum* strain 0083, 0222, Modesto and 95/03938 whole cell antibodies were purified from the serum of the rabbits (IgG; Section 2.5.1.2) and from the yolk of the chickens' eggs (IgY; Section 2.5.1.3) by precipitation with PEG. The antibodies were partially characterised by ELISA (Section 2.5.2) and were used as the primary antibody in western blot analyses (Section 2.5.3).

4.10 Results and discussion

4.10.1 Whole cell protein profiles

Non-reducing SDS-PAGE of the whole cell proteins (WCPs) of NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates produced reproducible patterns containing 30 to 40 discrete bands (Figure 4.2 - 4.4). Analysis of the WCP profiles of the six NAD-dependent *H. paragallinarum* reference strains, as well as field isolates previously serotyped by R.R. Bragg (University of Pretoria, South Africa) using a haemagglutination-inhibition test, facilitated fundamental documentation of serotype-specific WCP fingerprints. Visual comparison of these characteristic profiles, in addition to identification and molecular mass determination of prominent, individual proteins, enabled broad allocation of the NAD-independent *H. paragallinarum* field isolates to the appropriate serotype, and to a limited extent, their corresponding serovar.

It should be noted that the degree of complexity of the WCP profiles did not permit the unique serotype-determining proteins to be annotated (Figures 4.2 – 4.4). The comparative simplicity of the OMP patterns afforded illustration of these protein bands (Figures 4.5 – 4.7).

4.10.1.1 NAD-dependent *H. paragallinarum* reference strains and field isolates

Non-reducing SDS-PAGE, in conjunction with a silver protein stain detection system, of the WCPs of NAD-dependent *H. paragallinarum* reference strains (Figure 4.2) and field isolates (Figure 4.3) revealed overall similar, yet complex protein profiles containing several common, prominent protein bands (Table 4.1; 4.2). Moreover, several unique protein bands were identified, largely in the 20.1 to 43 kDa range, their molecular masses were determined (Table 4.1; 4.2), and they were utilised as distinctive markers for type allocation.

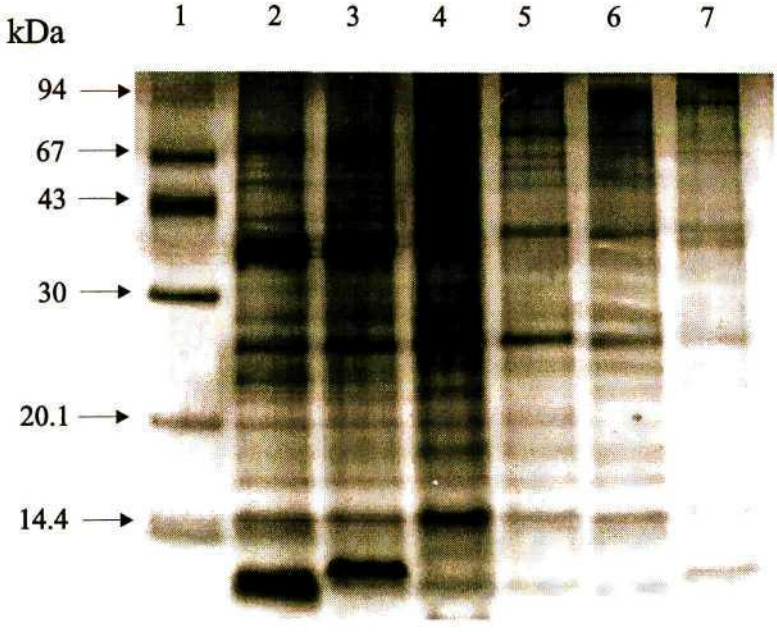


Figure 4.2 Non-reducing SDS-PAGE of the WCPs of NAD-dependent *H. paragallinarum* reference strains. Samples were boiled in non-reducing treatment buffer and loaded onto a 10% tricine gel. **Lane 1,** Molecular mass markers (phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa), **lanes 2-7,** *H. paragallinarum* strains [**lane 2,** 0083 (serovar A-1); **lane 3,** 221 (serovar A-1); **lane 4,** 2403 (serovar A-2); **lane 5,** ATCC 29545 (serotype A); **lane 6,** 0222 (serovar B-1); **lane 7,** Modesto (serovar C-2)].

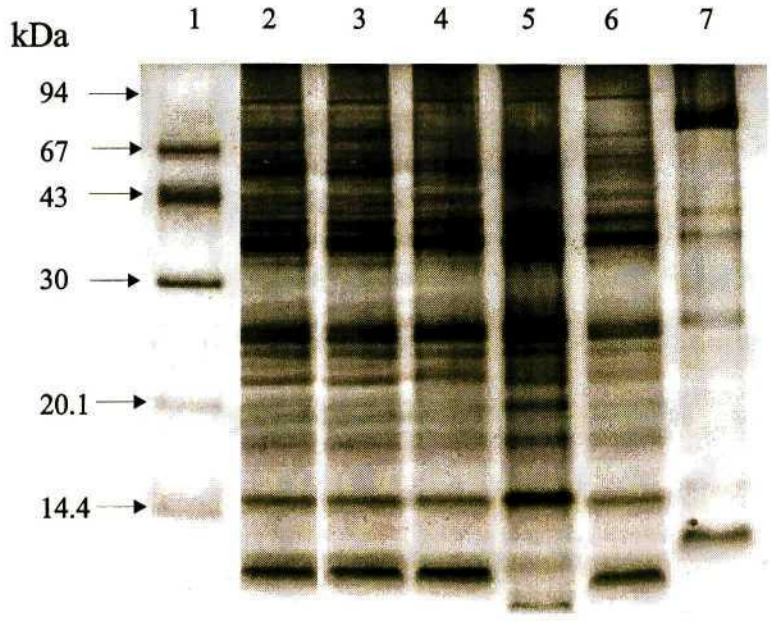


Figure 4.3 Non-reducing SDS-PAGE of the WCPs of NAD-dependent *H. paragallinarum* field isolates. Samples were boiled in non-reducing treatment buffer and loaded onto a 10% tricine gel. **Lane 1,** Molecular mass markers (as in Figure 4.2), **lanes 2-7,** *H. paragallinarum* strains **[lane 2,** 32 (serotype A); **lane 3,** 39 (serotype A); **lane 4,** 41 (serotype A); **lane 5,** 37 (serovar C-2); **lane 6,** 43 (serovar C-2); **lane 7,** 46 (serovar C-3)].

Table 4.1 Analysis of the WCPs of NAD-dependent *H. paragallinarum* reference strains for identification of unique and common prominent proteins.

Protein band number	Molecular mass (kDa)	NAD-dependent *H. paragallinarum* reference strains					
		0083 (A-1)	221 (A-1)	2403 (A-2)	ATCC 29545 (A)	0222 (B-1)	Modesto (C-2)
1	87.3	c	c	c	c	c	c
2	79.0	c	c	c	c	c	c
3	77.3	c	c	c	c	c	c
4	69.6	c	c	c	c	c	c
5	66.0	c	c	-	c	c	c
6	57.0	c	c	c	c	c	-
7	40.8	**u**	**u**	-	-	-	-
8	39.2	-	-	-	**t_1**	-	**t_1**
9	38.3	**t_2**	**t_2**	**t_2**	-	**t_2**	-
10	36.5	c	c	c	-	-	-
11	35.2	c	c	c	-	-	c
12	28.4	c	c	c	c	c	-
13	26.4	-	-	-	**u**	-	-
14	25.9	c	c	c	-	c	c
15	24.6	-	-	c	c	c	-
16	23.2	**u**	**u**	-	-	-	-
17	22.8	c	c	c	-	c	c
18	19.7	c	c	c	c	c	c
19	18.3	c	c	c	c	c	c
20	16.8	c	c	c	c	c	c
21	14.7	c	c	c	c	c	c
22	12.8	-	**u**	-	-	-	-
23	12.0	**u**	-	-	-	-	**u**
24	11.5	-	-	c	c	c	c

NOTE: c = common protein; u = unique protein; - = absent protein, t_1, t_2 = type 1 or 2 protein

Table 4.2 Analysis of the WCPs of NAD-dependent *H. paragallinarum* field isolates for identification of unique and common prominent proteins.

Protein band number	Molecular mass (kDa)	NAD-dependent *H. paragallinarum* field isolates					
		32 (A)	39 (A)	41 (A)	37 (C-2)	43 (C-2)	46 (C-3)
1	88.2	-	-	-	-	-	**u**
2	82.3	c	c	c	c	c	c
3	75.6	c	c	c	c	c	-
4	61.0	c	c	c	c	c	-
5	55.8	c	c	c	c	c	-
6	43.0	**u**	**u**	**u**	-	**u**	-
7	41.6	-	-	-	**u**	-	-
8	39.9	$\mathbf{t_1}$	$\mathbf{t_1}$	$\mathbf{t_1}$	-	$\mathbf{t_1}$	-
9	38.2	c	c	c	c	c	c
10	37.5	c	c	c	-	c	-
11	36.4	c	c	c	-	c	c
12	35.4	c	c	c	c	c	-
13	34.9	c	c	c	c	c	-
14	33.5	c	c	c	c	c	-
15	29.0	-	-	-	-	-	**u**
16	26.7	-	-	-	-	-	**u**
17	25.9	-	-	-	c	-	-
18	25.4	c	c	c	-	c	c
19	24.7	c	c	c	c	c	-
20	23.7	c	c	c	c	c	-
21	21.9	c	c	c	c	c	c
22	19.7	c	c	c	c	c	c
23	17.8	c	c	c	c	c	c
24	14.4	c	c	c	c	c	c
25	13.1	-	-	-	-	-	**u**
26	10.9	c	c	c	c	c	-

Blackall *et al.* (1989a) identified two protein profile types, characterised by the relative molecular mass of a prominent polypeptide. Strains belonging to protein profile type 1 expressed a major protein at approximately 42 kDa, whereas type 2 isolates were characterised by the presence of a prominent protein at 40 kDa, which replaced the 42 kDa protein. In the present study, affirmation of the initial deductions of Blackall *et al.* (1989a) was demonstrated, in that the NAD-dependent *H. paragallinarum* reference strains could be assigned to one of two protein profile types. However, the present study differed in that the relative sizes of the proteins of interest were not 42 (type 1) and 40 kDa (type 2), but rather 39 and 38 kDa respectively, as confirmed later by Blackall *et al.* (1990d). In addition, the present study contrasted to previous findings in the allocation of the reference strains to the established protein profile types 1 and 2.

In the present study (Tables 4.1 and 4.2), *H. paragallinarum* strains ATCC 29545, 32, 39, 41 (all serotype A), 43 and Modesto (both serovar C-2) were found to express a major marker protein at 39 kDa, characteristic of protein profile type 1. On the other hand, *H. paragallinarum* strains 0083, 221 (both serovar A-1), 2403 (serovar A-2) and 0222 (serovar B-1) were typical of protein profile type 2 due to the presence of a 38 kDa protein. These results were only in agreement with those reported by Blackall *et al.* (1989a; 1990d) regarding the protein profile type assignment of strains 2403, 0222 and Modesto. Nevertheless, the validity of the above results was partially confirmed by the degree of reproducibility obtained following repeated electrophoretic runs, carried out under the same experimental conditions, and that observed between different strains within a single serovar.

As previously mentioned, the WCPs profiles of the NAD-dependent *H. paragallinarum* reference strains and field isolates within the protein profile types 1 and 2 were found to be reproducible for different strains of the same serovar. Type 2 *H. paragallinarum* serovar A-1 strains 0083 and 221 produced identical protein profiles and could be differentiated from type 2 serovar A-2 strain 2403 by the presence of protein bands at 40.8 and 23.2 kDa in the former. Furthermore, these strains could be differentiated from a related type 2 strain 0222 on this basis (Table 4.1). In a similar manner, strains of protein profile type 1 were subdivided into serovar-specific fingerprints. The ATCC 29545 strain, of serotype A, may be distinguished from the serovar C-2 Modesto strain due to the presence of a unique 26.4 kDa band in the former and a unique 12.0 kDa protein expressed by the latter. As expected, serotype A strains 32, 39 and 41 produced identical WCP

profiles distinct from those of serovar A-1, A-2 and the ATCC 29545 strain, prompting the hypothesis that the former strains, in addition to the ATCC 29545 strain, may independently belong to either serovar A-3 or A-4. Unexpectedly, serovar C-2 strain 43 produced a protein profile identical to that of strains 32, 39, and 41. This result suggests that the serovar C-2 field isolate could have been assigned to the incorrect serotype. Alternatively, this observation may provide the first evidence of the limitations of WCP profiling for serotype determination.

The WCP profiles of *H. paragallinarum* serovar C-2 and C-3 field isolates 37 and 46 respectively, were distinctive and could not be included in either protein profile type 1 or 2. Isolate 37 may be identified by unique proteins at 41.6 and 25.9 kDa, and displayed distinctly different WCP profiles to that of the classical serovar C-2 reference strain Modesto. This provides evidence of antigenic variation within a serovar and highlights possible ambiguities encountered during WCP typing. Isolate 46 is characterised by unique bands at 88.2, 29.0, 26.7, and 13.1 kDa. The 29.0 kDa protein band was of particular interest and attempts to further characterise a corresponding protein in the NAD-independent isolates is described in Section 4.10.8.

The serovar-specific WCP fingerprints generated were subsequently employed as templates to which the WCP profiles of the NAD-independent *H. paragallinarum* field isolates were compared to enable type assignment.

4.10.1.2 NAD-independent *H. paragallinarum* field isolates

Non-reducing SDS-PAGE, in conjunction with a silver stain detection system, of the WCPs of NAD-independent *H. paragallinarum* field isolates (Figure 4.4) revealed complex protein profiles which were identical, but differed significantly from those of the NAD-dependent strains. As the NAD-independent isolates were obtained from two geographically separated locations (Gauteng Province and Kwazulu-Natal), the resulting reproducible and indistinguishable patterns provide evidence to the clonal origins of this *H. paragallinarum* variety.

Table 4.3 summarises the most prominent proteins (observed in Figure 4.4), with their corresponding molecular masses, expressed by the NAD-independent *H. paragallinarum* field isolates. In addition, a comparison of these proteins with those of the NAD-dependent

H. paragallinarum reference strains and field isolates was made in an attempt to assign the NAD-independent isolates to one of several previously fingerprinted serotypes and serovars. Protein bands were characterised according to whether they were unique i.e. absent in previously established serotype fingerprints (u), ubiquitous i.e. common to more that one established serotype (c), or serotype-specific (s).

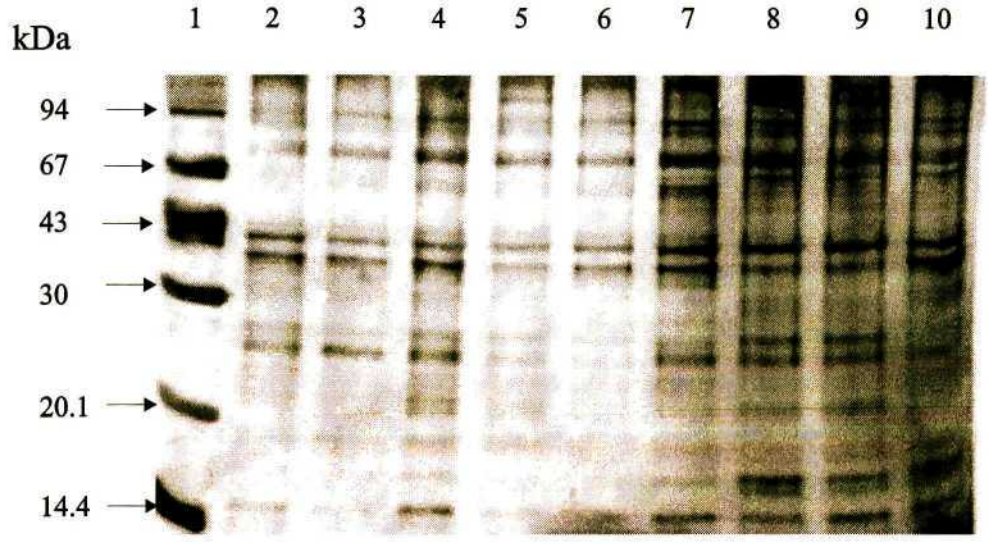


Figure 4.4 Non-reducing SDS-PAGE of the WCPs of NAD-independent *H. paragallinarum* field isolates. Samples were boiled in non-reducing treatment buffer and loaded onto a 10% tricine gel. **Lane 1,** Molecular mass markers (as in Figure 4.2); **lanes 2-10,** *H. paragallinarum* strains [**lane 2,** 95/03938; **lane 3,** 93/09472; **lane 4,** 93/00449; **lane 5,** 96/11756; **lane 6,** 96/12954; **lane 7,** RH 2390; **lane 8,** 1130 (serotype A); **lane 9,** 1742 (serotype A); **lane 10,** 541 (serovar C-3)].

The NAD-independent *H. paragallinarum* field isolates did not exhibit a WCP profile characteristic of either type 1 or 2, due to the absence of a 39 or 38 kDa protein. As neither serovar C-2 (based on field isolate 37) or C-3 protein profiles corresponded to either of the established WCP types (Section 4.10.1.1), the NAD-independent isolates were tentatively assigned to one of these serovars. Consequently, a comparison of individual NAD-independent field isolate WCPs with those of the NAD-dependent *H. paragallinarum* reference strains and field isolates was effected, revealing several standard proteins expressed by most *H. paragallinarum* organisms (Table 4.1 - 4.3). The ubiquitous nature of these proteins implied their role as integral functional components of the surviving bacterium. In addition, individual proteins were recognised which were unique to the NAD-independent isolates providing further

evidence of differentially expressed proteins in comparison to an individual NAD-dependent counterpart. More importantly, however, a number of protein bands were identified which were specific to microorganisms of a particular serotype. The distinguishing proteins were approximately 43, 37, 27, 26, and 23 kDa in size, suggesting that the NAD-independent isolates may belong to one of the serovars of serotype A or to either serovar C-2 or C-3 (Table 4.3).

Table 4.3 Analysis of the major WCPs of the NAD-independent *H. paragallinarum* field isolates and comparison to established reference strain protein profiles.

Protein band number	**Molecular mass (kDa)**	**NAD-independent *H. paragallinarum* field isolates**	**Common (c), unique (u) or serotype-specific (s) protein bands as compared to NAD-dependent *H. paragallinarum* reference strains**
1	92.3	c	u
2	77.1	c	c
3	73.7	c	u
4	62.5	c	u
5	56.7	c	c
6	55.7	c	c
7	42.7	c	s (A)
8	40.3	c	s (A-1)
9	37.0	c	s (A)
10	27.3	c	s (A, C-3)
11	25.8	c	s (C-2)
12	23.9	c	c
13	23.0	c	s (A-1)
14	21.8	c	c
15	18.2	c	c
16	17.2	c	c
17	15.2	c	c

Application of WCP profiling to the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates was met with limited success in that antigenic differences between strains of different serotype and, to a certain degree, of different serovar were noted. This was in agreement with previous studies (Blackall *et al.*, 1989a) where Laemmli (1970) SDS-PAGE gels were used. It was thought that the Tris-tricine SDS-PAGE system (Schägger and von Jagow, 1987) would give improved resolution of the WCP bands, thereby showing differences between the different strains. However, despite better resolution these differences noted were not sufficiently marked such that field isolates could be irresputably assigned to a serotype. It was therefore decided to focus on the surface-exposed OMPs which were proposed by Rapp *et al.* (1986) as candidates for recognising strain variations within a particular species. Subsequently, comparably more reliable serotype-specific fingerprints may be created.

4.10.2 Outer membrane protein profiles

The OMPs were isolated using one of two methods: a) sonic disruption followed by differential centrifugation and selective solubilisation in Triton X-100, and b) phase separation employing Triton X-114.

OMPs were isolated using a rapid outer membrane protein (ROMP) procedure (Carlone *et al.*, 1986) in which the detergent sodium *N*-lauroyl sarcosinate was replaced with Triton X-100 (Blackall *et al.*, 1990d). This technique exploited the difference in size between the cell membranes and the large unbroken cells and cell debris, generated following sonication, using differential centrifugation. Subsequently, the Triton X-100-insoluble property of the outer membrane was manipulated to effect the solubilisation of the Triton X-100-soluble cytoplasmic membranes, resulting in the release of OMPs at a concentration of 2 to 4 mg/ml. These preparations contained only a limited number of whole cell proteins.

This is the first report of the isolation of the OMPs of this avian *Haemophilus* using the detergent Triton X-114. As the OMPs are anchored to the cytoplasmic membrane by regions of hydrophobic amino acids or amphiphilic groups covalently linked to the polypeptide chains (Brusca and Radolf, 1994), they partitioned into the lower Triton X-114-rich phase. Triton X-114 phase partitioning was reported to be a deceptively powerful technique in that proteins separated to the detergent phase were enriched approximately 20-fold (Maher and

Singer, 1985). These reports were confirmed by SDS-PAGE analysis of the detergent-containing proteins (results not shown) when compared to the OMP profile obtained following Triton X-100 extraction (Figure 4.4 - 4.6), as near identical protein patterns were exhibited.

The two techniques were thus equally successful in terms of dissociation, resolution and reproducibility in the isolation of the OMPs from whole cell bacteria. However, isolation using the ROMP procedure employing Triton X-100 was preferred since this method was comparably rapid requiring approximately 2.5 hs for completion.

The application of OMP profiling to all isolates employed in the present study, with the exception of the NAD-dependent strains 0083, 0222 and Modesto, represents a novel study aimed at developing a reliable serotype-specific protein fingerprint. Non-reducing SDS-PAGE of the Triton X-100 isolated OMPs of NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates produced reproducible patterns containing a range of major and minor OMPs (Section 4.10.2.1 and 4.10.2.2). The selectivity and hence success of the isolation of OMPs was revealed when a comparison of the WCP and OMP protein profiles was made. This study revealed individual major WCPs that were absent in the OMP profiles, while the converse was also true, in that several minor WCPs appeared as major OMPs following SDS-PAGE analysis. Qualitative interpretation of the OMP patterns of the reference strains and field isolates was done in a further attempt to establish a serotype-specific fingerprint, following the limited success obtained for serotype allocation of the NAD-independent *H. paragallinarum* field isolates on a whole cell level.

4.10.2.1 NAD-dependent *H. paragallinarum* reference strains and field isolates

Non-reducing SDS-PAGE, in conjunction with a silver protein stain detection system, of OMPs of NAD-dependent *H. paragallinarum* reference strains (Figure 4.4) and field isolates (Figure 4.5) revealed overall similar profiles with fewer protein bands compared to the whole cell profiles. A number of common major and minor OMPs were observed (Table 4.4; 4.5), of which the 93, 87 and 66 kDa proteins were expressed by all NAD-dependent isolates studied. In addition, 73 and 25 kDa proteins were identified which were shared by most, but not all, isolates. Comparative analysis of all the common proteins shared by strains of different serotypes was not implemented, for it was considered cumbersome and would offer no contribution to the ultimate

aim of the present study i.e. identification of sero-determinants as potential targets in vaccine design. As a result a thorough investigation was carried out into the identification of the serotype-specific antigens for the generation of protein fingerprints and subsequent serotype allocation of the problematic NAD-independent *H. paragallinarum* field isolates.

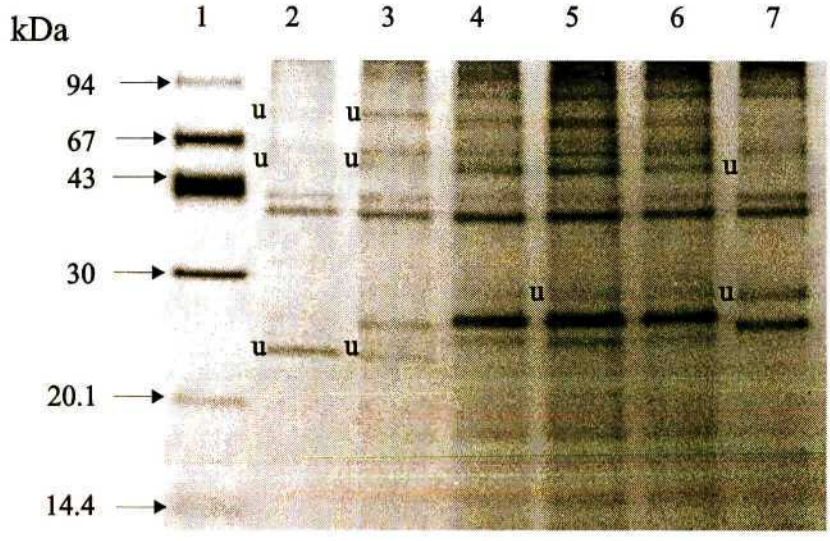


Figure 4.5 Non-reducing SDS-PAGE of the OMPs of NAD-dependent *H. paragallinarum* reference strains. Samples were boiled in non-reducing treatment buffer and loaded onto a 10% tricine gel. **Lane 1,** Molecular mass markers (as in Figure 4.2); **lanes 2-7,** *H. paragallinarum* strains **[lane 2,** 0083 (serovar A-1); **lane 3,** 221 (serovar A-1); **lane 4,** 2403 (serovar A-2); **lane 5,** ATCC 29545 (serotype A); **lane 6,** 0222 (serovar B-1); **lane 7,** Modesto (serovar C-2)]. The unique serotype-determining proteins (u) are indicated.

