The development of a molecular serotyping system and an investigation into the presence of prophages in *Avibacterium paragallinarum* serogroups

by

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"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

Albert Einstein

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Figure 4.11: Phage screening for C-repressor gene (CRG) of HP2-like phage with an expected band size of 385 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). Amplification was only observed for lanes 4, 5 and 8.

Figure 4.12: Phage screening for C-repressor gene (CRG) of HP2-like phage with an expected band size of 385 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). Amplification was only observed for lane 3.

Figure 4.13: Phage screening for rep gene (HDP) of HP2-like phage with an expected band size of 791 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). No amplification was observed for lanes 7, 8 and 9. Non specific binding was observed for lanes 4, 5, 6 and 10.

Figure 4.14: Phage screening for rep gene (HDP) of HP2-like phage with an expected band size of 791 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). No amplification was observed for lanes 1, 3 and 7.

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Figure 4.19: Phage screening for major tail subunit gene (MTS) of Mu-like phage with an expected band size of 299 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). Amplification was only observed for lanes 1, 2, 5, 6 and 8.

Figure 4.20: Phage screening for major tail subunit gene (MTS) of Mu-like phage with an expected band size of 299 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). No amplification was observed for any of the isolates.

Figure 4.21: Phage screening for Major head subunit gene (MHS) of Mu-like phage with an expected band size of 403 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). No amplification was observed for lane 4. Non specific binding was observed for lane 7.

Figure 4.22: Phage screening for major head subunit gene (MHS) of Mu-like phage with an expected band size of 403 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (NT); Lane 6: 484 (NT); Lane 7: 155085 (C-3). Amplification was observed for lanes 1, 4 and 6.

Figure 4.23: Phage screening for tail fiber gene (MTF) of Mu-like phage with an expected band size of 1 403 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). No amplification was observed for lanes 4 and 7. Non specific binding was observed for lane 8 and 10.

Figure 4.24: Phage screening for tail fiber gene (MTF) of Mu-like phage with an expected band size of 1 403 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). Amplification was observed for all of the isolates. The DNA concentration was too low to sequence the non specific amplification.

Figure 4.25: Phage screening for transposase gene (TRP) of Mu-like phage with an expected band size of 989 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). Amplification was observed for lane 8. Non specific binding was observed for lane 8.

Figure 4.26: Phage screening for transposase gene (TRP) of Mu-like phage with an expected band size of 989 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). No amplification was observed for any of the isolates.

Figure 4.27: A neighbour-joining tree depicting the phylogenetic relationship of the HTT gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the HP2-like phage genes posted on GenBank (Accession number: JN627908.1).

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LIST OF ABBREVIATIONS

bp	(nucleotide) base pair
BTA	Blood Tryptose Agar
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ERIC PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction
HA	Haemagglutinin
HI	Haemagglutination inhibition
IC	Infectious Coryza
Mabs	Monoclonal antibodies
NAD	Nicotinamide adenine dinucleotide
NCBI	National Centre for Bioinformatics
NJ	Neighbour-joining
NT	Non-typable *Avibacterium paragallinarum* field isolates
PCR	Polymerase Chain Reaction
Φ	Phage
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RT	Room Temperature

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Avibacterium paragallinarum is the causative agent of Infectious Coryza (IC), which is an upper respiratory tract disease that occurs primarily in chickens (Blackall *et al*., 1990). This organism was previously named *Haemophilus paragallinarum*, and was reclassified as *Avibacterium paragallinarum* by Blackall and co-workers (2005), based on results obtained through16S rDNA sequencing. This disease causes a 10%-40 % decrease in egg production, which leaves a significant economic impact on the poultry industry (Blackall *et al*., 1990). The presence of IC has been observed in various other countries than South Africa which includes Australia, Canada, Egypt, Great Britain, Holland, India, Argentina, USA and Mexico just to name a few (Vergas & Terzolo, 2004).

NAD^+-dependent *A. paragallinarum* consists of three serologically distinct groups namely serogroups A, B and C. The serogroups are further divided into 9 different serovars namely A1-A4; B1 and C1-C4 (Blackall *et al.,* 1990). NAD^+-independent strains of *A. paragallinarum* have also been identified for all of these serogroups (Bragg *et al*., 1993; Miflin *et al*., 1999; Garcia *et al*., 2004). The test used to establish the serogroups and serovars is the haemagglutination (HA) and haemagglutination-inhibition (HI) tests. There are currently molecular techniques available for the successful and rapid diagnosis of IC. One of these techniques is a species-specific PCR termed HPG2-PCR (Chen *et al.,* 1996).

Based on work done by Roodt and co-workers (2012), prophage sequences were detected in the genome of *A. paragallinarum* serovars C-2 for the first time. It would be interesting to determine whether there are any prophages present in the other *A. paragallinarum* serovars, as this can be a possible explanation for the occurance of different serovars. This can also serve as an explanation for differences in virulence between serovars.