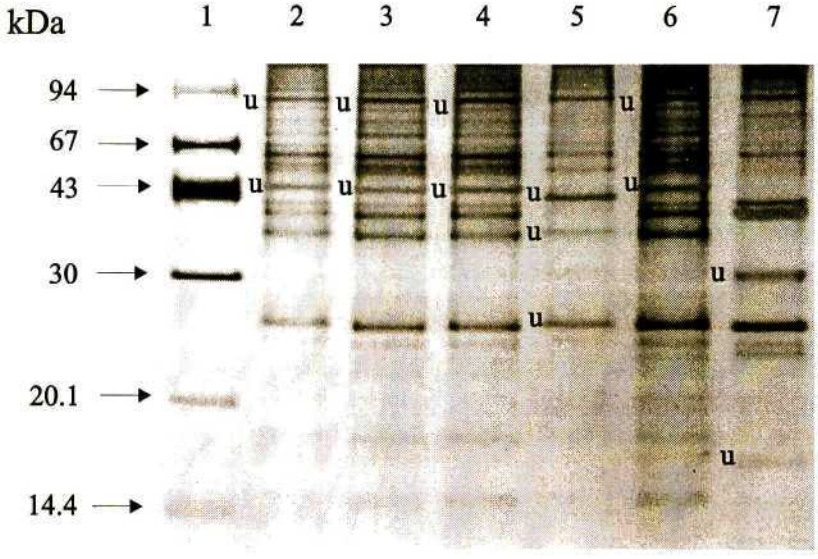


Figure 4.6 Non-reducing SDS-PAGE of the OMPs of NAD-dependent *H. paragallinarum* field isolates. Samples were boiled in non-reducing treatment buffer and loaded onto a 10% tricine gel. **Lane 1,** Molecular mass markers (as in Figure 4.2); **lanes 2-7,** *H. paragallinarum* strains **[lane 2,** 32 (serotype A); **lane 3,** 39 (serotype A); **lane 4,** 41 (serotype A); **lane 5,** 37 (serovar C-2); **lane 6,** 43 (serovar C-2); **lane 7,** 46 (serovar C-3)]. The unique serotype-determining proteins (u) are illustrated.

Table 4.4 Analysis of the OMP profiles of NAD-dependent *H. paragallinarum* reference strains for identification of unique and common prominent proteins.

Protein band number	Molecular mass (kDa)	NAD-dependent *H. paragallinarum* reference strains					
		0083 (A-1)	221 (A-1)	2403 (A-2)	ATCC 29545 (A)	0222 (B-1)	Modesto (C-2)
1	93.8	c	c	c	c	c	c
2	86.8	c	c	c	c	c	c
3	80.8	**u**	**u**	-	-	-	-
4	73.1	c	c	c	c	c	-
5	71.3	-	-	c	c	-	c
6	65.5	c	c	c	c	c	c
7	59.6	c	c	c	c	c	c
8	55.9	**u**	**u**	-	-	-	**u**
9	54.4	-	-	c	c	c	-
10	53.3	c	c	c	-	-	-
11	51.5	-	-	c	c	c	c
12	41.7	c	c	c	c	c	c
13	39.6	$\mathbf{t_1}$	-	-	-	-	$\mathbf{t_1}$
14	38.7	-	$\mathbf{t_2}$	$\mathbf{t_2}$	$\mathbf{t_2}$	$\mathbf{t_2}$	-
15	28.9	-	-	-	**u**	-	**u**
16	26.3	-	-	c	c	c	-
17	26.1	c	c	-	-	-	c
18	25.0	c	c	c	c	c	c
19	24.0	**u**	**u**	-	-	-	-
20	18.5	c	c	c	c	c	c
21	16.8	c	c	c	c	c	c
22	15.0	c	c	c	c	c	c

Table 4.5 Analysis of the OMP profiles of NAD-dependent *H. paragallinarum* field isolates for identification of unique and common prominent proteins.

Protein band number	Molecular mass (kDa)	NAD-dependent *H. paragallinarum* field isolates					
		32 (A)	39 (A)	41 (A)	37 (C-2)	43 (C-2)	46 (C-3)
1	93.3	c	c	c	c	c	c
2	87.5	c	c	c	c	c	c
3	84.6	**u**	**u**	**u**	-	**u**	-
4	81.6	c	c	c	c	c	c
5	74.1	c	c	c	c	c	c
6	72.3	c	c	c	c	c	c
7	71.5	c	c	c	-	c	-
8	65.8	c	c	c	c	c	c
9	62.0	c	c	c	c	c	c
10	57.1	c	c	c	c	c	-
11	54.2	c	c	c	-	c	-
12	43.8	**u**	**u**	**u**	-	**u**	-
13	41.4	-	-	-	**u**	-	-
14	40.3	$\mathbf{t_1}$	$\mathbf{t_1}$	$\mathbf{t_1}$	-	$\mathbf{t_1}$	-
15	38.9	-	-	-	-	-	$\mathbf{t_2}$
16	38.6	c	c	c	-	c	-
17	36.0	-	-	-	**u**	-	-
18	35.6	c	c	c	-	c	-
19	29.8	-	-	-	-	-	**u**
20	26.5	-	-	-	**u**	-	-
21	26.3	c	c	c	-	c	c
22	25.0	c	c	c	-	c	c
23	24.1	c	c	c	-	c	c
24	18.1	c	c	c	-	c	-
25	16.7	-	-	-	-	-	**u**
26	14.7	c	c	c	-	c	-

Attempts were made to assign the NAD-dependent *H. paragallinarum* reference strains and field isolates to one of two protein profile types described by Blackall *et al.* (1990d) and applied earlier to the WCP patterns (Section 4.10.1). In the present study, *H. paragallinarum* strains 0083 (serovar A-1), 32, 39, and 41 (all serotype A), 43 and Modesto (both serovar C-2) were found to express a major OMP at approximately 39 kDa, characteristic of protein profile type 1. In contrast, *H. paragallinarum* strains 221 (serovar A-1), 2403 (serovar A-2), ATCC 29545 (serotype A), 0222 (serovar B-1) and 46 (serovar C-3) were assigned to protein profile type 2 due to the presence of an OMP at approximately 38 kDa. The clear dominance and apparent significance of this protein in type determination prompted Blackall *et al.* (1990d) to assign the numerical acronym OMP C to this protein of variable molecular size.

The above results correlate with those obtained for these strains at a WCP level, with the exception of strains 0083, ATCC 29545 and field isolate 46, the former two being previously allocated to type 2 and 1 respectively, while the latter was untypable. However, the allocation of strain 0083 to protein profile type 1 corroborated the findings of Blackall *et al.* (1990d). Therefore, the degree of agreement between the assignment of the *H. paragallinarum* reference strains and field isolates to either protein profile type 1 or 2 was comparable at both a WCP and OMP level, and to a certain extent with the findings of Blackall *et al.* (1989a; 1990d). However, variability in the type assignment of strains 0083 and ATCC 29545, in addition to fluctuations in the molecular size of the determining 38 and 39 kDa proteins of individual strains, and hence the inability of these profile types to differentiate between strains of different serotype, limited the application of this protocol for type allocation. Consequently, a study of unique, serotype-determining proteins was undertaken.

The outer membrane protein profiles of different strains within type 1 or 2 of the same serotype were notably different, while those of the same serovar were found to be predominately reproducible. In agreement with the WCP profiles, serotype-determining OMP differences were identified, largely, in the 20.1 to 43 kDa range. *H. paragallinarum* serovar A-1 strains 0083 and 221, corresponding to protein profile type 1 and 2, produced identical OMP profiles, with the exception of the variable position of OMP C which fluctuated between approximately 39 and 38 kDa respectively. In general, however, serotype A-1 strains 0083 and 221 may be fingerprinted on the basis of the 80.8 and 24.0 kDa proteins. Serotype A strains 32, 39 and 41,

and serovar C-2 strain 43 produced identical OMP profiles, confirming equivalent findings at the whole cell level, and distinguishing them from fellow type 1 strain 0083 (and related serovar A-1 strain 221) by unique bands at approximately 84.6 and 43.8 kDa. In addition, these strains may be differentiated from type 1 serovar C-2 strain Modesto, which expresses a distinguishing combination of proteins with a molecular size of approximately 55.9 and 28.9 kDa. On a similar basis, strains of protein profile type 2 were characterised into serovar-specific OMP patterns. *H. paragallinarum* serovar A-2 strain 2403 was distinguished from related serovar A-1 reference strains by a 26.1 kDa protein, but is separated from serotype A ATCC 29545 strain by the absence of a 28.9 kDa OMP. The OMP profile of serovar B-1 strain 0222 was unexpectedly, yet remarkably, similar to the profile of a combination of the ATCC 29545 and 2403 strains, to the extent that 0222 isolates did not appear to express a serotype-determining protein and discrimination between these strains proceeded via a process of protein band elimination. *H. paragallinarum* serovar C-3 field isolate 46 expressed unique proteins at 29.8 and 16.7 kDa distinguishing it from fellow type 2 isolates. This major 29.8 kDa OMP corresponded to a minor WCP and, as the NAD-independent counterpart is believed to be the most prevalent strain infecting local flocks of birds, an attempt was made to further characterise this protein (Sections 4.10.3 - 4.10.8).

Once again, the OMP profile of *H. paragallinarum* serovar C-2 strain 37 was distinctive and could not be assigned to either type 1 or 2 protein profile due to the absence of both a 39 or 38 kDa protein. Isolate 37 was distinguished from the previously mentioned *H. paragallinarum* reference strains (including fellow serovar C-2 strain Modesto) and field isolates by unique proteins at 41.4, 36.0 and 26.5 kDa.

The OMPs of the NAD-dependent *H. paragallinarum* reference strains and field isolates were sufficiently different to design serotype-specific, and to a certain extent serovar-specific, OMP fingerprints which are less complex and unambiguous than previously constructed WCP templates. On this basis, the NAD-independent *H. paragallinarum* field isolates had been tentatively assigned (Section 4.10.1.2) to one of either serovars of serotype A or to either serovar C-2 (based on field isolate 37) or C-3. The following section shall describe attempts to allocate these NAD-independent isolates to one of the above types.

4.10.2.2 NAD-independent *H. paragallinarum* field isolates

Non-reducing SDS-PAGE, in conjunction with a silver protein stain detection system, of the OMPs of NAD-independent *H. paragallinarum* field isolates (Figure 4.7) revealed reproducible profiles with slight qualitative and a certain number of quantitative differences amongst the strains from varying geographical locations.

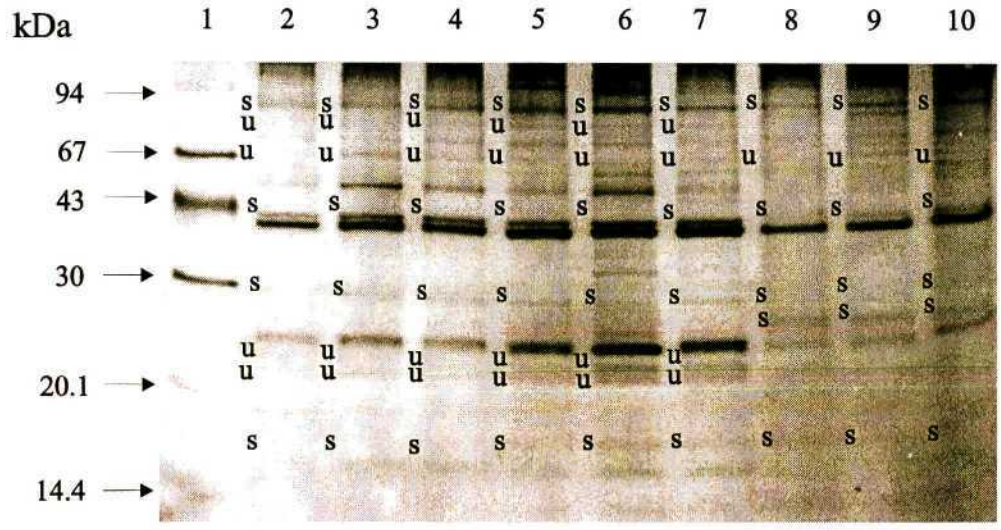


Figure 4.7 Non-reducing SDS-PAGE of the OMPs of NAD-independent *H. paragallinarum* field isolates. Samples were boiled in non-reducing treatment buffer and loaded onto a 10% tricine gel. **Lane 1,** Molecular mass markers (as in Figure 4.2); **lanes 2-10,** *H. paragallinarum* strains **[lane 2,** 95/03938; **lane 3,** 93/09472; **lane 4,** 93/00449; **lane 5,** 96/11756; **lane 6,** 96/12954; **lane 7,** RH 2390; **lane 8,** 1130 (serotype A); **lane 9,** 1742 (serotype A); **lane 10,** 541 (serovar C-3)]. Protein bands unique to NAD-independent isolates (u), as well as serotype-specific proteins (s) are illustrated.

The NAD-independent *H. paragallinarum* field isolates exhibited an OMP profile characteristic of type 1 due to the dominant and reproducible expression of OMP C at approximately 39 kDa. These findings are in contrast to those found with the WCPs which lacked a 39 or 38 kDa type 1 or 2 protein. It could be postulated that NAD-independent isolates belong to one of the type 1 serovars namely A-1, A-3, A-4 or C-2, which is consistent with the conclusions from whole cell profiling (Section 4.10.1.2) with the omission of OMP type 2 serovar C-3. Within protein profile type 1, however, differences were noted in the banding patterns amongst isolates obtained from

poultry units in the Gauteng Province (1130, 1742, and 541) and in Kwazulu-Natal (95/03938, 93/09472, 93/00449, 96/11756, 96/12954, and RH 2390). As field isolates 1130, 1742 (all serotype A) and 541 (serovar C-3) had previously been characterised using a haemagglutination-inhibition test (R.R. Bragg, University of Pretoria, South Africa), it was expected that they would produce differing profiles. The identical nature of these protein profiles was alarming in that it demonstrated the limited application for serotyping using OMP fingerprints, clearly demanding genotypic techniques for differentiation. On the other hand, however, the reproducibility of these profiles once again proved evidence that all the NAD-independent strains originated from a single bacterial cell, itself derived from an NAD-dependent microorganism which overcame the requirement of NAD for growth. Despite differences in the protein profiles of strains from different geographical locations, it is still believed that they are clonal, with these protein differences being attributed to differential protein expression as a result of a combination of environmental factors, number of infected hosts, and host-bacterial cell interactions.

Consequently, a comparison of the individual OMPs of the two groups of NAD-independent field isolates with each other and with the fingerprinted NAD-dependent *H. paragallinarum* reference strains and field isolates was made (Table 4.6). Several proteins expressed by most *H. paragallinarum* organisms were observed, in addition to a number of NAD-independent-specific determinants at 79.0, 67.3, 51.0, 23.4 and 22.1 kDa. With the exception of the 67.3 kDa protein, these proteins are unique to isolates obtained from local poultry farms, while a protein of 26.8 kDa distinguishes the Gauteng isolates. Furthermore, a slight protein banding difference corresponding to 85.6 and 84.4 kDa was observed, but is thought to be the same OMP of variable size. Possible methods of differential (acquisition and/or loss of) protein expression within strains of NAD-independency have been described above. Comparison with formerly serotyped reference isolates revealed a number of serotype-specific protein bands. The common OMP of variable size (85.6 and 84.4 kDa) was limited to serotype A strains. However, the fluctuating molecular mass of this protein in a region of low resolution on the gel, and the protein not being typically sero-determining (Nicolet *et al.*, 1980), led to this option being discarded. It thus remained to differentiate between serovar C-2 and C-3 for the assignment of the NAD-independent field isolates. Both the Kwazulu-Natal and Gauteng

Province isolates qualitatively displayed serovar C-3 specific OMPs at 29.6 and 17.2 kDa, favouring the allocation of all the NAD-independent isolates studied as serovar C-3.

Table 4.6 Analysis of the major OMPs of the NAD-independent *H. paragallinarum* field isolates and comparison to established reference strain protein profiles.

Protein band number	**Molecular mass (kDa)**	**NAD-independent *H. paragallinarum* field isolates**		**Common(c), unique (u) or serotype-specific (s) protein bands as compared to NAD-dependent *H. paragallinarum* reference strains**
		Kwazulu-Natal	**Gauteng Province**	
1	86.8	c	c	c
2	85.6	**u**	-	s (A)
3	84.4	-	**u**	s (A)
4	79.0	**u**	-	u
5	72.7	c	c	c
6	67.3	c	c	u
7	51.0*	c	c	c
8	41.2	c	c	s (C-2)
9	39.5	c	c	$\mathbf{t_1}$
10	29.7	c	c	s (C-3)
11	26.8	-	**u**	s (C-2)
12	25.3	c	c	c
13	23.4	**u**	-	u
14	22.1	**u**	-	u
15	17.2*	c	c	s (C-3)
16	15.6	c	c	c

* based on analysis of the original SDS-PAGE gel, protein bands are not evident on the scanned image

The application of OMP profiling to the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates proved efficacious in that reference fingerprints were

constructed as templates for serotype determination of field isolates. Genotypic analyses of the conserved ribosomal RNA sequences (Chapter 5) of strains of varying NAD dependency, is clearly essential for confirmation of phenotypic conclusions. Following WCP and OMP profiling, attempts were made to further characterise the surface-exposed OMPs both structurally and functionally (Section 4.10.3 - 4.10.8).

4.10.3 Identification of heat-modifiable OMPs

NAD-dependent *H. paragallinarum* samples exhibiting OMP profiles representative of each serotype and serovar previously characterised (Section 4.10.2.1), as well as two field isolates which displayed typical NAD-independent OMP profiles, were studied for possible identification of heat-modifiable OMPs. Non-reducing SDS-PAGE, in conjunction with a silver protein stain detection system, of the heat-treated OMPs of these reference and field isolates revealed a few obvious heat-modifiable OMPs (Figure 4.8).

No differences were detected in the OMP profiles between samples of identical strains incubated at either 25°C or 37°C for a period of 30 min. For this reason, samples heated at 37°C were chosen to illustrate both incubation times. For all strains tested, with the exception of serovar C-2 strain 37, a heat-modifiable OMP of variable molecular size (39 or 38 kDa) was identified which was found to correspond to the protein profile type 1- and 2-determining protein, namely OMP C (Section 4.10.2). This protein was present in greatly reduced amounts when the preparation was heated at 37°C, however as the temperature was increased to 100°C OMP C appeared as a dominant band. These observations suggest that the appearance of OMP C upon boiling was not due to a more complete unfolding of the protein, but may be attributed to a peptidoglycan-disassociation (Rosenbusch, 1974). Consequently, OMP C may be characterised as a matrix-type protein, thus confirming the conclusions drawn from comparable studies conducted by Blackall *et al.* (1990d).

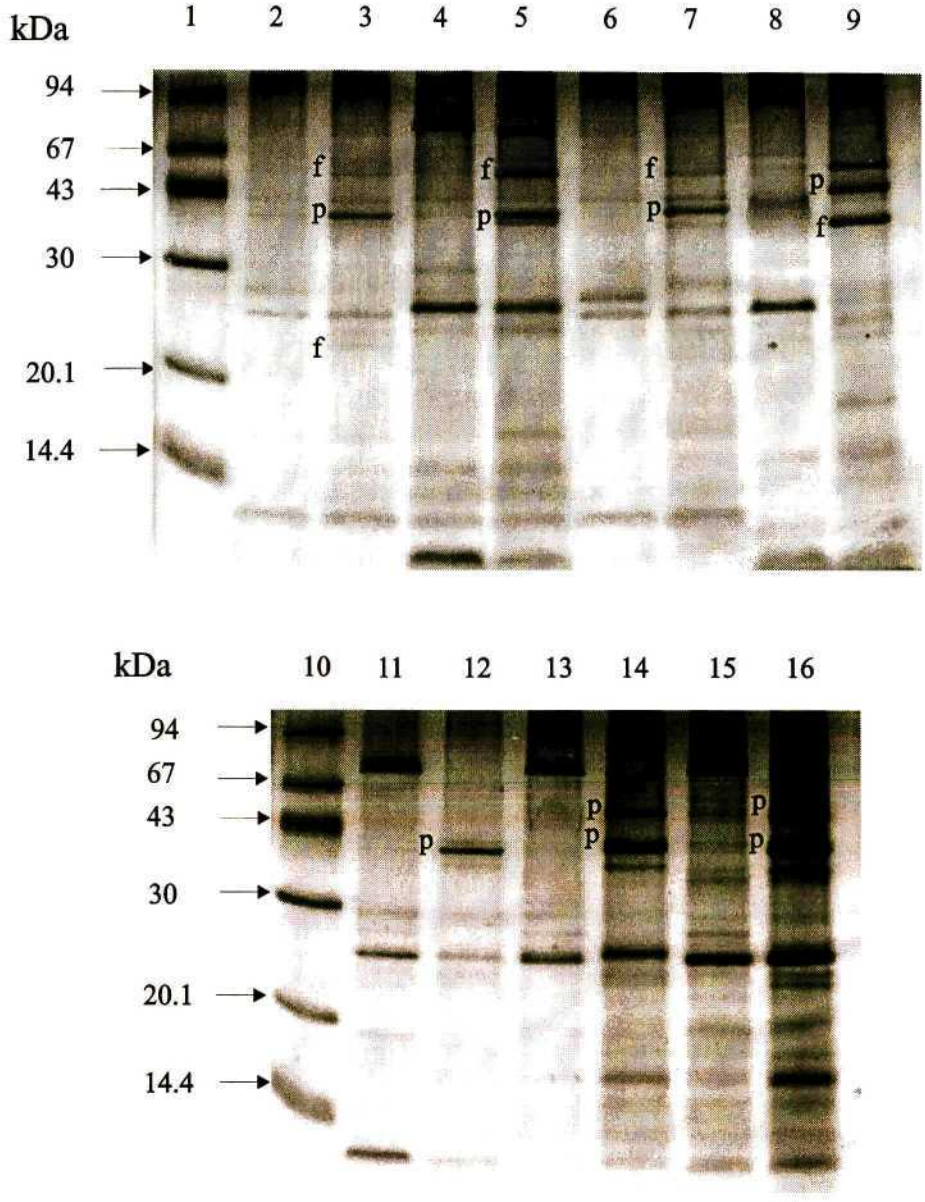


Figure 4.8 Non-reducing SDS-PAGE of heat-treated OMPs of selected NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates. Each sample was incubated at 37°C or 100°C for 30 min in non-reducing treatment buffer and loaded in adjacent wells of a 10% tricine gel. **Lanes 1, 10** Molecular mass markers; **lanes 2-9, 11-16,** *H. paragallinarum* strains **[lanes 2 and 3,** 0083 (serovar A-1); **lanes 4 and 5,** ATCC 29545 (serotype A); **lanes 6 and 7,** Modesto (serovar C-2); **lanes 8 and 9,** 37 (serovar C-2); **lanes 11 and 12,** 46 (serovar C-3); **lanes 13 and 14,** 93/03938; **lanes 15 and 16,** 96/11756]. Proteins that are folded (f) or associated with peptidoglycan (p) in the native state are indicated.

Further studies of the heat-treated OMPs of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates revealed several additional heat-modifiable OMPs. Table 4.7 summarises the most prominent heat-modifiable OMPs (observed in Figure 4.8) and their molecular mass changes following heat-treatment. In comparison to established

serotype- and serovar-specific OMP profiles (Section 4.10.2), these bands were characterised according to whether they were previously labelled as either common (c) or unique (u), or whether they were absent (-) from these profiles (Figure 4.5 - 4.7). Furthermore, attempts were made to describe the heat-modifiable OMPs as either peptidoglycan-associated (p) or arranged in a non-covalently-associated, folded (f) conformation in the native state (Section 4.5).

Table 4.7 Heat-modifiable OMPs of NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates.

***H. paragallinarum* strain**	**Protein band (kDa)**		**Common (c), unique (u) or absent (-) protein bands as assigned in previously typed OMP profiles**	**Peptidoglycan-associated (p) or folded (f) OMP conformation prior to boiling**
	Heat-treatment			
	37°C	**100°C**		
0083	20.0	22.6	u	f
	26.7	51.0	c	f
	36.1	36.1	-	p
ATCC 29545	29.0	51.0	c	f
	36.1	36.1	-	p
Modesto	26.8	51.0	c	f
	-	37.0	-	p
37	25.2	35.6	u	f
	41.5	41.5	u	p
46	-	37.1	-	p
95/03938	-	37.1	-	p
	48.3	48.3	c	p
96/11756	37.1	37.1	-	p
	48.3	48.3	c	p

The NAD-dependent reference isolate 37 exhibited two heat-modifiable OMPs, both of which define the serovar C-2-specific protein fingerprint. The unique 35.6 kDa protein was demonstrated to assume a folded structure in its native state, assuming a molecular size of 25.2 kDa at low temperatures. The other heat-modifiable OMP (41.5 kDa) was found to be peptidoglycan-associated as it was expressed in lowered concentrations when heated to 37°C.

NAD-dependent reference strains 0083, ATCC 29545 and Modesto expressed a 51 kDa protein which, prior to heat-treatment at 100°C, was folded into a conformation with an apparent lower molecular mass of between approximately 26 and 29 kDa. The NAD-independent field isolates expressed a common peptidoglycan-associated OMP at approximately 48.3 kDa which prior to heating was characteristically present in greatly reduced amounts. More importantly, however, was the recognition of a common peptidoglycan-associated, heat-modifiable OMP approximately 36 to 37 kDa in size, in all strains with the exception of field isolate 37. This OMP was noticeably absent from all the NAD-dependent and -independent reference strain and field isolate OMP profiles (Figure 4.5 - 4.7) which were prepared for SDS-PAGE analysis by heat-treatment at 100°C for 90 s. It is therefore postulated that the discrepancy between these two profiles may be attributed to a very high degree of association between the peptidoglycan and the OMP, requiring prolonged heat-treatment to effect dissociation.

A similar major, heat-modifiable envelope protein with a molecular mass of 36.5 kDa following dissociation from the peptidoglycan, was reported in strains of *E. coli* (Rosenbusch, 1974). This protein is believed to be responsible for the lattice structure covering the peptidoglycan, thereby conferring structural integrity to the outer membrane. Typical characteristics include a resistance to tryptic hydrolysis, however, it is believed that this protein appears partially on the outer surface of the rigid cell wall. The similarity of this membrane protein to the OMP of strains of *H. paragallinarum* in terms of heat-modifiability favours the extrapolation of the structural characteristics of the *E. coli* protein to the *H. paragallinarum* OMP. These investigators proposed two possible mechanisms for the phenomenon of heat-modifiability observed, with the the method that will be mentioned last being favoured. Firstly, associated proteins are thought to undergo gradual dissociation from the sample application well-bound peptidoglycan, thereby accounting for retardation of the protein in the matrix following treatment at low temperatures.

Alternatively, proteins complexed to the peptidoglycan have been demonstrated to be unable to bind the negatively charged SDS molecules tightly. As a result these proteins do not carry a nett negative charge proportional to their size, and as such are separated on the basis of both charge and size. The reduced negative charge accounts for a lower degree of attraction to the anode and subsequently a reduced electrophoretic mobility.

Either of these theories may account for the heat-modifiability of OMP C, as major protein bands found at approximately 78 kDa in strains ATCC 29545, 46, 95/03938, and 96/11756 disappeared upon heating to 100°C. The 78 kDa band disappeared due to the dissociation of the peptidoglycan-protein complexes, and was replaced by a protein at 39 or 38 kDa. As a high molecular mass dominant protein band was not evident in strain 37 when heat-treated to 37°C, a mechanism of peptidoglycan-disassociation of the 41.5 kDa protein could not be postulated. Similarly, the mechanism for the heat-dependent changes of OMP C observed in strains 0083 and Modesto is unknown.

It has been reported that the generation of distinguishing serotype-specific protein fingerprints may be aided by the identification of heat-modifiable OMPs (Rapp *et al.*, 1986). The 48.3 kDa protein uniquely present as a heat-modifiable OMP in NAD-independent field isolates was also expressed by the NAD-dependent field isolate 46 of serovar C-3 in a greatly reduced non-heat-modifiable form. Further comparison revealed the profiles of these strains to be qualitatively identical. These results serve to confirm the allocation of the NAD-independent field isolates to serovar C-3.

This section represented attempts to further characterise the OMPs, structurally and functionally. Two common, predominant heat-modifiable OMPs, approx. 36 and 51 kDa in size, were characterised which were both peptidoglycan-associated and most likely matrix-type proteins. Subsequently, it is essential to determine whether these common proteins are susceptible to proteolytic digestion and are therefore surface-exposed (Section 4.10.4) and immunogenic (Section 4.10.8) - all critical characteristics of the efficient production of an effective vaccine.

4.10.4 Identification of surface-exposed OMPs

Trypsin and chymotrypsin treatment of the WCPs of selected NAD-dependent and -independent reference strains and field isolates for the identification of surface-exposed OMPs, was met with varied success. Protease treatment with chymotrypsin was more effective than trypsin treatment, and as such only the results yielded by treatment with the former enzyme will be presented. In addition, immunoblot analysis of the chymotrypsin-treated and untreated whole cells using rabbit anti-*H. paragallinarum* strain 95/03938 antibodies (Figure 4.9) proved more efficient in revealing the surface-exposed OMPs in comparison to a silver stain detection system (results not shown), with the added advantage of identifying antibody-accessible OMPs.

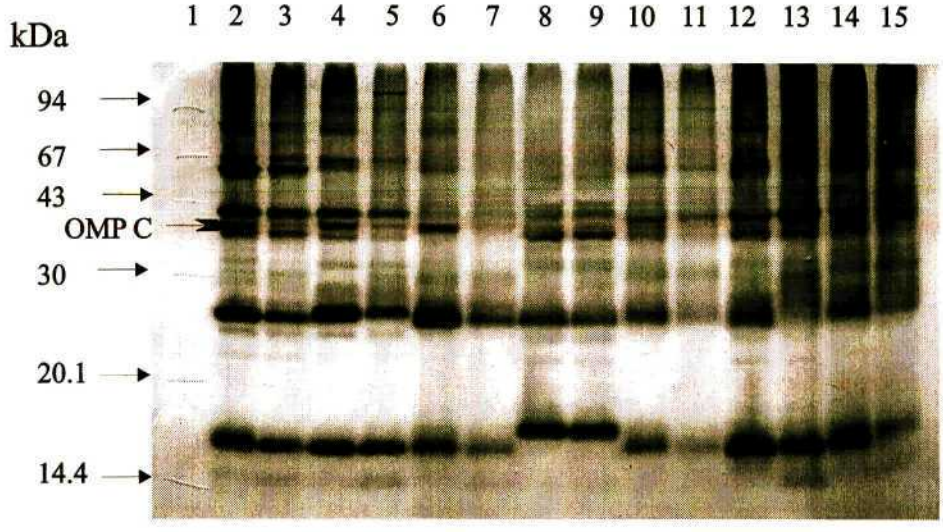


Figure 4.9 Western blot analysis of chymotrypsin-treated WCPs of selected NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates. Untreated controls and chymotrypsin-treated samples were boiled in non-reducing treatment buffer and loaded in adjacent wells of a 10% tricine gel. **Lane 1,** Molecular mass markers (as in Figure 4.2); **lanes 2-15,** *H. paragallinarum* strains [**lanes 2 and 3,** 0083 (serovar A-1); **lanes 4 and 5,** ATCC 29545 (serotype A); **lanes 6 and 7,** Modesto (serovar C-2); **lanes 8 and 9,** 43 (serovar C-2); **lanes 10 and 11,** 46 (serovar C-3); **lanes 12 and 13,** 93/00449; **lanes 14 and 15,** 541 (serovar C-3)]. The apparent position of OMP C is illustrated.

Recognition of the surface-exposed OMPs proceeded by visual comparison of the protein profile of untreated controls and protease digested samples (Figure 4.9). The disappearance of a WCP band indicates cleavage and hence identifies a surface-exposed OMP. OMP C of reference strains 0083, ATCC 29545 and Modesto was chymotrypsin-sensitive as considerably reduced amounts of this protein were present in preparations treated with the protease. This finding may be central in

vaccine design as OMP C is a common type-determining protein (Section 4.10.1) with characteristics typical of OMPs. Examples include a susceptibility to heat treatment (Section 4.10.3) and a surface location such that it is able to interact with, amongst others, antibody molecules in the external environment.

Several major and minor bands in the molecular mass range 20.1 to 67 kDa were affected by chymotrypsin digestion to considerably lesser extents than OMP C, ranging from being mildly sensitive to insensitive. These observations have led to uncertainty as to whether some of these proteins are surface-exposed. Noticeably proteins at approximately 58.6 kDa (in strain 0083 and isolate 46), 41.3 kDa (in strains 0083, ATCC 29545 and isolate 43), 37.8 and 36.8 (in 0083 and, in ATCC 29545 and 43 respectively) and finally the ubiquitous proteins at 26 and 17.2 kDa (16.4 kDa in field isolate 43) appeared as dominant antibody-accessible OMPs (Figure 4.9) which were insensitive to chymotrypsin cleavage and perhaps buried in the cell envelope. However, the possibility that these proteins are not surface-exposed could not be completely discounted as many OMPs have been demonstrated to be tightly associated to the peptidoglycan, requiring prolonged exposure at high temperatures to effect their dissociation. It is thus unlikely that the short protease incubation employed in the present study would be sufficient to bring about this dissociation. Furthermore, relative protease resistance may be attributed to a lack of exposed cleavage sites.

The application of chymotrypsin digestion to *H. paragallinarum* whole cells was successful in that OMP C was shown to be surface-located. Further studies employing antibodies, raised against a purified OMP, as the probe for protease-treated digests will provide valuable information regarding the surface-location of that protein. In the present study, the use of antibodies generated against individual whole cell reference strain and field isolate proteins to probe isolated OMP samples may provide further information towards identifying immunogenic and hence possible surface-exposed OMPs (Section 4.10.7).

4.10.5 Identification of lipopolysaccharides

Lipopolysaccharides are structural components of the outer membrane, commonly referred to as endotoxins in several gram-negative bacteria (Towner and Cockayne, 1993). These are responsible for inducing nasal exudate production and inflammation (Matsumoto, 1988). As

LPSs have been implicated as virulence determinants, it was of interest to identify them within the whole cell preparations to assess their role as unique serotype determinants or merely common bands when compared to the WCP profiles previously described (Section 4.10.1 and 4.10.2). Furthermore, analysis of the LPS profiles of selected strains of different serovars was conducted as an alternative method towards further typing of the NAD-independent field isolates.

Attempts to prepare LPSs using a non-solvent based method (Hitchcock and Brown, 1983), combining bacterial lysis of whole cell samples and proteinase K digestion of the lysed WCPs, was met with limited success and proved to be unnecessary in relation to the defined profiles obtained by periodic acid-staining of untreated whole cells (Figure 4.10). Reducing SDS-PAGE, in conjunction with a periodic acid silver stain detection system, of the whole cell proteins of selected NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates revealed complex profiles of grey-stained LPSs, contaminated with brown-stained proteins and glycoproteins.

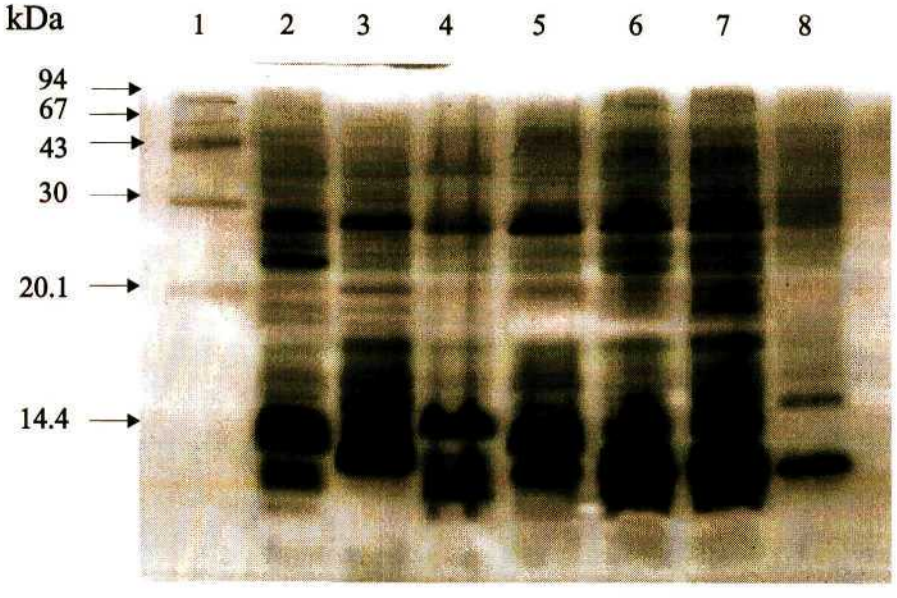


Figure 4.10 Reducing SDS-PAGE of selected NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates for identification of LPSs. Samples were boiled in lysing buffer, digested with proteinase K and loaded onto 15% tricine gel. **Lane 1,** Molecular mass markers (as in Figure 4.2); **lanes 2-8,** *H. paragallinarum* strains [**lane 2,** 0083 (serovar A-1); **lane 3,** ATCC 29545 (serotype A); **lane 4,** Modesto (serovar C-2); **lane 5,** 43 (serovar C-2); **lane 6,** 93/00449; **lane 7,** 96/11756; **lane 8,** 541 (serovar C-3)].

Comparative analysis of the periodic acid- and protein-silver stained profiles (Figures 4.2 - 4.4) enabled the identification of certain major and minor whole cell bands as LPSs, most notably in the region of 14.4 kDa and smaller. This suggests that LPSs are not likely to contribute to the serotype-determining WCP differences reported earlier, as they are concentrated largely in the 20.1 to 43 kDa range.

Closer analysis of the LPS bands in the region of the gel around 14 kDa, revealed the potential application of LPS profiling to extend the type allocation of *H. paragallinarum* field isolates. Reference strain 0083 and field isolate 43 possessed a dominant band at approximately 14.0 kDa, with the 2 strains being differentiated from each other by a broad, diffuse band of closely-spaced LPSs or a well-defined narrower band around 11 kDa respectively. This phenomenon of ordered spaced bands represents LPSs having different numbers of repeating O-polysaccharide side chains (Towner and Cockayne, 1993). In contrast, strain Modesto exhibits a major LPS at approximately 14.5 kDa and a ladder of about 5 smaller LPSs, which differentiate it from the ATCC 29545 strain which only possesses the major LPS at 12.9 kDa. The LPS profiles of the locally isolated NAD-independent *H. paragallinarum* isolates 93/00449 and 96/11756 appeared to be identical and visual comparison could not assign them to either of the serotype A or C profiles. The failure to assign the NAD-independent isolates to one of the employed serovars was encouraging, as a strain representative of serovar C-3 was not studied due to sample limitations. As such there is a possibility that these NAD-independent isolates may belong to serovar C-3.

This study serves to demonstrate the existence of low molecular mass LPSs which are likely to be rich in O-polysaccharide repeating units thus characterising them as smooth (Towner and Cockayne, 1993). In addition these observations provide preliminary evidence of the potential of LPS profiling for confirmation of type assignment of isolates of *H. paragallinarum.* Future experiments should employ OMP preparations to establish the cellular location of the LPSs and to evaluate their antigenicity as targets for a vaccine-conditioned immune response.

4.10.6 Determination of the titre of polyclonal anti-*H. paragallinarum* antibodies

Whole *H. paragallinarum* bacterial cells from NAD-independent field isolate 95/03938, and NAD-dependent reference strains 0083, 0222 and Modesto were immunogenic in both rabbits and chickens, eliciting an immune response which was monitored by ELISA (Section 2.5.2). The

increase in the antibody titre, expressed as the antibody concentration at which corresponding absorbance-values are significantly higher than the pre-immune controls, was followed for both IgG (Figure 4.11) and IgY (Figure 4.12) preparations for all four strains over the course of the immunisation period.

Rabbits, immunised with *H. paragallinarum* strains 0083, 0222, Modesto, and 95/03938, produced antibodies which did not appear to recognise the immobilised antigen very well (Figure 4.11 A-D). Antibodies, produced 6 weeks post immunisation, gave signals slightly higher to those generated by the pre-immune antibodies. These observations are indicative of a considerable amount of non-specific protein-protein interaction between the highly antigenic bacterial cells and the pre-immune antibodies. The antibody titre was estimated at 250 μg of antibody per ml for strains 0083 (Panel B) and 0222 (Panel C), and 500 μg of antibody per ml for strains 95/03938 (Panel A) and Modesto (Panel D). Therefore the overall magnitude of the antibody response of rabbits subjected to the different *H. paragallinarum* strains was very similar.

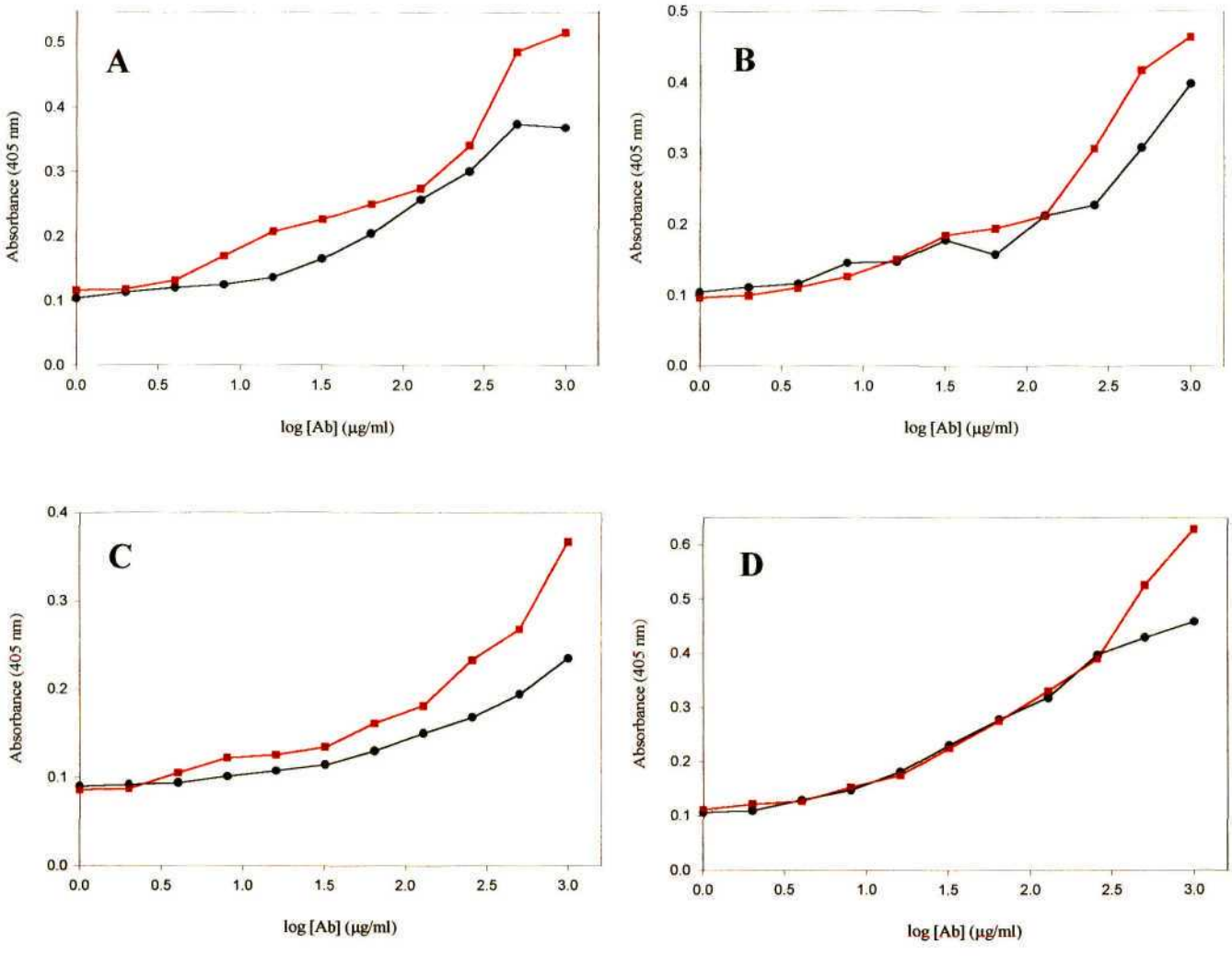


Figure 4.11 Monitoring of rabbit anti-*H. paragallinarum* IgG production by ELISA six weeks post-immunisation. Separate microtitre plates were coated with *H. paragallinarum* strains 95/03938 **(A)**, 0083 **(B)**, 0222 **(C)** or Modesto **(D)** (0.1 µg/ml) and incubated separately with serial doubling dilutions (from 1 mg/ml to 1 µg/ml) with pre-immune IgG (●) and the corresponding antibody (■). Binding of antibodies was visualised by incubation with sheep anti-rabbit HRPO as the secondary antibody, followed by ABTS/H_2O_2 substrate (Section 2.5.2). Each point is the mean absorbance at 405 nm of duplicate samples.

The immune response elicited in chickens by *H. paragallinarum* bacterial cells was not consistent among the different strains (Figure 4.12). Chickens immunised with *H. paragallinarum* reference strains 0083 (Panel B) and 0222 (Panel C) produced a response consistent with that observed in rabbits, in that the antibodies did not recognise the immobilised antigen very well. The titre of the anti-*H. paragallinarum* strain 0083 and 0222 IgY antibodies was estimated at 100 µg of antibody per ml. Furthermore, these strains appeared to exhibit a time-independent response over the

post-immunisation period of 16 weeks. In contrast, chickens immunised with *H. paragallinarum* strains 95/03938 (Panel A) and Modesto (Panel D) produced a very good antibody response and a titre of 4 and 2 μg, respectively, of antibody per ml was estimated. With respect to timing, a comparison with the immune response generated in rabbits cannot be made as this immunisation period was terminated after 6 weeks. The only comment that can be made is that the immune response in chickens is clearly time-dependent with the later weeks (weeks 14-16) giving higher signals than the earlier weeks (week 8). Week 8, in turn, produced a greater response when compared to the pre-immune antibodies.

The apparent low immunogenicity of the *H. paragallinarum* whole cells may be explained as follows. Preparation of the inoculant required harsh conditions to effect a thick emulsion, during which it is likely that a large proportion of bacterial cells were lysed. Subsequently, in addition to the surface-exposed proteins, a large amount of foreign material, normally enclosed within the bacterial cell, is presented to the immune system for antibody production. The resulting preparation thus contains a high percentage of antibodies directed against the interior proteins, which are unable to recognise the proteins expressed on the surface of the intact bacterium which is presented in an ELISA. These antibodies may find suitable application for the identification of antigenic proteins by western blotting, as the WCPs and OMPs have been denatured and electrophoretically separated (Section 4.10.7).

As previously mentioned, these antibodies were raised at Allerton Regional Veterinary Laboratory for use in a serodiagnostic assay and clearly have a limited application in the present study. Nevertheless, the results obtained form the basis for future investigations. The antibodies raised in chickens against *H. paragallinarum* strains 95/03938 and Modesto gave a very good response. A number of reasons may be postulated for the poor response to strains 0083 and 0222, which include variation between chickens, pre-exposure of the animal to infection or an apparent low degree of immunogenicity. Attempts to improve the antibody response towards these strains was beyond the scope of this study, yet should be considered in future investigations.