Accurate serotyping is very important in the case of IC, as incorrect serotyping of *A.paragallinarum* isolates can result in vaccine failures (Soriano *et al*., 2004a). Therefore, accurate detection plays an important role in the control of this disease. Various approaches are discussed in this review, which includes molecular serotyping of *A. paragallinarum*, as well as the role that prophages play in other bacterial species and what this might mean for *A. paragallinarum.*

1.2. Infectious coryza

Infectious coryza (IC) is an upper respiratory tract disease that occurs primarily in chickens (Yamamoto, 1984). It is generally an acute, but can be a chronic, disease (Yamamoto, 1984). IC results in about 20%-50% morbidity and 5%-20% mortality in infected chickens (Chen *et al.,* 1993). This disease is of economic importance especially in the poultry industry as it causes a decrease in egg production and an increase in unthrifty chickens (Chen *et al*., 1996).

Clinical symptoms associated with this disease include nasal discharge, facial swelling, lacrimation, anorexia and diarrhea (Blackall, 1999). This results in growth retardation, weight loss and an increased number of culls (Yamamoto, 1984). Some of the listed symptoms are displayed in the photograph illustrated by Figure 1.1.

IC is transmitted through drinking water, it is airborne over short distances and replacement stocks are a major source of infection (Blackall *et al*., 1990). The disease characteristically has a short incubation period of 24-48 hrs (Yamamoto, 1984). Clinical signs in susceptible birds that were exposed to infected birds usually show signs of the disease in 1-3 days. Birds of all ages are susceptible, with less severity in juvenile birds (Yamamoto, 1984). In mature birds, especially laying hens, the incubation period is shortened and the duration of the disease is longer (Yamamoto, 1984).

Figure 1.1: Photographic illustration of a chicken showing clinical symptoms associated with Infectious Coryza.

1.3. *Avibacterium paragallinarum*

1.3.1. Classification

The first haemophilic organisms that caused upper respiratory tract disease in chickens were isolated by De Blieck (1932), and were termed *Bacillus haemoglobnophilus coryza gallinarum* (Yamamoto, 1984). This organism was described as gram negative, non-motile short rods as well as coccobacilli, by Beach & Schalm (1936), as depicted in the microscopic preparations in figure 1.2. It is a slow growing, fastidious organism that shows the tendency to form filaments (Blackall, 1999). The causative agent of IC was later renamed *Haemophilus gallinarum* (Elliot & Lewis, 1934). Later it was renamed to *Haemophilus paragallinarum* due to being X-factor independent and V-factor dependent (Page 1962; Blackall & Yamamoto, 1989). In 2005 the name was changed again and termed *Avibacterium paragallinarum* (Blackall *et al*., 2005).

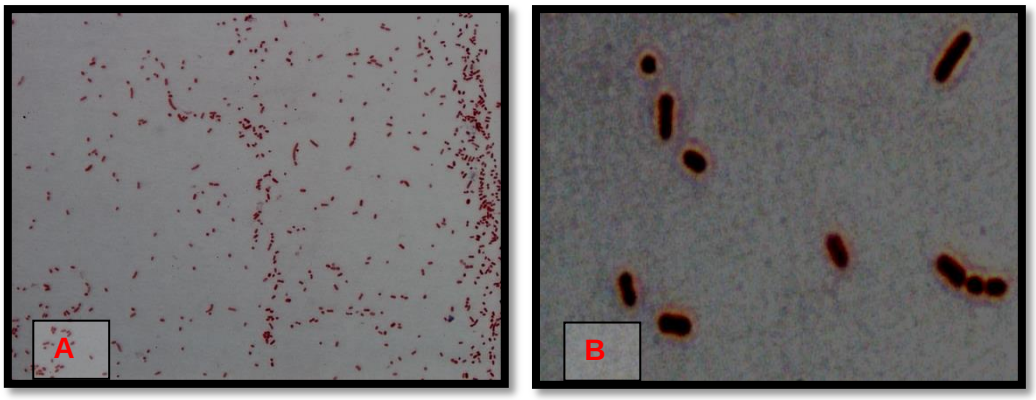

Figure 1.2: Microscope pictures illustrating *Avibacterium paragallinarum*. A: a 40 x magnification showing gram negative cocci and B: a 100 x magnification showing coccobacilli as well as short rods.

During the first characterization of the disease done by McGaughey (1932) it was reported that the isolates required V-factor (NAD) but not X-factor (hemin) for growth *in vitro*. Beach & Schalm (1936) and Delaphane and co-workers (1938) reported that the isolates required both V-factor and X-factor for growth *in vitro*.

Work done by McGaughey (1932) was largely overlooked and *H. gallinarum* was accepted as the causative agent of IC, which requires both V-factor (NAD^+) and X-factor (hemin) for growth. In the 1960's several studies reported isolates of the causative agent of IC which required only V-factor and not X-factor for growth *in vitro* (Page, 1962). This new species that caused IC was termed *H. paragallinarum* and is X-factor independent and V-factor dependent (Blackall & Yamamoto, 1989).