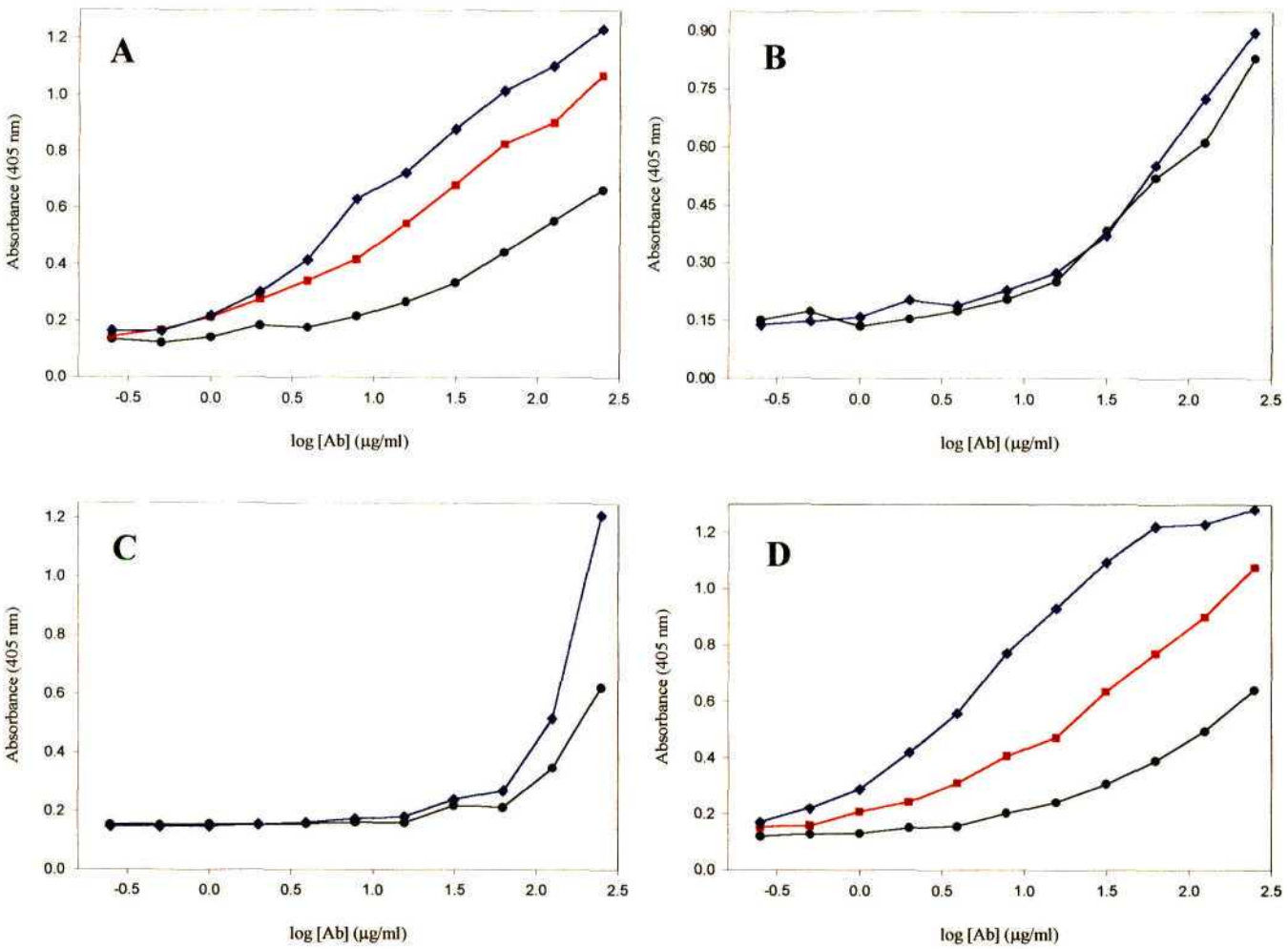


Figure 4.12 Monitoring of chicken anti-*H. paragallinarum* IgY production by ELISA at various weeks post-immunisation. Separate microtitre plates were coated with *H. paragallinarum* strains 95/03938 **(A)**, 0083 **(B)**, 0222 **(C)** or Modesto **(D)** (0.1 µg/ml) and incubated with serial doubling dilutions (from 250 to 0.01 µg/ml) of pre-immune IgY (●) or the corresponding antibody from week 8 (■) or weeks 14-16 (◆) combined. Binding of antibodies was visualised by incubation with rabbit anti-chicken HRPO as the secondary antibody, followed by ABTS/H_2O_2 substrate (Section 2.5.2). Each point is the mean absorbance at 405 nm of duplicate samples.

As the anti-*H. paragallinarum* antibodies raised in rabbits produced a more uniform response relative to the pre-immune control for all four strains studied, these antibodies were employed for the identification of antigenic proteins in western blotting in preference to those raised in chickens which exhibited a variable response relative to the control.

4.10.7 Immunoblot analysis of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates

4.10.7.1 Immunoblots of the whole cell proteins

Non-reducing SDS-PAGE of the WCPs, in conjunction with an immunochemical detection system employing polyclonal anti-*H. paragallinarum* reference strain 0083 (serovar A-1), 0222 (serovar B-1), and Modesto (serovar C-2) as well as field isolate 95/03938 IgG antibodies, revealed overall similar, yet complex profiles. These profiles are comparable to the whole cell protein profiles visualised using a silver protein stain (Figure 4.2 - 4.4). This is in agreement with reports by Roggen *et al.* (1992) which demonstrated that protein profiles of *H. ducreyi* probed with polyvalent sera, obtained from patients naturally-infected with the pathogen, produced overall similar profiles to those obtained following silver staining. Furthermore, a large degree of cross-reactivity was observed between *H. paragallinarum* strains allocated to different serovars when the denatured WCPs were probed with antibodies raised against a single strain of a specified serovar.

Consequently, the combination of WCP profile complexity, reproducibility in comparison to silver-stained protein profiles, and a high degree of cross-reactivity between strains of different serovar, made further serotyping of the *H. paragallinarum* field isolates on a whole cell level extremely difficult. As a study of antigenic OMPs, exposed to the external environment of the host, was believed to be more beneficial to serotype allocation, immunoblot analysis of the WCPs was abandoned in favour of similar analysis of the OMPs. Therefore, in an attempt to further develop serotype- and serovar-specific fingerprints the surface-located OMP preparations were probed with anti-*H. paragallinarum* whole cell antibodies generated against strains of the three serotype groups and a typical NAD-independent field isolate.

4.10.7.2 Immunoblots of outer membrane proteins

H. paragallinarum field isolates are commonly serotyped using a haemagglutination-inhibition test (Bragg *et al.*, 1996; Eaves *et al.*, 1989; Kume *et al.*, 1983a). Antibodies raised against classical reference strains [0083 (serovar A-1), 0222 (serovar B-1) and Modesto (serovar C-2)] are employed to recognise and bind to surface molecules of potassium thiocyanate-treated bacterial cells, thus preventing their association with the glutaraldehyde-fixed erythrocytes and thereby inhibiting haemagglutination. One of the limitations of this method is the inability to

provide any information regarding the individual recognising epitopes, with the outer membrane proteins being strong candidates. For this reason, these antibodies were employed in an extension of the HI tests (performed at Allerton Regional Veterinary Laboratory), as probes for the identification of electrophoretically separated OMPs.

Non-reducing SDS-PAGE of the OMPs, in conjunction with an immunochemical detection system, revealed overall similar profiles (Figure 4.13) which showed fewer bands compared to the WCP immunoblot profiles. A large number of common major and minor OMPs were recognised when selected NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates were probed with one of the four antibody preparations and a pre-immune control.

The immunoblots showed three pronounced antigenic proteins of slightly variable molecular mass shared by all isolates when probed with all five IgG preparations (Figure 4.13 A - E). The similarity of the OMP profiles obtained with the pre-immune control compared to those obtained with the test antibody preparations is indicative of the low titres of the test antibodies. High concentrations of these antibodies, and hence of the pre-immune IgG, were required to obtain a signal. Such large amounts of antibody result in high levels of protein-protein interaction accounting for the high degree of similarity observed. The protein targeted at approximately 38-39 kDa corresponds to OMP C which is the central type 1 and 2-determinant. This protein has been demonstrated to be heat-modifiable (Section 4.10.3) and exposed on the outer membrane surface of some bacterial strains (Section 4.10.5). Subsequent characterisation of this common OMP as immunogenic may have extensive implications in vaccine design. Should OMP C play a critical role in adherence to the epithelial cell wall or maintenance of a bacterial infection, it may serve as a target for vaccine design, regardless of the serovar of the infecting strain. Additional common OMPs at approximately 25 and 16 kDa were also demonstrated to be recognised in all strains selected by all antibody preparations tested. These proteins have not been characterised as extensively as OMP C, however, their high degree of immunogenicity and cross-reactivity suggests that they are also likely to satisfy the requirements of a good vaccine target.

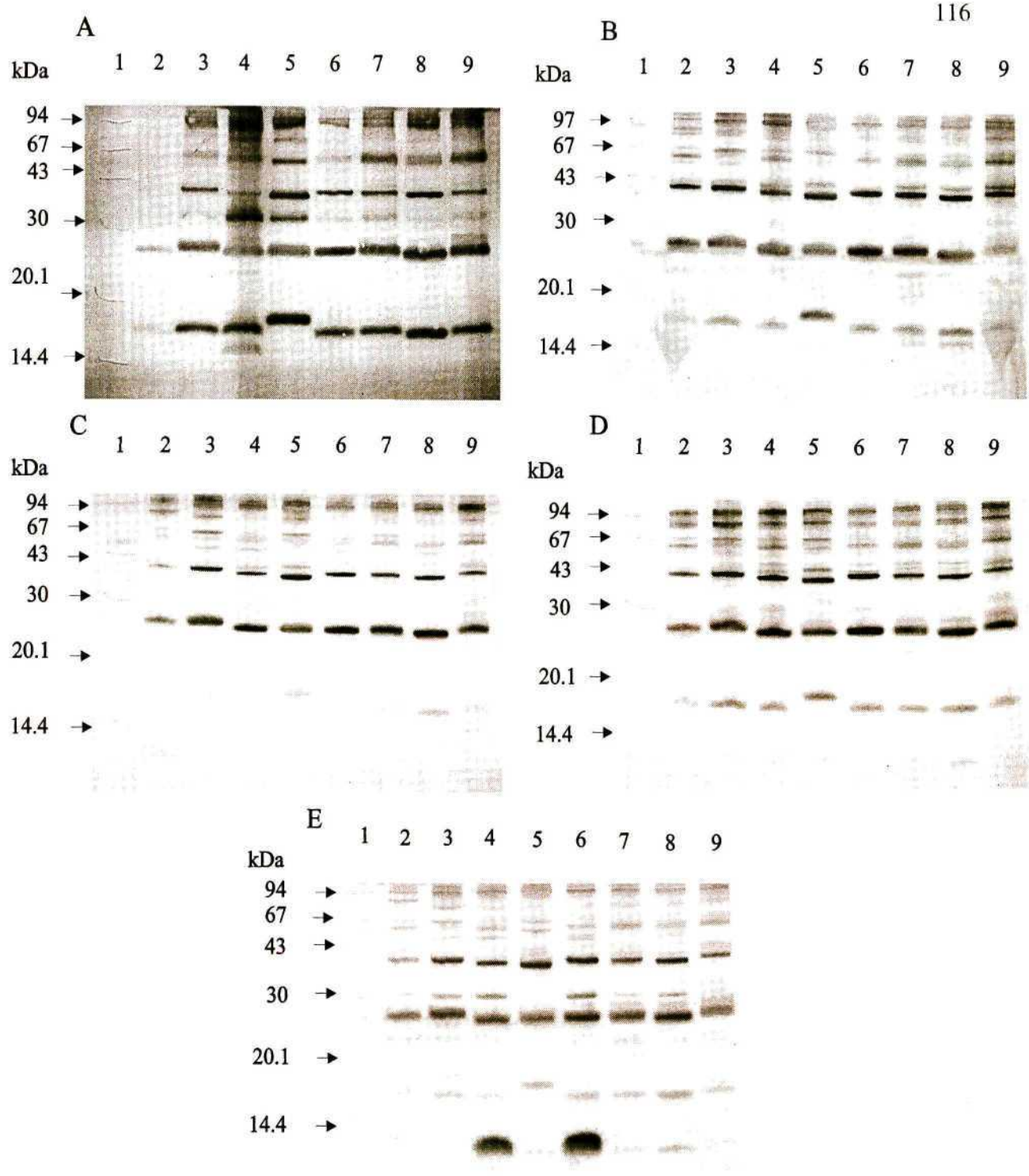


Figure 4.13 Western blot analysis of the OMPs of selected NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates. Samples were resolved by non-reducing Tris-tricine SDS-PAGE (Section 2.3), electroblotted onto nitrocellulose and probed with **(A)** pre-immune rabbit IgG antibodies (35.0 µg) or rabbit IgG antibodies against *H. paragallinarum* strains **(B)** 95/03938 (28.8 µg); **(C)** 0083 (serovar A-1; 36.0 µg); **(D)** 0222 (serovar B-1; 39.6 µg); **(E)** Modesto (serovar C-2; 30.5 µg). **Lane 1,** Molecular mass markers (as in Figure 4.2); **lanes 2-9,** *H. paragallinarum* strains [**Lane 2,** 0083 (serovar A-1); **lane 3,** ATCC 29545 (serotype A); **lane 4,** Modesto (serovar C-2); **lane 5,** 43 (serovar C-2); **lane 6,** 46 (serovar C-3); **lane 7,** 93/00449; **lane 8,** 96/11756, **lane 9,** 541 (serovar C-3)].

The reproducibility observed in the OMP profiles when probed with antibodies raised against one of serotype A, B or C, indicates a high degree of antigenic cross-reactivity which is very encouraging in terms of vaccine control of infections in the field. Should the common proteins identified be pivotal in bacterial survival, it is likely that a vaccine containing strains of one serotype should be able to provide adequate host protection by inducing antibodies against these common proteins. This hypothesis is only encouraging in theory, however, as conditions in the field have proved otherwise. Immunity induced by bacterins is serotype-specific with vaccines providing protection against homologous challenge and not against heterologous challenge (Rimler *et al.*, 1977a; Kume *et al.*, 1980c; Blackall and Reid, 1987). Field trials revealed a limited degree of cross-protection amongst serovars within a serotype. As previously mentioned, it is likely that that the high degree of antigenic cross-reactivity observed is merely an artefact due to non-specific protein-protein interaction, as evidenced by the degree of similarity obtained when the OMPs are probed with a pre-immune control. Consequently, the existence of serotype-specific proteins (their existence has been proven in Section 4.10.2 but their functions have not been firmly established) that facilitate host colonisation must be determined to enable the inclusion of a representative strain of the predominant infecting serovar in local vaccines.

Clearly, the current protocol is limited in terms of serotype determination due to a high degree of common, cross-reactive antigens. However, on closer examination of the immunoblot profiles generated when the OMP profiles were probed with anti-serovar C-2 strain Modesto antibodies, a few unique or strongly recognised bands were identified, apparently absent or present in reduced concentrations in the preparations probed with anti-strain 0083, -0222 and -95/03938 IgG antibodies. A protein at approximately 30 kDa was strongly recognised in all strains studied with the exception of serovar C-2 strain 43. Slight recognition of this protein, believed to be serovar C-3 specific, was observed in strains Modesto, 46 and the NAD-independent isolates, when probed with anti-*H. paragallinarum* 95/03938 antibodies. These results proved to be confusing as a 30 kDa band was only visible in the silver stained OMP preparation of isolate 46 and all the NAD-independent isolates. It was thus postulated that this protein is in fact present in most strains at low concentrations. However, in serotype C strains, especially serovar C-3 isolates, it is expressed in greater amounts capable of eliciting an immune response. As such this band shall remain a serovar C-3-determinant. Subsequently, attempts were made to sequence the corresponding NAD-independent *H. paragallinarum* field isolate 96/11756 protein

(approximately 30 kDa in size) for comparison with a data base of previously sequenced proteins (Section 4.10.8). An additional difference was recorded in the banding intensity at 12 kDa, where an exceptionally strong signal was observed in strains Modesto and 46 in comparison to the other strains when probed with anti-*H. paragallinarum* strain Modesto antibodies. Furthermore, this band was not detected when strains Modesto and 46 were probed with each antibody directed against strains 0083, 0222, or 95/03938. As the pre-immune control did not detect this band, its appearance is not due to a non-specific interaction. Nevertheless, these differences are not sufficient for the serotype allocation of the NAD-independent *H. paragallinarum* field isolates.

The inconsistencies in the above profiles are not conducive to the construction of serotype-specific fingerprints. However, the basis of such a typing scheme has been established. Improvements to this protocol include the production of a library of serovar-specific antibodies to generate unambiguous profiles as templates for comparing antibody-probed OMPs of field isolates of unknown serovar. Furthermore, comparative analysis of western blot profiles generated by probing OMPs with sera from infected flocks of birds which have recovered from infection and those which did not, would provide information regarding the virulence-determinants.

4.10.8 N-terminal sequencing

As a first step towards serovar-specific antibodies, the N-terminal amino acid sequence of the 30 kDa OMP from the NAD-independent *H. paragallinarum* field isolate 96/11756 was determined (Figure 4.14) as proposed above. This protein was found to exhibit considerable homology with the N-terminal sequences of *H. influenzae* type *b* P5 protein (Munson *et al.*, 1993), *P. multocida* OMP 35 (Lugtenberg *et al.*, 1986), *E. coli* and *Salmonella typhimurium* OmpA (Sugawara and Nikaido, 1992), and the cystic fibrosis transmembrane conductance regulator (Morales *et al.*, 1996; Figure 4.14).

H. paragallinarum OMP 30	A P Q A N T
Cystic fibrosis transmembrane conductance regulator	A P Q A N F
H. influenzae type *b* P5	A P Q E N T
P. multocida OMP 35	A P Q P N T
E. coli OmpA	A P K D N T
S. typhimurium OmpA	A P K D N T

Figure 4.14 Comparison of the N-terminal amino acid sequence of the 30 kDa OMP from the NAD-independent *H. paragallinarum* field isolate 96/11765 with homologous proteins. Regions of homology are shown in black while regions of heterology are illustrated in red.

The OmpA family of proteins, of which the *H. influenzae* type *b* P5 protein is a member, are conserved components of the outer membrane of many gram-negative bacteria, responsible for maintaining the integrity of the membrane (Weiser and Gotschlich, 1991). Visual comparison of the N-terminal sequences of the *H. paragallinarum* 30 kDa protein revealed a high degree of homology with the OmpA proteins, conferring related structural and functional properties to this protein. Furthermore, N-terminal sequence analysis may contribute towards the serological classification of the NAD-independent isolates provided the corresponding 30 kDa protein of serovar C-3 field isolate 46 generates an identical N-terminal sequence. Due to the limited availability of sample, this theory could not be explored, however, the potential contribution towards future studies regarding type characterisation of NAD-independent field isolates is clear.

4.11 Conclusions

Employing a combination of SDS-PAGE and silver protein staining, reference fingerprints were constructed as templates for serotype allocation of the untyped *H. paragallinarum* field isolates. Visual comparison of the WCP and OMP profiles of the local NAD-independent *H. paragallinarum* field isolates with those of the established reference strains, revealed a close resemblance to the NAD-dependent field isolate 46. As such, these NAD-independent isolates were tentatively assigned to serovar C-3.

Attempts were made to further characterise the OMPs, with intent to identify immunogenic, surface-exposed OMPs which contribute towards the virulence of an infectious coryza infection.

The type 1 and 2-determinant, OMP C, was demonstrated to be heat-modifiable and surface-exposed in most strains studied. Furthermore, this common OMP was found to be immunogenic and partly responsible for cross-reactivity observed between strains of different serovar. In addition, a common heat-modifiable OMP approximately 36 kDa in size, strongly associated to peptidoglycan, was found in nearly all strains studied. Analysis of the endotoxins of the *H. paragallinarum* strains revealed a number of low molecular mass O-polysaccharide repeating units, hence characterising strains of *H. paragallinarum* as smooth with reference to outer membrane LPSs. The LPS profiles generated differed slightly for different strains.

Unfortunately, comments on the immunogenicity of the OMPs of the strains employed in the present study were limited due to the nature of the antibodies. Rabbit anti-serotype antibodies had a very low titre and as such the resulting immunoblots were very similar to those treated with pre-immune antibodies. Nevertheless, antibodies raised in chickens against strains 95/03938 and Modesto showed promise, identifying a few unique OMPs and providing evidence of the potential efficacious application of immunoblot analysis for serotype determination.

The identification of serovar-specific proteins remains to be a subjective technique, strongly influenced by environmental factors. As such, the interpretation of the WCP and OMP profiles for typing of *H. paragallinarum* isolates remains a matter of conjecture, requiring confirmation at a molecular level (Chapter 5). The characterisation of the major surface-exposed OMPs is critical towards recognising potential protein targets for inclusion in a revised vaccine, responsible for stimulating a conditioned immune response. The observations made in the present study provide the ground work towards characterising unique serovar-determinants.

CHAPTER 5

Analysis of the 16S rRNA gene sequences for genotypic characterisation of *H. paragallinarum*

5.1 Introduction

Although the study of the WCP and OMP profiles, generated following non-reducing Tris-tricine SDS-PAGE and visualised with a silver protein stain, proved efficacious for typing the NAD-independent *H. paragallinarum* field isolates, these techniques are limited to the analysis of the phenotype rather than the genotype. Reservations regarding phenotyping have been expressed by, amongst others, Zhao *et al.* (1992), Russell *et al.* (1994) and Nagai *et al.* (1995) to the extent that Olsen and Woese (1993) described techniques including those based on whole cell properties, as ' ...too simple and too volatile to be reliable...'. These limitations are largely due to the inconsistent expression of the phenotypic traits which are strongly under the control of environmental influences (Snipes *et al.*, 1992), and to an inadequate sensitivity level for accurate strain differentiation within a species (Snipes *et al.*, 1989). These drawbacks prompted the application of molecular techniques, namely ribotyping and Restriction Fragment Length Polymorphism (RFLP) analysis of PCR-generated 16S rDNA, for the type classification of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates, in an extension of the phenotypic characterisations made previously (Chapter 4).

The best characterised phylogenetically informative molecules are the ribosomal RNAs (Olsen and Woese, 1993), encoded for by the operon *rrn* (Figure 1.5; Jorden and Leaves, 1997). Sequences of the 16S rRNA were demonstrated to be evolutionary conserved amongst similar and distantly related microorganisms (Towner and Cockayne, 1993). Polymerase chain reaction amplification of the 16S rRNA gene sequences using the universal primers, 27f and 1525r (Figure 5.2), generates a probe specific for the conserved sequences. Ribotyping fingerprints are produced following hybridisation of the single-stranded, labeled 16S rDNA probe with any region of complementarity on restriction enzyme digested chromosomal DNA fragments. Alternatively, direct restriction enzyme digestion of the amplified 16S rDNA enables the identification of RFLPs. As some bacterial species are genetically homogenous, all strains within that species will

produce a single rRNA gene restriction pattern. On the other hand, some species show genetic heterogeneity such that different strains within the same species may give different gene restriction patterns (Grimont and Grimont, 1991). The considerable degree of phenotypic variation within strains of NAD-dependent *H. paragallinarum* and compared to their NAD-independent counterparts, suggested that molecular analysis of the 16S rRNA sequences would likely reveal genetic mutations which may serve as epidemiological markers.

Subsequently, the 16S rRNA gene sequence of *H. paragallinarum* strain NCTC 11296 T (Figure 5.1) was studied. A number of restriction enzymes were selected on the basis of frequency of cutting within the 16S rRNA gene sequence. Enzymes of low frequency and thus few cleavage sites were chosen to increase the probability of identifying any nucleotide additions or deletions effecting changes in the restriction enzyme recognition sequences, manifested by the disappearance of one band and replacement with a chromosomal DNA fragment of a different size. Enzymes that would find suitable application are illustrated in Figure 5.1, with those employed in the present study highlighted in red. Previously, *Hin*dIII, *Hpa*II and *Ssp*I have found successful application for ribotyping of selected NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates (Miflin *et al.*, 1995). Subsequently, these enzymes were employed in the present study in efforts to confirm the serotype classifications previously carried out. However, the use of the enzyme *Sma*I in ribotype analysis represents a novel study. Furthermore, attempts were made for the first time in the present study to identify RFLPs, following restriction enzyme digestion of PCR-amplified 16S rDNA, from the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates (Table 3.1).

1 ATCGAAGAGT T T↓*Sau3A* GATC NTGG CTCAGATTGA ACGCTGGCGG CAGGCTTAAC ACATGCAAGT

61 CGAACGGTAA CGGGTTGA A↓*HindIII*A GCTT GCTTTC AATGCTGACG AGTGGCGGAC GGGTGAGTAA

121 TGCTTGGGAA TCTGGCTTAT GGAGGGGGAT AACCATTGGA AACGATGGCT AATACCGCAT

181 AGAATCGGAA GATTAAAGGG TGGGACTTTT TAGCCACCTG CCATAAGATG AGCCCAAGTG

241 GGATTAGGTN GTTGGTGAGG TNAAGGCTCA CCAAGCCTN C↓*Sau3A* GATC TCTAGC TNGTCTNAGA

301 GGA TGG↓*BalI* CCA G CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG

361 GG AAT↓*Ssp I* ATT GCG↓*Hha I* C NATGGGGG GAACCCTGAC GCAGCNATGC CGCGTGAATG

AAGA AGG↓*Stu I* CCT

421 TCGGGTTGTA AAGTTCTTTC GGTGGTGAGG AAGGTTNGTG TGTTAATAGC ACACTAATTT

481 GACGTTAGCC ACAGAAGAAG CA C↓*Hpa II* CGG CTAA CTCCGTGCCA GCAGCCGCGG TNATACGGAG

541 GGTGCGAGCG TTNATCGGAA TAACTGGGCT TAAAGGGCAC GCAGGCGGTA AATTAAGTGA

601 GATGTGAAAT CCCCGAGCTT AACTTAGGAA TTGCATTTCA GACTNGTTTA CTAGAGTACT

661 TTAGGGAGGG NTA G↓*EcoRI* AATTC C ACGTGTAGCG GTGAAATGCG TAGAGATGTG GAGGAATACC

721 GAAGGCGAAG GCAGCCCCTT GGGAAGCTAC TGACGCTCAT GTGCNNAAGC GTGGGGAGCA

781 AACAGGATTN GATA C↓*EcoRII* CCTGG TAGTCCACGC TGTAAACGCT GTCGATTTGG GGATTGGGCT

841 TNNGGCTTGG TGCCCGTAGC TAACGTGATA AATCGACCGC CTNGGGAGTA CGGCCGCAAG

901 GTTAAAACTC AAATNAATTG ACGG GGGCC↓*ApaI* C GCACNAGCGG TGGAGCATGT GGTTTNATTC

961 GANNNAACGC GAAGAACCTT ACCTACTCTT GACACCTAA GAATCCTGTA GAGATACGGG

1021 AGTGCCTTCG GGAGCTTAGA GACAGGTGCT GCATGGCTGT CGTCAGCTCG TGTTGTGAAA

1081 TGTTGGGTTN AGTCCCGCAA CGA GCG↓*Hha I* C AAC CCTTATCCTT TGTTGCCAGC ACTTCGGGTG

1141 GGAACTCAAA GGAGACTGCC AGTGATNAAC TGGAGGAAGG TGGGGAT GACGT↓*AatII* C AAGTCAT

1201 CATGGCCCTT ACGAGTAGGG CTACACACGT GCTACAATGG TGCATACAGA GGGAAGCGAG

1261 CCTGCGAGGG GGAGCGAATC TCAGAAATG CATCTAAGT C↓*HpaII* CGG ATTGGAG TCTGCAACTC

1321 GACTCCATGA AGTCCGGAATC GTCAGTAATC GCAAATCAGA ATGTTGCGGT GAATACGTTC

1381 C↓*HpaII* C↓*SmaI* GG G CCTTG TACACACCGC CCGTCACACC ATGGGAGTGG GTTGTACCAG

AAGTAGATAG

1441 CTTAACCTTC GGGAGGGCGT TTACCACGGT ATGATTCATG ACT

Figure 5.1 ***H. paragallinarum*** **strain NCTC 11296 T 16S rRNA gene sequence (Dewhirst *et al.*, 1992).** Enzymes employed in the present study are highlighted in red, while further enzymes with potential application are illustrated in blue.

5.2 Polymerase chain reaction (PCR): synthesis of 16S rDNA

5.2.1 Growth of, and isolation of chromosomal DNA from, NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates

The NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates were grown in 100 ml of TMB (Section 3.1.3; 16 h, 37°C, 10% CO_2). Chromosomal DNA was extracted using standard DNA extraction methods (Blackall *et al.*, 1995) described in Section 3.3.2. The concentration of the chromosomal DNA was determined, following 1% (m/v) agarose gel electrophoresis (Section 2.6).

5.2.2 PCR reaction conditions

A DNA fragment, approximately 1.5 kb in size, corresponding to the conserved 16S rRNA gene sequence, was amplified with the universal primers 27f and 1525r (Figure 5.2; Lane, 1991).

Forward primer (27f):

5' AGA GTT TGA TCM TGG CTC AG **3'**

where M = C:A (1:1)

Reverse primer (1525r):

5' AAG GAG GTG WTC CAR CC **3'**

where W = A:T, R = A:G (both 1:1)

Figure 5.2 Nucleotide sequences of the universal PCR primers (Lane, 1991) used to amplify the 16S rRNA gene sequences from NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates.

5.2.2.1 Materials

As per Section 3.3.3.1 with the exception of the stock and working primer solutions.

Stock 27f primer solution. Freeze-dried 27f primer (347 µg) was reconstituted in TE buffer (Section 2.6; 535 µl).

Stock 1525r primer solution. Freeze-dried 1525r primer (320 µg) was reconstituted in TE buffer (Section 2.6; 581 µl).

Working 27f primer solution. Stock 27f primer solution (20 µl) was diluted to 100 µl with dist. H_2O.

Working 1525r primer solution. Stock 1525r primer solution (20 µl) was diluted to 100 µl with dist. H_2O.

5.2.2.2 Procedure

The PCR mixture contained PCR reaction buffer (5 µl), $MgCl_2$ (2 µl), dNTP working solution (5 µl), primers 27f and 1525r (1 µl of each), and template DNA (10-50 ng of chromosomal DNA) diluted to a final volume of 50 µl with deionised H_2O. The mixture was heated to 98°C for 2.5 min in a Perkin-Elmer 2400 thermocycler, before 1.25 U *Taq* DNA polymerase was added. The samples were amplified for 25 cycles. Each cycle consisted of a denaturation step at 94 °C for 30 s, followed by annealing at 58°C for 30 s and extension at 72°C for 1 min. The final extension step was at 72°C for 10 min to allow complete extension of the DNA product. Verification of the correct size of the amplicon was established by running the completed PCR reaction product (5 µl) through a 1% (m/v) agarose gel (Section 2.6). To ensure that no false positive PCR products were obtained a set of controls were included in which template DNA, *Taq* DNA polymerase, or the universal primers were omitted.

5.3 Ribotyping

Ribotyping involves the inter-species identification and intra-species typing of microorganisms based on the hybridisation of labeled rRNA-derived probes to gel-separated, restriction enzyme digested chromosomal DNA fragments. Small variations in conserved or semi-conserved regions are revealed using a commercially available detection kit, thereby generating species- and strain-specific fingerprints. Subsequently, ribopatterns of reference strains of established serovar, will serve as templates for visual comparison with the profiles obtained from field isolates, of undetermined serotype or serovar.

For successful and optimal ribotyping results, a considerable degree of attention must be given to each stage in the procedure, namely

1) generation of a suitable restriction enzyme fingerprint;
2) method of transfer of the electrophoresed DNA restriction fragments to a nylon membrane;
3) choice of an appropriate probe and the labeling thereof; and
4) accurate determination of the resulting fragment sizes (Grimont and Grimont, 1991; Towner and Cockayne, 1993).

An ideal fingerprint will consist of a few well-resolved DNA fragments with an even size distribution. The number of fragments obtained varies according to the DNA source and is dependent on the precise restriction enzyme used. It has been found that the results of studies which relied on the use of only one or two enzymes may be misleading and thus a thorough screening of strains with several different restriction enzymes should be implemented (Grimont and Grimont, 1991; Towner and Cockayne, 1993). In the present study, restriction enzymes *Hin*dIII, *Ssp*I and *Hpa*II were selected due to previous success obtained following their application in the ribotyping of *H. paragallinarum* isolates (Miflin *et al.*, 1995). Following examination of the 16S rRNA gene sequence, an additional enzyme was chosen which exhibited a low frequency of cutting and therefore produced a minimal number of well-resolved fragments on agarose gel electrophoresis.

Once an acceptable restriction enzyme fingerprint has been generated, it is necessary to transfer the fragments to a blotting membrane. In 1975, E.M. Southern devised a method to transfer alkali-denatured DNA from an agarose gel to a membrane. This technique, aptly named Southern transfer, is achieved by capillary action where the liquid associated with the gel is literally blotted out thereby effecting the irreversible binding of the single-stranded DNA fragments to the nylon membrane (Southern, 1975). More recently a protocol for vacuum transfer was developed, by which nucleic acid fragments were eluted from the gel and deposited onto the membrane following application of a low-pressure vacuum (Medveczky *et al.*, 1987). Optimal transfer is effected in less than 1 h and as it results in thinner, less diffused DNA bands, appears to be the method of choice for transfer.

Detection of the nylon membrane-bound, single-stranded DNA fragments proceeds by hybridisation with labeled DNA probes to specific sequences within the separated DNA fragments. Hence, the basic requirement for a nucleic acid probe is that it should recognise and bind to target complementary nucleotide bases, but fail to hybridise with any other nucleic acid molecules present in the restriction digested sample. Selection of a probe is an empirical process with the large data base of 16S rRNA gene sequences available offering the most powerful approach to tailored probe design. Small gene sequence variations within these highly homologous regions of the bacterial genome, form the basis of species and sub-species typing. As such, a PCR-generated DNA product amplifying the 16S rRNA sequences (Figure 5.1) was the choice of probe in the present study.

Nucleic acid probes may be labeled by one of two mechanisms; either directly or indirectly. In the former method a detectable label is attached, either internally or as an end-label, by a covalent bond to a functional group on the nucleic acid probe. Detection proceeds in two steps: hybrid formation between the target and the labeled probe, and signal generation by the covalently-linked reporter group. Examples of labels employed in direct labeling systems include ^{32}P (Southern, 1975), alkaline phosphatase (Renz and Kurz, 1984), and fluorescein (Amman *et al.*, 1990). On the other hand, the indirect method involves attachment of a modification group to the probe, which in turn exhibits a high degree of specificity for a labeled binding protein. In contrast to the direct method, three reaction steps are required for the detection of nucleic acid probe complexes in indirect labeling systems; namely, hybrid formation between the target and the modified probe; a specific non-covalent association between the modified probe and a binding protein coupled to a reporter group; and finally, detection of a signal generated by the reporter group. Examples of the complex-forming reactive groups in indirect labeling systems include biotin-dUTP with a streptavidin binding protein (Leary *et al.*, 1982), and digoxigenin-dUTP with an anti-digoxigenin binding protein (Kessler *et al.*, 1990). Both systems produce a detectable signal generated by an alkaline phosphatase receptor group. The biotin- and digoxigenin-based systems constitute the most sensitive and most popular indirect detection methods currently employed, offering a high degree of sensitivity and, in the latter, a comparatively low level of background staining (Towner and Cockayne, 1993). As a result the digoxigenin-based system was employed in the present study, and therefore detailed reaction mechanisms for probe labeling and detection of the probe-DNA complexes will be presented.

The steroid hapten digoxigenin, is coupled to 2'-deoxy-uridin-5'-triphosphate via an alkali-labile ester bond (DIG-11-dUTP; Figure 5.3). Labeling of the 16S rDNA probe with DIG-11-dUTP proceeds enzymatically according to the method of random primed labeling (Feinberg and Vogelstein, 1983), which is based on the hybridisation of random oligonucleotides to the denatured DNA template. A complementary DNA strand is synthesised by the Klenow enzyme (containing a polymerase and an exonuclease activity) which uses the 3' OH termini of the random nucleotides as primers for the addition of one of a mixture of deoxyribonucleosides containing DIG-11-dUTP. This results in the incorporation of the digoxigenin into the newly synthesised DNA, complementary to the 16S rDNA template strand, at a labeling efficiency rate of 1 in 1.3 i.e. 780 ng of labeled product out of 1 μg of template DNA, at 37°C overnight.

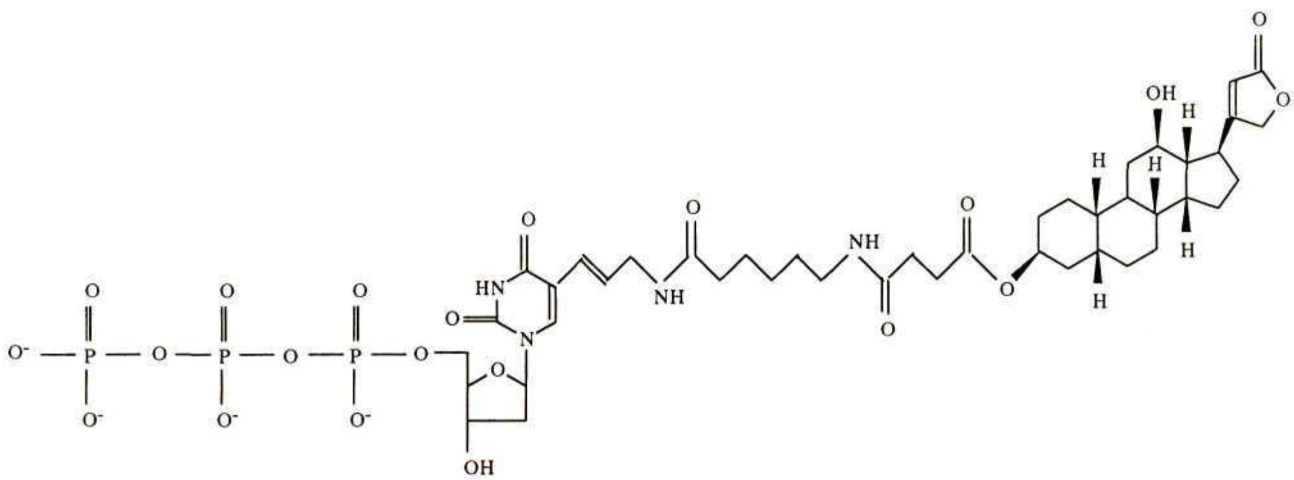


Figure 5.3 The chemical structure of digoxigenin coupled to dUTP (Boehringer Mannheim Technical Manual).

Following hybridisation of the DIG-labeled probe to complementary chromosomal DNA fragments, detection proceeds immunologically. An alkaline phosphatase-conjugated anti-digoxigenin antibody is allowed to recognise and bind to the labeled probe, with visualisation proceeding by the formation of a purple-coloured precipitate due to the colorimetric substrates NBT and BCIP.

The final consideration to successful and optimal ribotyping is to establish an accurate method for the determination of the sizes of the identified fragments, such that the typing system is reproducible between different laboratories. The use of different DNA fragment size markers and

different size determination algorithms, may result in excessively large variations in size determination. Subsequently, *Hin*dIII-cut lambda DNA standard markers, probed with a complementary DIG-labeled DNA strand, were employed in the present study while the accurate algorithm method of Elder and Southern (1983) was implemented for molecular size determination of the recognised DNA fragments.

In the present investigation, ribopatterns were generated for NAD-independent *H. paragallinarum* field isolates using the restriction enzymes *Hin*dIII, *Hpa*II, *Ssp*I and *Sma*I. Visual comparison of these profiles with established ribopatterns of *H. paragallinarum* reference strains (Miflin *et al.*, 1995) enabled type allocation of these field isolates.

5.3.1 Restriction enzyme digestion, agarose gel resolution and Southern transfer of the chromosomal DNA

5.3.1.1 Materials

All solutions were filtered through 0.45 µm filters or autoclaved (121°C, 15 min) before use.

*Hin*dIII buffer [10 mM Tris-HCl, 5 mM magnesium chloride, 100 mM sodium chloride, 1 mM 2-mercaptoethanol, pH 8.0]. Tris (0.012 g), $MgCl_2$ (0.005 g) and NaCl (0.058 g) were dissolved in 9.5 ml deionised H_2O and the pH was adjusted to 8.0 with HCl. 2-mercaptoethanol (698 µl) was added and the buffer was made up to 10 ml with deionised H_2O.

*Hpa*II buffer [10 mM Bis Tris propane-HCl, 10 mM magnesium chloride, 1 mM dithiothreitol, pH 7.0]. Bis Tris propane (0.028 g), $MgCl_2$ (0.010 g) and DTT (0.002 g) were dissolved in 9.5 ml deionised H_2O, the pH was adjusted to 7.0 with HCl and the buffer was made up to 10 ml with deionised H_2O.

*Ssp*I buffer [100 mM Tris-HCl, 50 mM sodium chloride, 10 mM magnesium chloride, 0.025% (v/v) Triton X-100, pH 7.5]. Tris (0.121 g), NaCl (0.029 g), $MgCl_2$ (0.010 g) and Triton X-100 (2.5 µl) were thoroughly mixed in 9.5 ml deionised H_2O, the pH was adjusted to 7.5 with HCl and the buffer was made up to 10 ml with deionised H_2O.

*Sma*I buffer [20 mM Tris-HCl, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9]. Tris (0.024 g), potassium acetate (0.049 g), magnesium acetate (0.022 g) and DTT (0.002 g) were dissolved in 9.5 ml deionised H_2O, the pH was adjusted to 7.9 with acetic acid and the buffer was made up to 10 ml with deionised H_2O.

Denaturation solution [0.5 M sodium hydroxide, 1.5 M sodium chloride]. NaOH (20 g) and NaCl (87.66 g) were dissolved in dist. H_2O (1 litre).

Neutralisation solution [0.5 M Tris-HCl, pH 7.5, 3 M NaCl]. Tris (60.55 g) was dissolved in 950 ml dist. H_2O and the pH was adjusted to 7.5 with HCl. NaCl (175.32 g) was thoroughly mixed in and the solution was made up to 1 litre with dist. H_2O.

SSC stock solution [3 M **S**odium chloride, 0.3 M **S**odium **C**itrate, pH 7.0]. NaCl (35.06 g) and Na-citrate (17.65 g) were dissolved in 185 ml dist. H_2O. The pH was adjusted to 7.0 with citric acid and the solution was made up to 200 ml with dist. H_2O.

SSC working solution. SSC stock solution (20 ml) was diluted to 200 ml with dist. H_2O.

5.3.1.2 Procedure

Chromosomal DNA (120 ng) from the NAD-independent *H. paragallinarum* field isolates was digested overnight with each of the restriction enzymes *Hin*dIII, *Hpa*II, *Ssp*I and *Sma*I. The reaction components and conditions for digestion are given in Table 5.1.

Table 5.1 Reaction components and incubation conditions for restriction enzyme digestion of the NAD-independent *H. paragallinarum* field isolates.

Restriction enzyme	Reaction mixture components				Incubation temperature (°C)
	DNA (µl)	Buffer (µl)	Enzyme (µl; units)	Final volume (µl)	
*Hin*dIII	4	2[a]	5; 50U	20	37
*Hpa*II	4	2[b]	2; 20U	20	37
*Ssp*I	4	2[c]	2; 24U	20	37
*Sma*I	4	2[d]	2; 40U	20	25

Note: Buffer composition;

[a] *Hin*dIII buffer: 10 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM 2-mercaptoethanol, pH 8.0,

[b] *Hpa*II buffer: 10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0,

[c] *Ssp*I buffer: 100 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 0.025% (v/v) Triton X-100, pH 7.5,

[d] *Sma*I buffer: 20 mM Tris-HCl, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9.

The chromosomal DNA fragments were separated through a 1% (m/v) agarose gel (Section 2.6) at 100 V for 1 h and photographed. The gel was then submerged in denaturation solution and incubated (2 x 15 min) on an orbital shaker at room temperature to ensure that the DNA remained single-stranded and accessible to the probe. The gel was rinsed in dist. H_2O and incubated in neutralisation solution (2 x 15 min) at room temperature. During this step, the magnagraph transfer nylon membrane was cut to a suitable size and prewetted (15-30 min) in SSC working solution. The agarose gel was then set up for capillary transfer of the denatured DNA fragments to the nylon membrane. This involved layering the upper surface of a clean glass plate with SSC-soaked Whatman No. 4 filter paper, followed by the agarose gel, the nylon membrane, and three additional pieces of wet filter paper. Care was taken to ensure that no air bubbles were introduced in the set-up, especially between the gel and the membrane, as they decrease the efficiency of transfer. Finally a layer of dry paper towel approximately 4 cm in height and a light weight (2 kg) were positioned on top of the filter paper and transfer by capillary action was

allowed to proceed for a minimum of 6 hrs. The transferred DNA fragments were cross-linked to the membrane by exposure to an ultraviolet light for a couple of seconds.

5.3.2 Gel extraction and purification of the 16S rDNA

Electrophoretically separated DNA may be extracted and purified from an agarose gel using a QIAEX II gel extraction kit® (Qiagen; Hilden, Germany). Extraction of the DNA proceeds by solubilisation of the agarose by Buffer QX1®, which contains a high concentration of a chaotropic salt responsible for disrupting the hydrogen bonds between sugar groups in the agarose polymer. In addition, the high salt concentration dissociates the DNA binding proteins from the DNA fragments. The highly electrolytic environment established causes a modification in the structure of water, forcing the DNA (40 bp to 50 kb in size) to adsorb to the QIAEX II silica gel particles®. The binding efficiency is also affected by pH, occurring optimally at 7.5. Contrary to adsorption, elution of the attached DNA is favoured under basic conditions at low salt concentrations. Furthermore, elution is temperature sensitive with DNA fragments smaller than 4 kb eluting efficiently at room temperature, while for larger fragments the incubation temperature should be increased to 50°C (QIAEX Handbook, 1995).

DNA purified with the QIAEX II gel extraction kit® is suitable for various applications, including restriction digestion, labeling, PCR, ligation and transformation. In the present study, this kit was employed for the extraction and purification of the PCR-amplified 16S rDNA fragment for subsequent DIG-labeling, to serve as the ribotyping probe.

5.3.2.1 Materials

10 mM Tris-HCl, pH 8.5. Tris (0.121 g) was dissolved in 95 ml dist. H_2O, the pH was adjusted to 8.5 with HCl and the buffer was made up to 100 ml.

3 M sodium acetate, pH 5.0. Sodium acetate (40.81 g) was dissolved in 95 ml dist. H_2O, the pH was adjusted to 5.0 with acetic acid, and the solution was made up to 100 ml with dist. H_2O.

5.3.2.2 Procedure

PCR reaction product (Section 5.2.2; 10 µl) was electrophoresed through a 1% (m/v) agarose gel (Section 2.6) for the resolution of DNA fragments in the size range 0.5 to 10 kb. On completion of the electrophoretic run, the 1.5 kb DNA band was visualised under a transilluminator and excised from the agarose gel using a clean, sharp scalpel. The size of the gel slice was minimised by removing excess agarose, and then transferred to a 1.5 ml microfuge tube and weighed. Buffer QX1® was added to the agarose slice containing the DNA fragment, in the ratio of 3:1 i.e. 300 µl of buffer per 100 mg of gel. QIAEX II gel particles® were vortexed for 30 s, added to the suspension (10 µl for less than 2 µg DNA, 30 µl for 2-10 µg DNA) and incubated at 50 °C for 10 min with intermittent mixing. During the incubation, the pH of the solution was monitored and should it have increased to become more basic, altered to approximately 7.5 with sodium acetate, pH 5.0. The sample was centrifuged (10 000 x g, RT, 30 s), the supernatant removed and the pellet washed with Buffer QX1® (500 µl). The pellet was resuspended by vortexing, centrifuged (10 000 x g, RT, 30 s) and the supernatant, which contained any residual agarose contaminants, was removed. All salt contaminants were removed from the pellet by repeated washing in Buffer PE® (2 x 500 µl), vortexed and centrifuged (10 000 x g, RT, 30 s). The resulting pellet was air-dried for approximately 20 min whereupon the pellet had turned white. The DNA was eluted from the silica particles following resuspension in 10 mM Tris-HCl (pH 8.5; 20 µl for 5 min at RT). The suspension was centrifuged (10 000 x g, RT, 30 s) and the supernatant containing the extracted, purified DNA was removed. The efficiency of extraction was evaluated following agarose gel electrophoresis (Section 2.6) of a small volume (3 µl) of the purified DNA.

5.3.3 Digoxigenin (DIG)-labeling of the purified 16S rDNA

5.3.3.1 Materials

All solutions were filtered through 0.45 µm filters or autoclaved (121°C, 15 min) before use.

Hexanucleotide mixture [500 mM Tris-HCl, 100 mM magnesium chloride, 1 mM dithioerythritol, pH 7.2, containing 2 mg/ml BSA and 1.56 mg/ml hexanucleotides]. Tris (0.610 g), $MgCl_2$ (0.100 g), DTT (0.002 g) were dissolved in 9.5 ml deionised H_2O, the pH was adjusted to 7.2 with HCl and the buffer was made up to 10 ml with deionised H_2O. Random hexanucleotides (1.56 mg) and BSA (2 mg) were dissolved in 1 ml of this buffer.

dNTP labeling mixture [500 mM Tris-HCl, pH 7.5, containing 1 mM of each dATP, dCTP, and dGTP, 0.65 mM dTTP, and 0.35 mM alkali-labile DIG-dUTP]. Tris (0.610 g) was dissolved in 850 µl deionised H_2O, the pH was adjusted to 7.5 with HCl and the buffer was made up to 1 ml with deionised H_2O. dATP (0.491 mg), dGTP (0.507 mg), dCTP (0.467 mg), dTTP (0.338 mg) and DIG-dUTP (0.387 mg) were thoroughly mixed in.

0.2 M EDTA, pH 8.0. EDTA-Na_2 (0.44 g) was dissolved in 9.5 ml dist. H_2O, the pH was adjusted to 8.0 with acetic acid and the solution was made up to 10 ml with dist. H_2O.

4 M lithium chloride. LiCl (1.696 g) was dissolved in dist. H_2O (10 ml).

70% (v/v) ethanol. Ethanol (70 ml) was diluted to 100 ml with deionised H_2O.

5.3.3.2 Procedure

Purified 16S rDNA (Section 5.3.2; 2 µg) and *Hin*dIII-cut lambda DNA (Section 2.6.1; 1 µg) were each diluted to a final volume of 15 µl with deionised H_2O in a sterile microfuge. The template DNA was denatured by heat-treatment in a boiling water bath for 10 min and chilled quickly on ice to ensure that the DNA remains single-stranded. Hexanucleotide mixture (2 µl) and dNTP labeling mixture (2 µl) were added to the tube, on ice, and Klenow enzyme (1 µl of a 2 unit/µl solution) was mixed in well. The reaction was incubated at 37°C overnight, after which labeling was terminated by the addition of 0.2 M EDTA (2 µl). The labeled nucleic acid probe was precipitated with 4 M LiCl (0.1 volumes) and ethanol (2.5-3 volumes) at -20°C for a period of 8 hs. The precipitated probe was pelleted by centrifugation in a microfuge (13 000 x g, RT, 15 min), the supernatant was removed and the pellet was washed with ice-cold 70% (v/v) ethanol. The purified DIG-labeled probe was resuspended in TE buffer (Section 2.6.1; 50 µl for the 16S rDNA probe and 20 µl for the *Hin*dIII-cut lambda DNA markers) at 4°C overnight.

5.3.4 Quantification of DIG-labeling efficiency

5.3.4.1 Materials

DNA dilution buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0, containing 50 µg/ml herring sperm DNA]. Tris (0.012 g) and EDTA (0.004 g) were dissolved in 9.5 ml deionised H_2O, the pH was

adjusted to 8.0 with HCl and the buffer was made up to 10 ml with deionised H_2O. Herring sperm DNA (50 µg) was added to 1 ml of the buffer, which was used as a diluent of the unlabeled and labeled control DNA.

Unlabeled control DNA. pBR328 (200 mg/ml) that had been linearised with *Eco*RI.

Labeled control DNA. Digoxigenin-labeled pBR328 DNA at a total DNA concentration of 25 µg/ml, of which one-fifth is DIG-labeled.

5.3.4.2 Procedure

Unlabeled control DNA was labeled as described in Section 5.3.4, and shall be referred to as self-labeled control DNA, as opposed to kit labeled control DNA, in the present Section. DIG-labeled and self-labeled control DNA were diluted to a final concentration of 1 ng/µl with DNA dilution buffer. The amount of DIG-labeled 16S rDNA and marker DNA was estimated from Table 5.2, and adjusted to 1 ng/µl with DNA dilution buffer.

Table 5.2 The amount DIG-labeled DNA synthesised relative to the amount of template DNA following overnight labeling (Boehringer Mannheim Technical Manual).

Amount of template DNA (ng)	10	30	100	300	1000	3000
Amount of synthesised DIG-labeled DNA (ng)	50	120	260	500	780	890

A dilution series of the 1 ng/µl DIG-labeled DNA samples were made from 100 pg/µl through 10 pg/µl and 1 pg/µl to 0.1 pg/µl, with DNA dilution buffer. Each dilution, of each of the four labeled samples (DIG- and self-labeled control DNA, DIG-labeled 16S rDNA and *Hin*dIII-cut lambda DNA marker), were spotted onto a piece of nylon membrane and fixed by exposure to

ultraviolet light. Detection of the labeled-controls and -probes proceeded immunologically using the anti-DIG alkaline phosphatase-conjugate and was visualised with the colorimetric substrate NBT and BCIP (Section 5.3.6). The concentration of the DIG-labeled 16S rDNA and standard marker probes was determined by visual comparison of the spot intensities between the controls and the experimental samples.

5.3.5 Hybridisation of the DIG-labeled 16S rDNA and marker probes

5.3.5.1 Materials

All solutions were filtered through 0.45 μm filters or autoclaved (121°C, 15 min) before use.

Tris buffer [100 mM Tris-HCl, 150 mM NaCl, pH 7.5]. Tris (12.11 g) and NaCl (8.77 g) were dissolved in 950 ml dist. H_2O, the pH was adjusted to 7.5 with HCl and the solution was made up to 1 litre with dist. H_2O.

10% (m/v) blocking® solution. Blocking reagent (50 g) was dissolved, with heating, in Tris buffer (500 ml).

Hybridisation buffer [5 x SSC, 0.1% (m/v) *N*-lauroylsarcosinate, 0.02% (m/v) SDS, 1% (m/v) blocking solution]. SSC stock solution (Section 5.3.2.1; 50 ml), *N*-lauroylsarcosinate (0.2 g), SDS (0.04 g) and blocking solution (20 ml) were combined and made up to a final volume of 200 ml with deionised H_2O.

Wash solution 1 [2 x SSC containing 0.1% (m/v) SDS]. SDS (0.1 g) was dissolved in SSC stock solution (Section 5.3.2.1; 10 ml) and the mixture was made up to 100 ml with deionised H_2O.

Wash solution 2 [0.5 x SSC containing 0.1% (m/v) SDS]. SDS (0.1 g) was dissolved in SSC stock solution (Section 5.3.2.1; 2.5 ml) and the mixture was made up to 100 ml with deionised H_2O.

5.3.5.2 Procedure

The nylon membrane, to which the chromosomal DNA fragments were cross-linked, was incubated in hybridisation solution (20 ml) for a minimum of 1 h at 65-68°C. The

double-stranded 16S rDNA and marker probes were denatured by heating in a boiling water bath for 10 min and chilled directly on ice. The probe was diluted in hybridisation solution to a final concentration of 20 ng/ml and incubated with the membrane overnight at 65-68°C. The membrane was then washed with wash solution 1 (2 x 5 min) at room temperature to remove the unbound probe and subsequently washed in wash solution 2 (2 x 15 min) at 68°C.

5.3.6 Immunological detection and colorimetric visualisation of the DIG-complexes

5.3.6.1 Materials

Washing buffer [0.3% (v/v) Tween-20 in Tris buffer]. Tween-20 (0.3 ml) was diluted to 100 ml with Tris buffer (Section 5.3.5.1).

1% (v/v) blocking solution. 10% (m/v) blocking solution (Section 5.3.6.1; 10 ml) was diluted to 100 ml with Tris buffer (Section 5.3.5.1).

Detection buffer [100 mM Tris-HCl, 100 mM sodium chloride, pH 9.5]. Tris (6.055 g) and NaCl (2.922 g) were dissolved in 450 ml dist. H_2O and the pH was adjusted to 9.5 with HCl. The solution was made up to 500 ml with dist. H_2O and autoclaved (121°C, 15 min).

5.3.6.2 Procedure

All steps, with the exception of colour development, were carried out at room temperature on an orbital shaker. The membrane was equilibrated in washing buffer (1 min) and incubated, by gentle agitation, in 1% (v/v) blocking solution for 1 h. The blocking solution was removed and the membrane was immersed in the anti-digoxigenin alkaline phosphatase-conjugate (30 min) previously diluted 1:5 000 in 1% (v/v) blocking solution. The antibody was discarded and the membrane was washed (2 x 15 min) in washing buffer to remove any unbound antibody and equilibrated in detection buffer (20 ml; 2 min). NBT (45 µl; 75 mg/ml 70% (v/v) dimethylformamide solution) and BCIP (35 µl; 50 mg/ml 100% dimethylformamide) were diluted to 10 ml with detection buffer and poured over the membrane for reaction in the dark. Colour development proceeded until purple-coloured bands were evident against a lightly stained background. The membrane was finally removed from the substrate solution, washed in dist. H_2O and dried between two sheets of filter paper to ensure good preservation of bands.

5.4 Restriction digestion of PCR-amplified 16S rDNA

Ribotyping has been demonstrated to be a highly discriminatory method for inter- and intra-species typing of a number of bacteria, including strains of related Haemophili, for example *H. influenzae* (Jordens and Leaves, 1997) and strains of *P. multocida*, a fellow upper respiratory tract infectant in flocks of birds (Snipes *et al.*, 1989). However, the relative complexity and lengthy, labour-intensive procedures involved led to the introduction of comparatively simple and rapid characterisation techniques. One such method combines PCR and restriction endonuclease digestion of the amplified 16S rDNA for the identification of RFLPs. These polymorphisms are reflected as changes in the restriction fragmentation pattern and are due to either a deletion or creation of a restriction site within the evolutionary conserved 16S rRNA gene of different strains within a single species. Amplified 16S rDNA restriction analysis (16S ARDRA) has successfully differentiated species of streptococci (Jayarao *et al.*, 1992) and strains of *S. iniae* (Eldar *et al.*, 1997).

Clearly, the most important criterion for successful 16S ARDRA analysis is the choice of restriction enzyme. As described previously (Section 5.3), enzymes are chosen on the basis of the number of their specific recognition bases that exist within the gene sequences of the 16S rRNA (Figure 5.1). The fewer recognition sites, the fewer restriction fragments generated and the greater the opportunity for constructing an easily identifiable and reproducible fingerprint specific for a particular strain.

16S ARDRA was applied to the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates. These efforts were carried out using the enzymes *Hin*dIII, *Hpa*II, *Ssp*I and *Sma*I, in an attempt to confirm the ribotype analysis of the NAD-independent strains. Additional enzymes were chosen to identify those that are most suited for the production of the most informative typing profiles. Furthermore, this study served to evaluate the application of 16S ARDRA for fast and efficient type characterisation of field isolates, and hence the inclusion of the most prevalent strain in the current vaccine. The restriction sites and the expected fragment sizes generated following the cleavage of *H. paragallinarum* NCTC 11296 T with each of seven restriction enzymes *Hin*dIII, *Ssp*I, *Hpa*II, *Sma*I, *Stu*I, *Apa*I and *Bam*HI are illustrated in Table 5.3.

Table 5.3 Restriction sites and the resulting fragment sizes following cleavage of the *H. paragallinarum* NCTC 11296 strain with each of seven restriction enzymes.

Restriction enzyme	Restriction site (base number)	Restriction fragment size (bp)
*Hin*dIII	79	79, 1404
*Hpa*II	503, 1300, 1381	81, 102, 503, 797
*Ssp*I	365	365, 1118
*Sma*I	1382	101, 1382
*Apa*I	929	554, 929
*Stu*I	417	417, 1066
*Bam*HI	-	-

5.4.1 Procedure

The 16S rRNA gene sequence of each of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates was amplified by a PCR employing the universal primers 27f and 1525r (Figure 5.2) to produce a DNA fragment 1.5 kb in size (Section 5.2). The amplified products (2.5 µl of each) were digested overnight with each of seven restriction enzymes *Hin*dIII, *Ssp*I, *Hpa*II, *Sma*I, *Stu*I, *Apa*I and *Bam*HI (Table 5.3). The restriction enzyme *Bam*HI was included as a negative control, as there was no apparent site in the 16S rRNA gene sequence illustrated in Figure 5.1. The reaction components and conditions for digestion are given in Table 5.4.

Table 5.4 Reaction components and incubation conditions for restriction digestion of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates.

Restriction enzyme	Reaction mixture components				Incubation temperature (°C)
	DNA (µl)	Buffer (µl)	Enzyme (µl; units)	Final volume (µl)	
*Hin*dIII	2.5	2[a]	2; 20	20	37
*Hpa*II	2.5	2[b]	2; 20	20	37
*Ssp*I	2.5	2[c]	2; 24	20	37
*Sma*I	2.5	2[d]	1; 20	20	25
*Apa*I	2.5	2[d]	2; 20	20	30
*Stu*I	2.5	2[a]	2; 20	20	37
*Bam*HI	2.5	2[a]	2; 20	20	37

Note: Buffer composition;

[a] *Hin*dIII buffer (Section 5.3.2.1): 10 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM 2-mercaptoethanol, pH 8.0,

[b] *Hpa*II buffer (Section 5.3.2.1): 10 mM Bis Tris propane-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0,

[c] *Ssp*I buffer (Section 5.3.2.1): 100 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 0.025% (v/v) Triton X-100, pH 7.5,

[d] *Sma*I buffer (Section 5.3.2.1): 20 mM Tris-HCl, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9.

The digestion products were electrophoresed through a 1.5% (m/v) agarose gel (Section 2.6) for analysis and the resulting fragments were compared with those expected, as illustrated in Table 5.3, for the identification of RFLPs.

5.5 Results and discussion

5.5.1 Quantification of the DIG-labeling efficiency

Visual comparison of the intensities of the spots on the nylon membrane, representative of dilutions from 1 ng/µl to 0.01 pg/µl, between the DIG-labeled control DNA and the experimental samples (Figure 5.4) enabled quantification of the labeling efficiency.

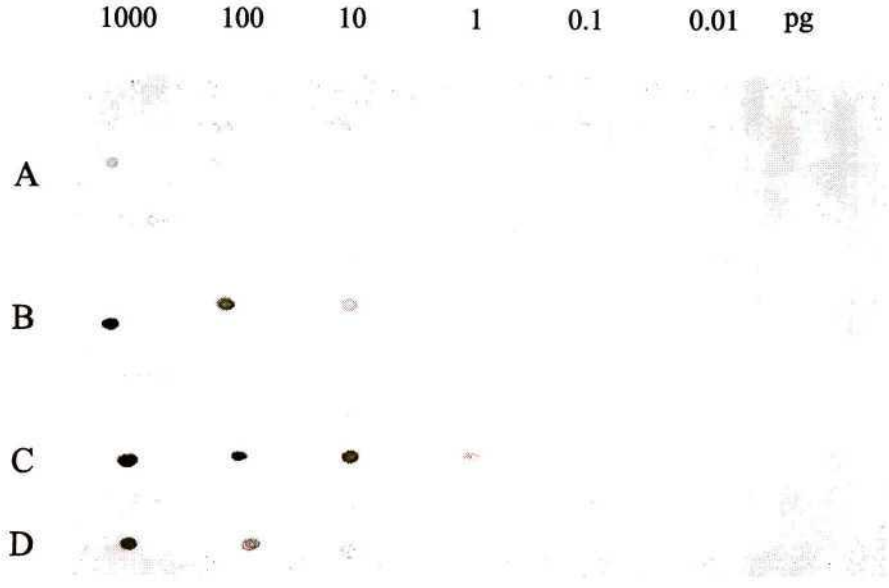


Figure 5.4 Dot blot analysis of the DIG-labeled 16S rDNA and *Hin*dIII-cut lambda DNA markers for evaluation of the efficiency of labeling. Ten-fold dilutions (from 1 ng/µl to 0.01 pg/µl) of **(A)** DIG-labeled control DNA, **(B)** self-labeled control DNA, **(C)** DIG-labeled 16S rDNA, and **(D)** DIG-labeled *Hin*dIII-cut lambda DNA markers, were spotted onto a nylon membrane, immunodetected and visualised with the substrate BCIP/NBT.

At a dilution of 10 pg/µl of the DIG-labeled control DNA (Figure 5.4A), the dot blot spot of the corresponding dilution of the DIG-labeled 16S rDNA probe was slightly less intense (Figure 5.4C) and as such the concentration of the diluted DIG-labeled probe was estimated at 9 pg/µl. Subsequently, the concentration of the undiluted probe was extrapolated as 15 ng/µl which corresponds to a total of 750 ng of labeled product (in a final volume of 50 µl) synthesised from 2000 ng of template DNA; a labeling efficiency of 1 in 2.7. In a similar manner, the concentration of the DIG-labeled *Hin*dIII-cut lambda DNA markers was determined to be 36 ng/µl i.e. 720 ng of labeled product (in a final volume of 20 µl) formed from 1000 ng of template DNA; a labeling efficiency of 1 in 1.4. The efficiency of labeling of the 16S rDNA probe and the *Hin*dIII-cut lambda DNA markers was satisfactory, correlating with the level expected (Boehringer Mannheim

Technical Manual). In addition, the degree of labeling obtained for the markers, in comparison to that observed for the 16S rDNA probe, suggested that 1 μg of template material is sufficient starting material for synthesis of an appreciable amount of labeled product. The level of labeling achieved implied that the application of these probes to ribotype analysis would produce a detectable signal.

5.5.2 Ribotyping of the NAD-independent *H. paragallinarum* field isolates

Studies of the epidemiology of infectious coryza outbreaks by restriction endonuclease analysis of the chromosomal DNA from eight representative NAD-dependent *H. paragallinarum* isolates have been reported (Blackall *et al.*, 1990b; 1991). The enzymes *Bam*HI, *Eco*RI, *Hin*dIII and *Sma*I were employed, revealing fragment patterns of varying complexity with *Hin*dIII generating the most fragments and allowing easiest recognition of genetic differences among the isolates. In the present study, REA by *Hin*dIII treatment of the chromosomal DNA of NAD-independent *H. paragallinarum* field isolates produced identical complex profiles (Figure 5.5). These intricate patterns made visual comparison with the profiles generated by Blackall and co-workers (1990b; 1991) for type characterisation an arduous task.

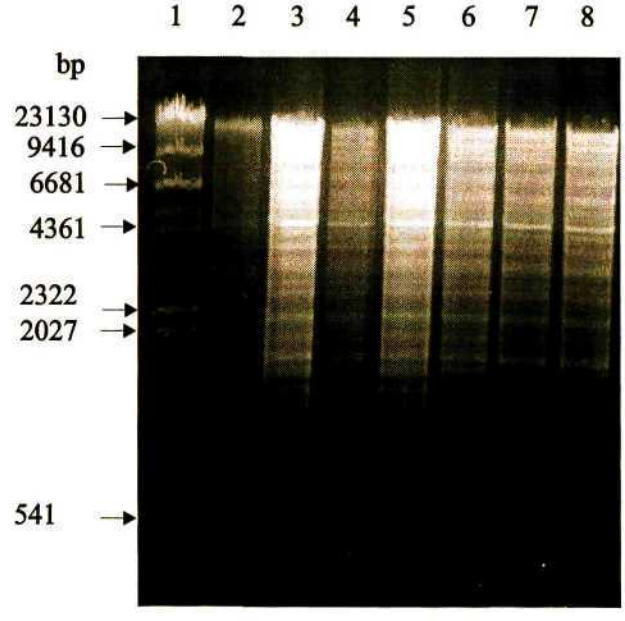


Figure 5.5 *Hin*dIII restriction endonuclease patterns of selected NAD-independent *H. paragallinarum* field isolates. Lane 1, *Hin*dIII-cut lambda DNA molecular weight ladder; **lanes 2-8,** *H. paragallinarum* strains **[lane 2,** 95/03938; **lane 3,** 93/09472; **lane 4,** 93/00449; **lane 5,** 96/12954; **lane 6,** RH 2390; **lane 7,** 1130 (serotype A) and ; **lane 8,** 541 (serovar C-3)].

Consequently, it was decided to apply the discriminatory ribotyping method to simplify the restriction endonuclease patterns, using a 16S rDNA probe to recognise complementary sequences of the 16S rRNA gene within the digested chromosomal DNA fragments. The restriction enzymes *Hin*dIII, *Hpa*II and *Ssp*I were used to confirm reports by Miflin *et al.* (1995) regarding the successful application of these enzymes for typing the infectious coryza causing agent, and to apply these enzymes to ribotype analysis of local NAD-independent isolates. An additional enzyme *Sma*I was chosen, despite the limited success reported by Blackall *et al.* (1991), due to its low frequency of cutting within the 16S rRNA sequences (Figure 5.1) and its availability in our laboratory.

Digestion of the chromosomal DNA from the NAD-independent *H. paragallinarum* field isolates with each of *Hin*dIII, *Hpa*II, *Ssp*I and *Sma*I resulted in several fragments that the 16S rDNA probe recognised and bound. Digestion with *Hin*dIII produced several reproducible, identical profiles for all strains tested (Figure 5.6). Four DNA fragments were identified approximately 2700, 1850, 1700 and 900 bp in size.

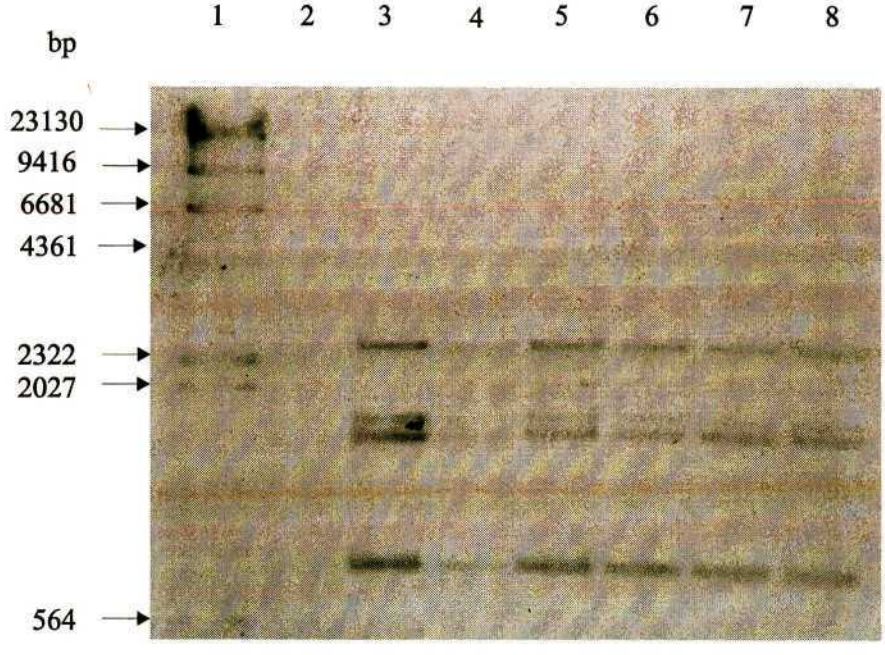


Figure 5.6 *Hin*dIII ribotypes for NAD-independent *H. paragallinarum* field isolates. **Lane 1,** *Hin*dIII-cut lambda DNA molecular weight ladder, probed with a complementary DIG-labeled strand; **lanes 2-8,** *H. paragallinarum* field isolates **[lane 2,** 95/03938; **lane 3,** 93/09472; **lane 4,** 93/00449; **lane 5,** 96/12954; **lane 6,** RH 2390; **lane 7,** 1130 (serotype A) and; **lane 8,** 541 (serovar C-3)]. The field isolates were probed with a PCR-generated, DIG-labeled 16S rDNA probe, immunodetected and visualised with the substrate BCIP/NBT.

Digestion with each of the restriction enzymes *Ssp*I and *Sma*I produced identical, reproducible profiles for each enzyme for all isolates studied, with each enzyme producing a characteristic profile (results not shown) and as such these results shall not be discussed. The identity of the ribopatterns of the NAD-independent *H. paragallinarum* field isolates serves to demonstrate the high degree of conservation in the 16S rRNA gene sequences and provides further evidence that these isolates are clonal, having originated from an NAD-dependent isolate at a particular point before spreading throughout the poultry industry.

The ribopatterns generated following *Hpa*II analysis were identical for all isolates tested, with the exception of serovar C-3 strain 541 (Figure 5.7). Isolates 95/03938, 93/09472, 93/00449, 96/12954 and RH 2390 exhibited 4 fragments approximately 6000 (broad doublet), 2000 and 700 bp in size; identical to the profile observed for serotype A strain 1130. The ribotype of strain 541 was similar in that DNA fragments at 2000 and 700 bp, as well as the lower molecular size band of the 6000 bp doublet, were present. However, the disappearance of the higher molecular size band of the 6000 bp doublet and the appearance of a larger fragment at approximately 9400 bp distinguishes the serovar C-3 isolate from the serotype A strain 1130 (classified by R.R. Bragg, University of Pretoria, South Africa using haemagglutination inhibition) and from the local NAD-independent field isolates. The observations made in the current investigation led to the definition of two ribotypes; ribotype 1 being characterised by a doublet at 6000 bp, while a singlet at 6000 bp and an additional band at approximately 9400 bp typifies ribotype 2. As the local NAD-independent field isolates and strain 1130 produce ribotype 1 profiles, it may be assumed that, on the basis of conserved 16S rRNA sequences, the local isolates belong to serotype A.

These findings are contradictory to those at the phenotypic level (Chapter 4). A study of the OMPs of the NAD-independent *H. paragallinarum* field isolates revealed two different protein banding patterns for the isolates obtained from poultry units in the Gauteng Province and those in Kwazulu-Natal. Furthermore, the OMP profiles of serotype A strain 1130 and serovar C-3 strain 541 appeared to be identical. In contrast, ribotype patterns were established independent of the geographical origin of the isolate and were able to differentiate isolates previously typed (using a haemagglutination-inhibition system) by R.R. Bragg (University of Pretoria, South Africa). Subsequently, there is uncertainty regarding phenotypic allocation of the local NAD-independent

field isolates to serovar C-3. The high degree of evolutionary conservation exhibited by the rRNA gene sequences, which are relatively unaffected by external factors, favours the outcome of the ribotyping results over those based on the WCPs and OMPs. Nevertheless, confirmation of these results, through the identification of RFLPs, remains to be demonstrated.

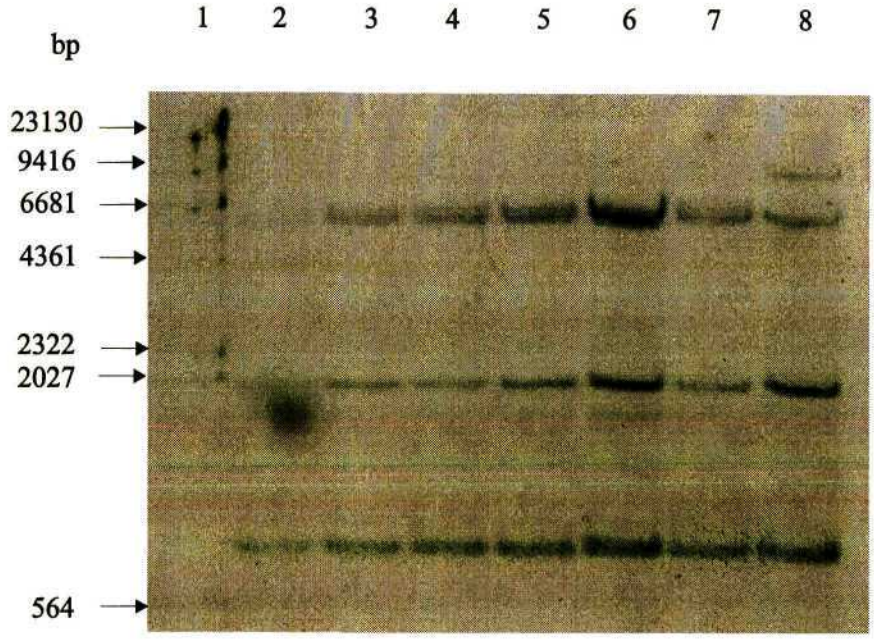


Figure 5.7 *Hpa*II ribotypes for NAD-independent *H. paragallinarum* field isolates. Lane 1, *Hin*dIII-cut lambda DNA molecular weight ladder, probed with a complementary DIG-labeled strand; **lanes 2-8,** *H. paragallinarum* field isolates **[lane 2,** 95/03938; **lane 3,** 93/09472; **lane 4,** 93/00449; **lane 5,** 96/12954; **lane 6,** RH 2390; **lane 7,** 1130 (serotype A) and; **lane 8,** 541 (serovar C-3)]. The field isolates were probed with a PCR-generated, DIG-labeled 16S rDNA probe, immunodetected and visualised with the substrate BCIP/NBT.

The NAD-independent *H. paragallinarum* field isolates employed in the study conducted by Miflin and co-workers (1995) were collected in Kwazulu-Natal between 1989 and 1993, and were characterised as serotype A by a haemagglutination-inhibition test. Application of *Hin*dIII, *Hpa*II and *Ssp*I for the ribotype analysis of all fifteen NAD-independent isolates produced a single, identical profile for each enzyme tested, with each enzyme giving a different pattern. The similar results obtained in the present study confirm these observations and exhibit the genetic relatedness of these isolates. As a group, the NAD-independent isolates gave very different ribotypes in comparison with the NAD-dependent field isolates of predetermined serotype. As the ribopatterns of the NAD-independent isolates obtained in this study and those obtained in the

study of Miflin *et al.* (1995) did not correspond to any of the NAD-dependent strains, it is likely that a set of reference strains specific for the NAD-independent isolates should be constructed to enable type allocation of newly isolated NAD-independent bacteria.

In both studies, the ribotypes generated by *Hpa*II digestion were the most informative. In the study by Miflin *et al.* (1995) four restriction fragments were identified approximately 6000 (triplet) and 2000 bp in size. It is postulated that should the *Hpa*II-digested chromosomal DNA fragments, from the NAD-independent isolates tested in the present study, be electrophoresed on a large agarose gel overnight (as performed by Miflin *et al.*, 1995), the broad doublet observed in the present study may be resolved into a triplet. Furthermore, the 2000 bp band was common in both studies and as the ribotype profile published by Miflin *et al.* (1995) was cut off below this band, it is not known whether the 700 bp fragment was found by these workers. As such it is possible that the ribopatterns reported in the present investigation and those reported by Miflin *et al.* (1995) are very similar. Should this be the case, these findings would support the allocation of the local NAD-independent isolates to serotype A.

The high degree of non-identity observed in the ribopatterns of the NAD-independent *H. paragallinarum* field isolates of serotype A and the NAD-dependent field isolates (serotype A) raise the question as to whether vaccines based on the latter will provide protection to flocks of birds infected with the former. If this is the case, the need for efficient typing systems for characterising the prevalent NAD-independent isolates is apparent. Limitations for typing based on the phenotypic characteristics of isolates have been highlighted (Chapter 4; Zhao *et al.*, 1992; Russel *et al.*, 1994; Nagai *et al.*, 1995; Olsen and Woese, 1993). Subsequently, the application of typing schemes based on genotypic characteristics were proposed (Snipes *et al.*, 1989; 1992; this study). Ribotyping, based on the highly conserved ribosomal RNA gene sequences, exhibits a high degree of discrimination amongst strains of different 'serotype' and seems to provide the answer to typing woes. However, due to the large amounts of starting material required, appreciably limited in the present study, and the lengthy, labour-intensive procedures, a simplified technique was sought. To this end, 16S ARDRA was applied to the typing of local *H. paragallinarum* isolates.

5.5.3 Restriction digestion of PCR-generated 16S rDNA

The 16S rRNA gene sequences (1483 bp in length) of 10 NAD-dependent *H. paragallinarum* reference strains and field isolates and 8 NAD-independent field isolates were amplified in a PCR employing the universal primers 27f and 1525r (Figure 5.2). Digestion of the amplified 16S rDNA product with each of seven different restriction enzymes; namely *Hin*dIII, *Hpa*II, *Ssp*I, *Sma*I, *Apa*I, *Stu*I and *Bam*HI, revealed a number of RFLPs.

In general, the NAD-independent isolates revealed identical restriction patterns. Following treatment of the NAD-dependent and -independent isolates with the restriction enzymes *Ssp*I, *Sma*I and *Apa*I reproducible profiles, consistent with fragment sizes reported for the NCTC 11296 T strain (Figure 5.1; Table 5.3), were obtained (results not shown) for each enzyme for all strains. This indicates that no new sites had been created and the 16S rRNA gene is highly conserved with respect to these enzymes. Furthermore, no *Bam*HI site (results not shown) had been created during evolutionary development in all strains studied with the exception of the ATCC strain, which revealed two digested bands at approximately 103 and 1380 bp. Although the above results provide data regarding the degree of conservation of the 16S rRNA gene sequences, they do not contribute towards the establishment of a typing system. In contrast, the results obtained with *Hin*dIII, *Hpa*II and *Stu*I were more informative and provide the basis for developing an efficient typing scheme.

*Hin*dIII 16S rDNA digestion of the NAD-dependent strains (Figure 5.8) and NAD-independent isolates (Figure 5.9) showed that the site at 79 bp was retained in all isolates (Table 5.5). An additional *Hin*dIII site was introduced in the ATCC 29545 strain such that *Hin*dIII restriction digestion revealed 2 further DNA fragments approximately 150 and 1330 bp in size. Nucleotide base changes in all the NAD-independent isolates resulted in a new *Hin*dIII site which, upon cleavage, generated fragments approximately 830 and 910 bp in length. The identity of all the 16S rDNA patterns of the NAD-independent isolates is in agreement with ribotyping results, which also revealed identical profiles following *Hin*dIII digestion (Section 5.3.2).

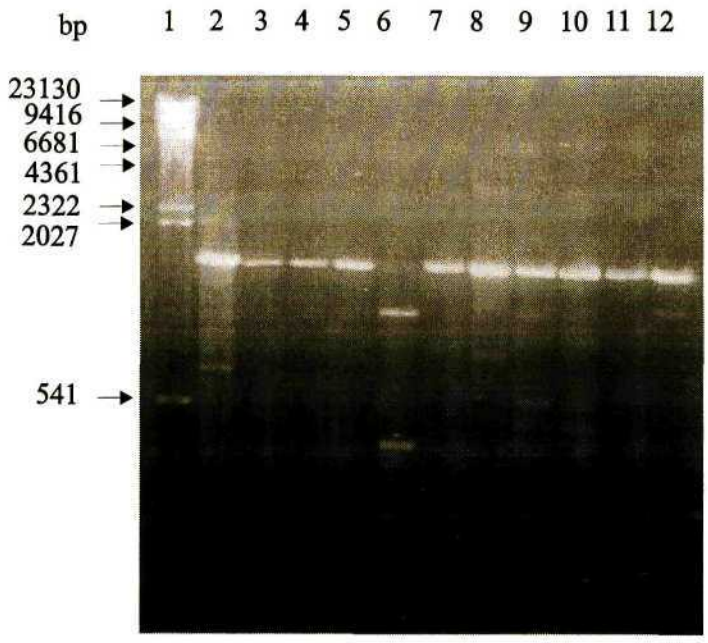


Figure 5.8 Restriction analysis of *Hin*dIII-digested 16S rDNA amplicons from NAD-dependent *H. paragallinarum* reference strains and field isolates. Lane 1, *Hin*dIII-cut lambda DNA molecular weight ladder; **lane 2,** uncut rDNA; **lanes 3-12,** *H. paragallinarum* strains **[lane 3,** 0083 (serovar A-1); **lane 4,** 221 (serovar A-1); **lane 5,** 2403 (serovar A-2); **lane 6,** ATCC 29545 (serotype A); **lane 7,** 0222 (serovar B-1); **lane 8,** Modesto (serovar C-2); **lane 9,** 39 (serotype A); **lane 10,** 41 (serotype A); **lane 11,** 37 (serovar C-2) and; **lane 12,** 46 (serovar C-3)].

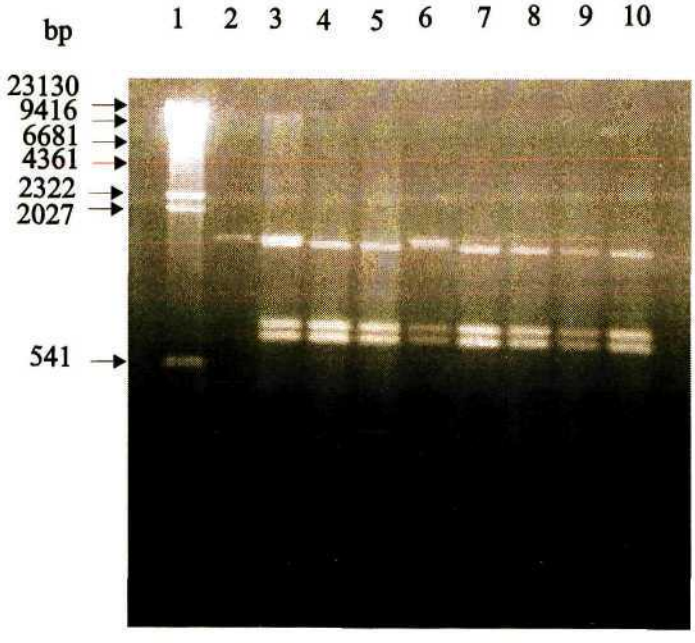


Figure 5.9 Restriction analysis of *Hin*dIII-digested 16S rDNA amplicons from NAD-independent *H. paragallinarum* field isolates. Lane 1, *Hin*dIII-cut lambda DNA molecular weight ladder; **lane 2,** uncut rDNA; **lanes 3-10,** *H. paragallinarum* strains **[lane 3,** 95/03938; **lane 4,** 93/09472; **lane 5,** 93/00449; **lane 6,** 96/11756; **lane 7,** 96/12954; **lane 8,** RH 2390; **lane 9,** 1130 (serotype A) and; **lane 10,** 541 (serovar C-3)].

Table 5.5 Identification of RFLPs in *Hin*dIII-digested 16S rDNA of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates.

Strain	DNA fragment (bp)	
	Conserved sites	RFLP
NAD-dependent		
0083 (serovar A-1)	79, 1404	
221 (serovar A-1)	79, 1404	
2403 (serovar A-2)	79, 1404	
ATCC 29545 (serotype A)	79, 1404	*c.* 150, 1330
0222 (serovar B-1)	79, 1404	
Modesto (serovar C-2)	79, 1404	
39 (serotype A)	79, 1404	
41 (serotype A)	79, 1404	
37 (serovar C-2)	79, 1404	
46 (serovar C-3)	79, 1404	
NAD-independent		
95/03938	79, 1404	*c.* 830, 910
93/09472	79, 1404	*c.* 830, 910
93/00449	79, 1404	*c.* 830, 910
96/11756	79, 1404	*c.* 830, 910
96/12954	79, 1404	*c.* 830, 910
RH 2390	79, 1404	*c.* 830, 910
1130 (serotype A)	79, 1404	*c.* 830, 910
541 (serovar C-3)	79, 1404	*c.* 830, 910

As the restriction profiles for all NAD-dependent reference strains and field isolates, representative of all Kume's serovars (Blackall *et al.*, 1990a) with the exception of serovar A-3 and A-4 (not available in the present study), exhibited identical profiles, *Hin*dIII is not suitable for typing *H. paragallinarum* outbreaks.

A greater degree of success was obtained following *Hpa*II digestion of the PCR-generated 16S rDNA amplicon of all isolates studied as a number of additional *Hpa*II restriction sites had been created or deleted in some of the NAD-dependent and -independent isolates. As the *Hpa*II recognition sequence is only four nucleotides in length (C↓CGG), the probability of a one base change generating a new *Hpa*II site is greater than for the generation of a new *Hin*dIII site, which requires a six nucleotide sequence (A↓AGCTT) for cleavage. NAD-dependent reference strains 2403 (serovar A-2), 0222 (serovar B-1) and Modesto (serovar C-2) and field isolates 39, 41 (both serotype A) and 37 (serovar C-2) did not exhibit any new *Hpa*II sites (Figure 5.10; Table 5.6). Similarly, the NAD-independent field isolates 93/09472, 93/00449, 96/12954, RH 2390, 1130 (serotype A) and 541 (serovar C-3) were conserved with respect to *Hpa*II (Figure 5.11; Table 5.6).

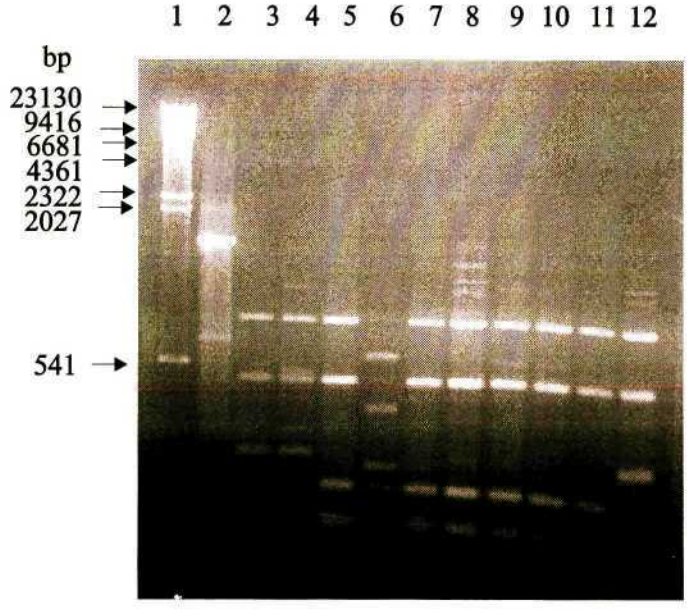


Figure 5.10 Restriction analysis of *Hpa*II-digested 16S rDNA amplicons from NAD-dependent *H. paragallinarum* reference strains and field isolates. Lane 1, *Hin*dIII-cut lambda DNA molecular weight ladder; **lane 2,** uncut rDNA; **lanes 3-12,** *H. paragallinarum* strains **[lane 3,** 0083 (serovar A-1); **lane 4,** 221 (serovar A-1); **lane 5,** 2403 (serovar A-2); **lane 6,** ATCC 29545 (serotype A); **lane 7,** 0222 (serovar B-1); **lane 8,** Modesto (serovar C-2); **lane 9,** 39 (serotype A); **lane 10,** 41 (serotype A); **lane 11,** 37 (serovar C-2) and; **lane 12,** 46 (serovar C-3)].

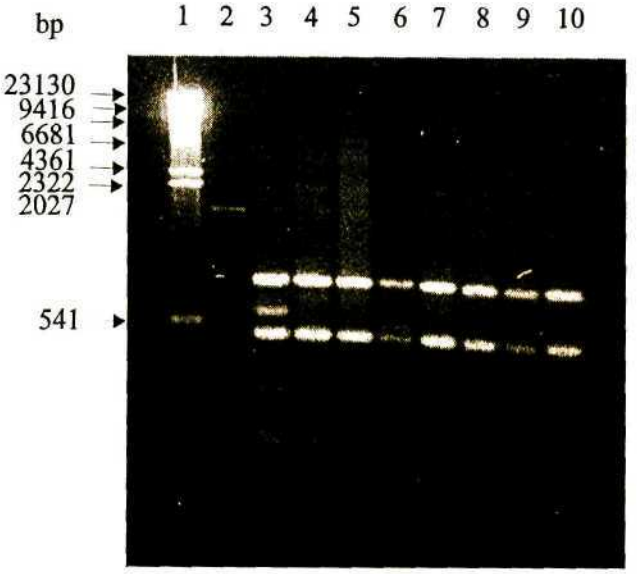


Figure 5.11 Restriction analysis of *Hpa*II-digested 16S rDNA amplicons from NAD-independent *H. paragallinarum* field isolates. **Lane 1,** *Hin*dIII-cut lambda DNA molecular weight ladder; **lane 2,** uncut rDNA; **lanes 3-10,** *H. paragallinarum* strains [**lane 3,** 95/03938; **lane 4,** 93/09472; **lane 5,** 93/00449; **lane 6,** 96/11756; **lane 7,** 96/12954; **lane 8,** RH 2390; **lane 9,** 1130 (serotype A) and; **lane 10,** 541 (serovar C-3)].

The appearance of a DNA fragment of approximately 200 bp in both serovar A-1 strains is likely due to a nucleotide base change at position 1381, such that this *Hpa*II site is deleted and subsequent cleavage at site 1300 generates the new fragment. However, confusion arose due a 102 bp fragment in strain 221 (which is formed by cleavage at the supposedly deleted site) and the appearance of two additional bands approximately 530 and 300 bp in length which could not be accounted for. These inconsistencies within the previously characterised serovar A-1 strains (Blackall *et al.*, 1990a) cast initial doubt on the application of *Hpa*II for typing serovar A-1 strains. In contrast, the recognition sequence centred around base 1381 appeared to be the only site conserved in the ATCC 29545 strain. It is possible that the sites at 503 and 1300 still exist, however, the creation of additional sites within those already in place were responsible for generating smaller fragments approximately 650, 400, 170 and 85 bp in size. Nevertheless, the resulting restriction pattern is clearly distinct and does not correspond to any of the other profiles obtained.

Serovar C-3 strain 46 also displayed a unique restriction pattern. The disappearance of DNA fragments at 102 and 81 bp suggests the deletion of the site at position 1381, however the appearance of a band 150 bp in length (clearly smaller that the serovar A-1 band) cannot be explained. The serovar C-3 profile may be interpreted in a two-fold manner. Firstly, the unique nature of this profile, to which none of the NAD-independent isolates correlated, suggests that the NAD-independent isolates are not serovar C-3; a conclusion made at the phenotypic level. On the other hand, *Hpa*II was unable to distinguish between NAD-independent isolates 1130 of serotype A and 541 of serotype C-3, in contrast to the results of ribotype analysis (Section 5.3.2). Clearly, this discrepancy requires further investigation. Nevertheless, the inability to distinguish between the two serotypes provides support for earlier suggestions that the NAD-independent isolates may not be suitably typed by visual comparison with the profiles of NAD-dependent strains but require their own set of reference strains. The identity of the banding patterns of the NAD-independent serotype A and serovar C-3 isolates with each other and with most of the local isolates, reaffirms the clonal origins of these isolates and suggests that the local isolates may belong to either serotype A or serovar C-3.

The NAD-independent isolates 95/03938 and 96/11756 exhibited two additional bands each at approximately 650 and 280, and 530 and 480 bp respectively (Table 5.6), over and above the conserved DNA fragments. The introduction of new restriction sites within the 16S rRNA gene sequences classes these isolates to a typing group other than serotype A or serovar C-3. However, it is possible that the remainder of the NAD-independent isolates studied may belong to either of these types due to the identity of their profiles.

Table 5.6 Identification of RFLPs in *Hpa*II-digested 16S rDNA of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates.

Strain	DNA fragment (bp)	
	Conserved sites	RFLP
NAD-dependent		
0083 (serovar A-1)	797, 503	*c.* 200
221 (serovar A-1)	797, 503, 102	*c.* 530, 300
2403 (serovar A-2)	797, 503, 102, 81	
ATCC 29545 (serotype A)	102	*c.* 650, 400, 170, 85
0222 (serovar B-1)	797, 503, 102, 81	
Modesto (serovar C-2)	797, 503, 102, 81	
39 (serotype A)	797, 503, 102, 81	
41 (serotype A)	797, 503, 102, 81	
37 (serovar C-2)	797, 503, 102, 81	
46 (serovar C-3)	797, 503	*c.* 150
NAD-independent		
95/03938	797, 503, 102, 81	*c.* 650, 280
93/09472	797, 503, 102, 81	
93/00449	797, 503, 102, 81	
96/11756	797, 503, 102, 81	*c.* 530, 480
96/12954	797, 503, 102, 81	
RH 2390	797, 503, 102, 81	
1130 (serotype A)	797, 503, 102, 81	
541 (serovar C-3)	797, 503, 102, 81	

Following *Stu*I digestion, a RFLP was found in all NAD-dependent (Figure 5.12) and -independent (Figure 5.13) *H. paragallinarum* reference strains and field isolates studied, with the exception of strains 2403 (serovar A-2) and 0222 (serovar B-1) which only produced two fragments of expected size, 417 and 1066 bp in length. Restriction digestion of the 1.483 kb 16S rDNA fragment with *Stu*I revealed a high degree of homology with the NCTC 11956 T strain

with regard to *Stu*I restriction sites. The ATCC 29545 strain appears to be the exception, as the site at base position 417 seems to have been deleted for no restriction pattern was produced upon digestion with *Stu*I. In all the other strains studied, it appeared that a *Stu*I restriction site was introduced at approximately base 280, generating additional fragments approximately 280 and 100 bp in length (Table 5.7) and accounting for the disappearance of the 417 bp fragment.

The additional band at 1200 bp in the Modesto strain may be attributed to an incomplete digestion of the 16S rDNA, having arisen from failure to digest the Modesto DNA at the newly created site, thereby resulting in a fragment approximately 1200 (1066 + 100) bp in size. Similarly, the appearance of the 417 fragment, in addition to the 280 and 100 bp bands, in the NAD-independent isolates may be attributed to incomplete digestion at the internal 280 base site, such that undigested 'comparably whole' fragments of 417 bp are detected. Once again, it was not possible to differentiate the NAD-independent strains 1130 and 541 using this enzyme, which produced profiles identical to each other and all the local NAD-independent isolates. As such the application of *Stu*I for type allocation is very limited.

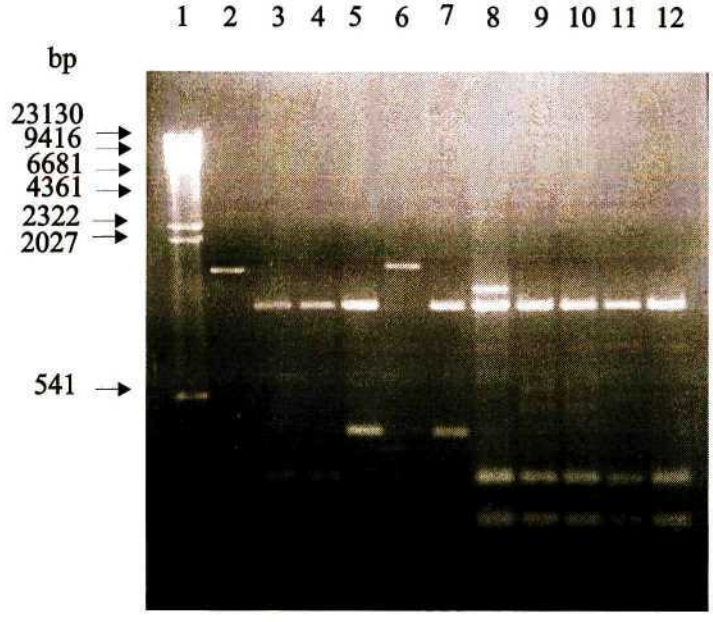


Figure 5.12 Restriction analysis of *Stu*I-digested 16S rDNA amplicons from NAD-dependent *H. paragallinarum* reference strains and field isolates. Lane 1, *Hin*dIII-cut lambda DNA molecular weight ladder; **lane 2,** uncut rDNA; **lanes 3-12,** *H. paragallinarum* strains **[lane 3,** 0083 (serovar A-1); **lane 4,** 221 (serovar A-1); **lane 5,** 2403 (serovar A-2); **lane 6,** ATCC 29545 (serotype A); **lane 7,** 0222 (serovar B-1); **lane 8,** Modesto (serovar C-2); **lane 9,** 39 (serotype A); **lane 10,** 41 (serotype A); **lane 11,** 37 (serovar C-2) and; **lane 12,** 46 (serovar C-3)].

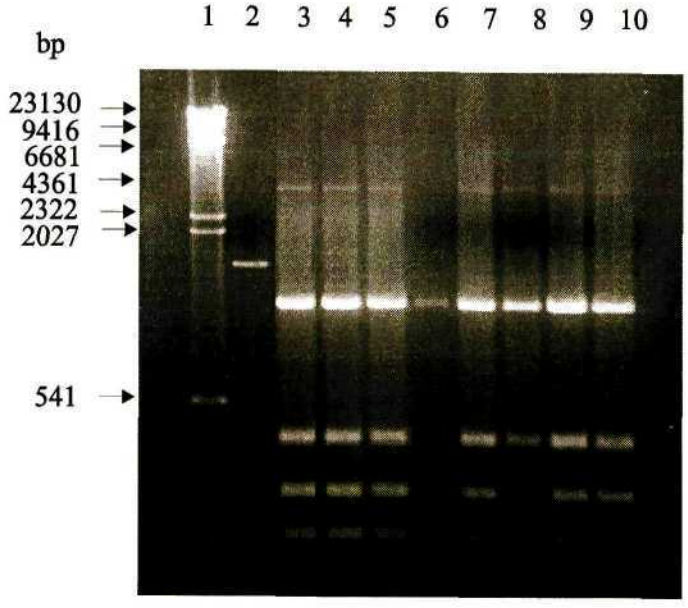


Figure 5.13 Restriction analysis of *Stu*I-digested 16S rDNA amplicons from NAD-independent *H. paragallinarum* field isolates. **Lane 1,** *Hin*dIII-cut lambda DNA molecular weight ladder; **lane 2,** uncut rDNA; **lanes 3-10,** *H. paragallinarum* strains [**lane 3,** 95/03938; **lane 4,** 93/09472; **lane 5,** 93/00449; **lane 6,** 96/11756; **lane 7,** 96/12954; **lane 8,** RH 2390; **lane 9,** 1130 (serotype A) and; **lane 10,** 541 (serovar C-3)].

Table 5.7 Identification of RFLPs in *Stu*I-digested 16S rDNA of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates.

Strain	DNA fragment (bp)	
	Conserved sites	RFLP
NAD-dependent		
0083 (serovar A-1)	1066	*c.* 280, 100
221 (serovar A-1)	1066	*c.* 280, 100
2403 (serovar A-2)	1066, 417	
ATCC 29545 (serotype A)	uncut	
0222 (serovar B-1)	1066, 417	
Modesto (serovar C-2)	1066	*c.* 1200, 280, 100
39 (serotype A)	1066	*c.* 280, 100
41 (serotype A)	1066	*c.* 280, 100
37 (serovar C-2)	1066	*c.* 280, 100
46 (serovar C-3)	1066	*c.* 280, 100
NAD-independent		
95/03938	1066, 417	*c.* 280, 100
93/09472	1066, 417	*c.* 280, 100
93/00449	1066, 417	*c.* 280, 100
96/11756	1066, 417	*c.* 280, 100
96/12954	1066, 417	*c.* 280, 100
RH 2390	1066, 417	*c.* 280, 100
1130 (serotype A)	1066, 417	*c.* 280, 100
541 (serovar C-3)	1066, 417	*c.* 280, 100

In the present study, no ideal restriction enzyme was identified which exhibited a unique restriction site in each strain of a different serovar, enabling a quick, undisputable serotyping of all field isolates. Nevertheless, this study served to form the basis for identifying a battery of restriction enzymes which produce a unique restriction profile for each serovar. *Hpa*II clearly results in a unique NAD-dependent serovar C-3 pattern, and as such should be employed to test any new field isolates. Moreover, *Hpa*II may be used for the initial characterisation of serovar

A-1 isolates. Conservation of the 16S rRNA gene with respect to *Stu*I is characteristic of strains 2403 and 0222, and thereafter it remains to identify a restriction enzyme to differentiate between these two strains. In this manner, restriction enzyme profiles characteristic of each serovar may be generated, with a separate set of reference strains for the NAD-dependent and -independent isolates.

It should be noted that a number of bands, in addition to those recognised as RFLPs (see e.g. Figure 5.8), were recorded in the field isolates. These fragments were not treated as RFLPs and, as such, it was postulated that they could be contributed to a heterogenous population of bacteria isolated from the infected chicken, which result in non-specific amplification during PCR. As the appearance of these bands was relatively consistent in all field isolates studied, they were disregarded.

5.6 Conclusions

Ribotype analysis of *Hpa*II-digested chromosomal DNA from local NAD-independent isolates, indicated that these isolates may be allocated to serotype A, due to comparative profiles with NAD-independent strain 1130. Isolates of NAD independency were conserved with respect to each other, however, in comparison to the NAD-dependent isolates the rRNA sequences were revealed to be considerably heterogenous. This observation was reaffirmed following 16S ARDRA studies, however, the restriction enzymes employed failed to differentiate between the NAD-independent isolates of serotype A and serovar C-3. Nevertheless, following the identification of restriction enzymes which produce serovar-specific fingerprints, this novel study indicates the potential application this technique has for the type allocation of *H. paragallinarum* strains.

CHAPTER 6

General Discussion

The etiological agent of infectious coryza, an acute upper respiratory tract disease of poultry, was diagnosed as the NAD requiring bacterium *H. paragallinarum.* Since the early reports of infectious coryza, this infection has displayed widespread variability in its severity and in its overall effect on layers (Schalm and Beach, 1936) with reduction in egg production ranging from minimal (Schalm and Beach, 1936) to 40% (Arzey, 1987; Sarakbi, 1987), with the potential to economically cripple an egg industry. Locally this disease became financially important after 1968, following significant drops in egg production in several large commercial laying flocks (Coetzee *et al.*, 1982). However, incidents of infectious coryza are of global concern, with the establishment of efficient control measures being a necessity.

Treatment procedures employing various sulfonamides and antibiotics were found to alleviate the severity and course of infectious coryza, however, none of these therapeutic agents were bactericidal and drug resistance developed (Reece and Coloe, 1985; Blackall, 1988). For this reason preventative control methods are favoured, most notably immunization protocols, in an effort to release the current stranglehold and curb any further detrimental effects this disease potentially exerts on the local poultry industry. Immunity induced by vaccines is dependent on the serotype and, to an extent, the serovar of the infecting bacterium such that protection is only offered against challenge of a homologous nature (Rimler *et al.*, 1977a; Kume *et al.*, 1980c; Blackall and Reid, 1987). Studies conducted in Brazil by Blackall *et al.* (1994) highlighted the necessity for active monitoring programmes to ensure that the prevalent serovars are contained within vaccines for endemic regions. Since the introduction of infectious coryza vaccines in South Africa in the early 1980s, there has been a continual decrease in the level of infectious control as the efficacy of current vaccines declined, precipitating profit losses. It is probable that the selective pressures imposed by long term use of commercial vaccines against infectious coryza have had a marked effect on the relative abundance of serovars in this country (Bragg *et al.*, 1996). Evidence to this effect is provided by a decrease in the number of serovar A-1 isolates reported following administration of a serotype A vaccine. This decline was accompanied by an

outbreak of virulent C-3 isolates, against which other serotype C strains provide partial protection. A similar scenario has been reported within serotype strains of *Neisseria meningitidis* (Moore and Broom, 1994), for as immunity against shared antigens decrease, so clones with a different array of surface antigens are capable of escaping immunosurveilance and initiating an epidemic.

The local situation has been further complicated by the emergence of a fast-growing, virulent variant of *H. paragallinarum* which does not display a requirement for NAD (Horner *et al.*, 1992; 1995). It is believed that the NAD-independent organisms are clonal in nature having evolved following differentiation of a single NAD-dependent *H. paragallinarum* bacterial cell of serotype A (Miflin *et al.*, 1995). In a study conducted by Bragg (1995) the existence of serotype A NAD-independent isolates was confirmed. Moreover, isolates of serovar C-3 were identified, derived following transfer of NAD-independence to an NAD-dependent serovar C-3 organism. The minimal growth requirements exhibited by these variants (Horner *et al.*, 1992) imparts a major competitive advantage to these organisms. It has allowed their rapid dissemination throughout the poultry industry which has rendered them the chief agent of infectious coryza, having largely replaced the NAD-dependent isolates (Horner *et al.*, 1995). NAD independency allows these strains to grow in areas of the sinus devoid of blood components, especially circulating protective antibodies, providing a possible mechanism for immunoevasion. Bragg *et al.* (1997) have expressed concern that the current vaccination protocols, based on NAD-dependent strains, may become ineffective against the NAD-independent isolates. With the existence of nine possible serovars within the three serotypes it has become clear that efficient biochemical, serological and molecular characterisation tests are required for monitoring the population dynamics of both the NAD-dependent and -independent isolates in the field and enabling the inclusion of the predominant infecting strain in the current commercial vaccine.

The *H. paragallinarum* isolates of varying NAD dependency may be biochemically differentiated from related avian pathogens (e.g. *H. avium* and *Ornithobacterium rhinotracheale*) depending on their carbohydrate fermentation patterns (Blackall and Reid, 1982; Blackall, 1983; Piechulla *et al.*,

1984), and from each other according to their differing co-factor requirements for growth (Rimler *et al.*, 1976; 1977; Horner *et al.*, 1992). Thereafter, identified *H. paragallinarum* variants may be conventionally subtyped into serotypes or their serovars depending on their inability to agglutinate treated or untreated red blood cells, as described in a haemagglutination inhibition test (Kato *et al.*, 1965). This inability is effected by *H. paragallinarum* epitope recognition by the type-specific antibodies (Iritani *et al.*, 1981a; Kume *et al.*, 1980c; Sawata *et al.*, 1982). Haemagglutination inhibition provides a reliable serotyping scheme with a specificity superior to prior agglutinin systems (Eaves *et al.*, 1989), however, little knowledge is gained regarding the outer membrane-located serotype-specific antigens (Sawata *et al.*, 1984a). Type allocation based on the outer membrane protein profiles has a dual application; providing a characteristic protein fingerprint for type assignment and facilitating visual identification and analysis of individual outer membrane proteins. In an extension of previous studies conducted (Blackall *et al.*, 1989a; 1990d; Horner *et al.*, 1995), the whole cell proteins and outer membrane proteins of the NAD-dependent and -independent *H. paragallinarum* reference strains and field isolates were examined. Furthermore, molecular characterisation of these isolates was attempted by exploiting the evolutionary conserved 16S ribosomal RNA gene sequences (Grimont and Grimont, 1991; Towner and Cockayne, 1993; Miflin *et al.*, 1995; Jordens and Leaves, 1997; Eldar *et al.*, 1997).

In the present investigation, the conventional *H. paragallinarum* diagnostic tools were dispensed with. Contributing factors include possible ambiguities (Blackall, 1988) in the visual interpretation of carbohydrate fermentation patterns, fastidious growth requirements of the cultured NAD-dependent organisms such that rapidly multiplying contaminants can mask their presence (Blackall and Yamamoto, 1990), and finally, flawed diagnosis due to the presence of related haemophilic bacteria which infect flocks of birds (Mutters *et al.*, 1995). *H. paragallinarum* diagnosis instead proceeded by means of a specific, sensitive and adequately versatile PCR-based technique (Chen *et al.*, 1996). In this manner all the strains and isolates studied were irrefutably demonstrated to be the causative agent of infectious coryza, a fundamental verdict to subsequent type characterisations. The use of PCR in diagnostics has previously found application for the detection of *Pseudomonas pseudomallei* (Lew and Desmarchelier, 1994) and *E. coli* (Schultsz *et al.*, 1994).

The natural progression of type allocation was the use of previously serotyped NAD-dependent *H. paragallinarum* reference strains (Kume *et al.*, 1983a; Blackall *et al.*, 1990a) and field isolates (R.R. Bragg, University of Pretoria, South Africa) to create a data base against which fingerprints generated from unknown field isolates may be compared. In the present study, serovar-specific templates were established on both a whole cell protein and outer membrane protein level for the type characterisation of the NAD-independent isolates. The WCP profiles of isolates growing in the absence of the co-factor NAD were identical, regardless of their geographical origins and were tentatively assigned into serotype A, or to either serovar C-2 or C-3. The constraint of complexity complexity and accompanying interpretative ambiguities of the WCP patterns limit their serotypic application.

Analysis of the comparatively simple, Triton X-100-isolated, OMPs of field isolate 46 revealed a major, apparently unique, protein of approximately 30 kDa. Qualitative and quantitative differences were noted in the OMP patterns of the NAD-independent isolates from Kwazulu-Natal when compared to the identical profiles exhibited by strains of serotype A and serovar C-3 (R.R. Bragg, University of Pretoria, South Africa), which were obtained from Gauteng poultry units. Nevertheless, visual comparative analysis of the OMP profiles with the formulated data bank disclosed a 30 kDa protein corresponding to that of field isolate 46, suggesting that all strains of NAD independency studied could be allocated to serovar C-3. The nature of the NAD-independent profiles observed may be interpreted in one of two manners. Firstly, the overall similarity of the NAD-independent isolates is indicative of clonal origins, with the serovar C-3 isolates having arisen following transfer of NAD independency from a serotype A (Miflin *et al.*, 1995) to a serovar C-3 isolate (Bragg *et al.*, 1997). In a similar manner, electrophoretic analysis also served to establish the clonal origins of strains of *H. pleuropneumoniae* (Rapp *et al.*, 1986) and *E. coli* (Ochman and Selander, 1984). Secondly, however, the inability of OMP analysis to differentiate between two isolates, previously differentiated using a haemagglutination inhibition system, implies a limited application of OMP profiling as the sole means for type characterisation of *H. paragallinarum* field isolates.

The favourable application of protein profiling appears to be dependent on the bacterial species. Barenkamp *et al.* (1981a; b) reported the efficacious use of OMP analysis for subtyping of

H. influenzae type *b* strains as well as their nontypable variants (Murphy *et al.*, 1992). Furthermore, the phenotypically- and genetically-related strains of *H. aegyptius* and *H. influenzae* biotype III were differentiated on this basis (Carlone *et al.*, 1985). Limitations of phenotypic subclassification have been expressed by a number of researchers (Zhao *et al.*, 1992; Russel *et al.*, 1994; Nagai *et al.*, 1995; Olsen and Woese, 1993). However, provided a high level of reproducibility and sensitivity to strain variation is achieved (Barenkamp *et al.*, 1981a), and the OMPs maintain stability following repeated culturing (Rapp *et al.*, 1986; Saravani *et al.*, 1992), OMP profiling should be employed as a useful and informative adjunct to traditional subtyping systems. With reference to *H. paragallinarum* subclassification it is believed that, in conjunction with haemagglutination inhibition and molecular characterisation techniques, and following the suggested construction of a set of NAD-independent based reference strains, OMP profiling will find continued application.

Since the usefulness of outer membrane components as vaccine constituents depends on their general occurrence in the disease-causing isolates, biochemical and immunological characterisation of the OMPs of *H. paragallinarum* isolates of varying NAD dependency was required. In this regard, the results obtained and the conclusions drawn pertain to common OMPs only, and constitute preliminary studies of a novel nature with respect to *H. paragallinarum*, establishing a foundation for future investigations. OMPs were structurally characterised according to their susceptibility to heat-treatment (Rosenbusch, 1974; Nakamura and Mizushima, 1976) and chymotrypsin cleavage (Pandher and Murphy, 1996). Type 1- and 2-determining OMP C was found to be a peptidoglycan-associated porin protein (Rosenbusch, 1974), confirming reports by Blackall *et al.* (1990d). This protein commonly functions as a receptor for bacteriophages, bacteriocins or other lethal agents (Beher *et al.*, 1980). This observation implies a surface-location of this OMP, which was corroborated following chymotrypsin treatment of the intact bacterium. Porin proteins are reported to be little conserved during evolution, exhibiting minimal homology between and within a species (Lee *et al.*, 1979; Lee and Schnaitman, 1980). This accounts for the variability observed in the molecular size of OMP C. In addition, a common heat-modifiable OMP, approximately 36 kDa in size, was recognised which in the native state maintains a very strong association with the peptidoglycan. An overall similarity of this protein with an envelope protein of *E. coli*, confers a functional contribution towards maintenance of the

structural integrity of the bacterial cell (Rosenbusch, 1974). These techniques were limited in their application for the characterisation of serotype-specific OMPs, finding greater use in identifying common determinants as potential candidates for inclusion in a subunit vaccine.

Subsequently, efforts to ascertain the immunogenic epitopes of the varying NAD-dependent and -independent isolates was necessary. Regrettably, the nature of the polyclonal antibodies raised for use in haemagglutination inhibition assays, did not allow reliable interpretation of immunogenic OMPs, as a large degree of non-specific protein-protein interaction was evident. OMP C was strongly recognised by all five IgG preparations, possibly accounting for a degree of the cross-reactivity observed amongst strains of the same serotype (Kume *et al.*, 1980c; Blackall and Reid, 1987). Should OMP C play a pivotal role in bacterial survival within the upper respiratory tract of the host, the current investigation may have identified a potential target for purification and inclusion in the vaccines currently in use, against which a conditioned immune response may be stimulated. It is postulated that circulating antibodies directed against the common determinant could provide sufficient host protection against an infectious coryza infection. This theory requires investigation, however, immunity induced by a common OMP would negate the need for complex, time-consuming monitoring programmes.

Should too many flaws arise in the practical application of this theory, immunoblotting may be employed to complement serodiagnosis by haemagglutination inhibition and OMP profiling. In the present study, evidence towards this application was provided following the identification of apparent serotype C-specific OMPs, visualised upon reaction with antibodies directed against serotype C-2 strain Modesto. Clearly, immunoblotting is reliant on the availability of discriminating antibodies. A suggested modification to the current protocol involves treatment of the IgG preparation with whole bacterial cells, such that antibodies against specific surface-exposed epitopes are adsorbed and removed. Further recommendations include revision of the immunisation protocol to yield antibodies of a higher titre, generation of a battery of serovar-specific antibodies, and comparative analysis of antibodies from uninfected and infected chickens, as well as sera from birds which had recovered from infectious coryza. A panel of monoclonal antibodies may find further application for typing local isolates, in a continuation of work conducted on South African vaccine and field isolates (Verschoor *et al.*, 1989; Bragg *et al.*,

1993) and Australian isolates (Blackall *et al.*, 1990e). The successful employment of a panel of monoclonals is strongly dependent on their preliminary characterisation such that the patterns obtained are highly reproducible and reliable, in itself an extensive, time-consuming study. It is possible that anti-peptide antibodies may overcome this problem.

Due to the level of complexity in interpretative analysis of the phenotypic properties of *H. paragallinarum* isolates, serodiagnosis is rather achieved using a combination of techniques. *Neisseria meningitidis* strains are classified into serogroups by capsular polysaccharide typing, into serotypes on the basis of outer membrane protein profiles, and into sub-serotypes or immunotypes by lipooligosaccharide analysis (Gu *et al.*, 1992). A preliminary study into the lipopolysaccharides of isolates of *H. paragallinarum* was conducted in the present study. Comments on the resulting profiles shall be reserved, with a conservative conclusion, that the LPS appear to be smooth. The application of LPS profiling for subtyping of *N. meningitidis* strains suggests that these antigens, which enclose an endotoxin function (Towner and Cockayne, 1993), conferring a degree of virulence (Kume *et al.*, 1980b) to the infecting bacterium, should be further investigated.

More recently a universal typing method, based on the genotype of the microorganism, has been implemented as a further adjunct to a combination phenotypic classification scheme (Grimont and Grimont, 1991; Snipes *et al.*, 1992). In an extension of the work carried out by Miflin *et al.* (1995), an analysis of the ribopatterns generated following *Hin*dIII, *Ssp*I and *Sma*I digestion of the chromosomal DNA of seven NAD-independent isolates, revealed genetically indistinguishable profiles. This high degree of conservation at the ribosomal RNA level further implies a clonal origin of these strains, in agreement with reports by Miflin *et al.* (1995) and Bragg *et al.* (1997), and supportive of similar conclusions at a phenotypic level. In comparison, *Hpa*II digestion defined two different riboprofiles, with serotype A strain 1130 and all the local NAD-independent isolates producing identical profiles. In contrast, serovar C-3 strain 541 produced a unique ribopattern and consequently the local isolates were typed as A. This finding is contradictory to that at a phenotypic level, however, it is favoured due to the minimal interference from external factors on the chromosomally-located rRNA gene sequences. Furthermore, phenotypically

indistinguishable strains 1130 and 541, have been differentiated on the basis of genotypic attributes.

The qualitative differences between genotypic and phenotypic data has been given extensive consideration by Olsen and Woese (1993). Change at a molecular level was described as selectively neutral, contributing to the 'quasi-random' nature of genotypic change, often referred to as an 'evolutionary clock'. Furthermore, variances in the genome may produce similar phenotypes. This finding has been reinforced by Snipes *et al.* (1989), following an intensive investigation into the relationship between serotype and ribotype. Some portions of the genome encode the production of similar antigens, thereby accounting for isolates with the same serotype, yet different genotype. In the present study serovar C-3 strain 541 provides an example of a typical NAD-independent phenotype, but a unique genotypic arrangement. An explanation for the situation in which a similar ribotype is reported but the serotype is distinct may be explained by the fact that serotyping is based on a boiled extract containing a variety of antigens. These antigens may have been encoded by genes in DNA fragments that bound the probe or they may have been encoded by genes in nonhybridizing fragments. Furthermore, the base sequences in the fragments of a given size that hybridize with the probe are not necessarily identical; certain portions may have sufficient similarities to recognise and bind the probe, but may contain portions of significant size that are different. To overcome this obstacle, ribotype analysis was complemented by restriction digestion of PCR amplified 16S rDNA sequences. However, isolates with a varying OMP profile often showed a high degree of evolutionary conservation in their rRNA sequences. Although only a limited knowledge was gained using 16S ARDRA, the novel application of this technique lays the groundwork for further research.

The present investigation has demonstrated the complexity of type determination, be it the serotype or ribotype, and it therefore suggests that a combination approach should be applied. An amalgamation of the results obtained from a haemagglutination inhibition assay, OMP profiling, and analysis of the conserved 16S rRNA sequences, either through ribotyping or 16S ARDRA, is proposed. In this regard the current study provides a basis for the implementation of such a scheme. As such the scope for future research in this field is great.

A primary concern would be the aquisition and type characterisation of all nine of the previously characterised (Kume *et al.*, 1983a; Blackall *et al.*, 1990a) classical reference strains, to establish a a data bank of OMP profiles, ribotype patterns and 16S rRNA RFLPs. The establishment of unique type-specific riboprofiles and RFLPs is, on its own, an extensive and intensive study, requiring that a battery of restriction enzymes be tested for their potential in type determination. Should some strains exhibit such a high degree of homology in their 16S rRNA sequences, greater variance between strains may be detected by studying the sequence and length heterogeneity of the intergenic spacer regions of the *rrn* operon (Kostman *et al.*, 1995; Jordens and Leaves, 1997). Despite reports concerning the limited application of this technique for strain differentiation (Jordens and Leaves, 1997) success in this regard has been detailed (Kostman *et al.*, 1992; Cartwright *et al.*, 1995). The favourable use of this method for *H. paragallinarum* strain identification remains to be demonstrated. In addition, the current dissertation has indicated that a data base of 'classical' NAD-independent reference strains needs to be set up. This necessity was coerced by the uncomparable profiles generated by the NAD-dependent and -independent isolates such that type allocation by visual analogy was not always possible. Implementation of the establishment of a serovar-specific data base, requires an effectual system for obtaining a number of isolates statistically representative of the organism endemic to a certain area.

The virulence-associated antigens of *H. paragallinarum* comprises a relatively uncharted field. It is suggested that future research concentrates on characterising determinants responsible for ensuring bacterial survival in the hostile environment of the host; namely those antigens against which protective antibodies are generated, the haemagglutinins and the adhesins. It is possible that these three determinants are 'one and the same' i.e. responsible for establishing the bacterium in the host milieu, and maintaining an infection, thereby conferring virulence. Due to their nature it is possible that these antigens also function as the haemagglutinin in the corresponding assays. The development of bactericidal drugs that specifically inhibit attachment to the mucosal surfaces or the inclusion of purified adhesins in a subunit vaccine against which protective antibodies may be generated, provides an alternative approach to current treatment or preventative control schemes.

The chief aim of this study was to establish an efficient typing systems for inclusion of the currently dominating *H. paragallinarum* infectant in the local vaccine. The results obtained in the present study lay the foundation for establishing such a system and propose employing a combination of techniques to this end. Although evidence from the current investigation is not conclusive in this regard, Bragg *et al.* (1997) reported a similar finding. Should current vaccination programmes continue to omit a strain of serovar C-3, commercial poultry farmers may be faced with an epidemic initiated by strains of this serovar. Furthermore, a platform for future studies regarding the outer membrane-located virulence determinants has been established. The potentially devastating hold infectious coryza may exert on a poultry industry, reinforces an immediate practical application for improved vaccine design, strongly motivating for further research in this field.

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