This bacterium belongs to the family *Pasteurellaceae* (Bisgaard, 1993). Blackall and co-workers (2005) conducted phylogenetic experiments on the 16S rDNA of the *Pasteurellaceae* family. The results showed that *H. paragallinarum, Pasteurella gallinarum, Pasteurella. avium* and *Pasteurella. volantium* formed a monophyletic group with 96.8% sequence similarity as seen in figure 1.3. Based on these findings *H. paragallinarum* was reclassified into a new genus *Avibacterium* (Blackall *et al*., 2005). Phenotypic and genotypic testing supported the reclassification of the separate and distinct nature of this subcluster into the new genus *Avibacterium* (Blackall *et al*., 2005).

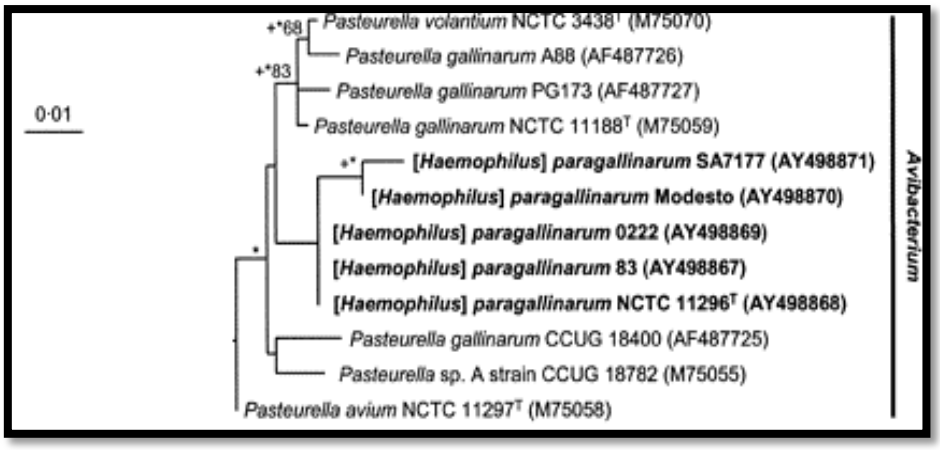

Figure 1.3: The phylogenetic relationships based on maximum likelihood analysis of 16S rRNA gene sequences of the members of the *Avibacterium* gen. nov. and members of the representative genera of the family *Pasteurellaceae*. Bootstrap analysis indicated by values higher than 50% and nodes supported in phylogenetic trees obtained by neighbor-joining and parsimony methods are indicated by + and *. Bar, 0.01 evolutionary distance (Blackall *et al.,* 2005).

1.3.2. Cultivation and growth conditions

Avibacterium paragallinarum is mainly isolated from within the sinus cavity of infected chickens (Yamamoto, 1984). An incision is made into the sinus cavity and isolation is by inserting a sterile swab deep into the sinus cavity of an infected chicken (Yamamoto, 1984). The swab is then streaked onto or inoculated into the required media. The reduced form of NAD, NADH, or the oxidized form NAD^+ must be included into the growth medium of NAD^+-dependent strains. A number of bacterial species can excrete NAD^+ and these strains can be used as feeder cultures to support the growth of *A. paragallinarum* (Page, 1962). *Staphylococcus* spp. are commonly used as feeder culture as they have the required ability to exrete NAD^+ (see figure 1.4).

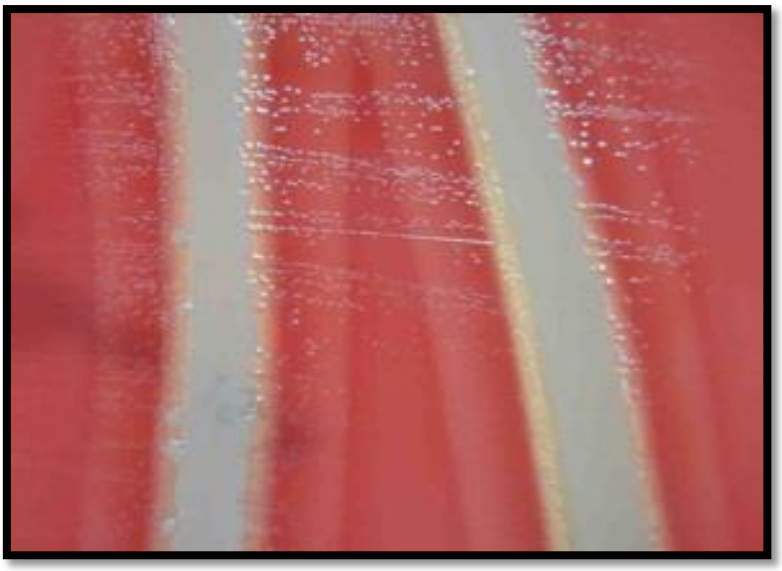

Figure 1.4: A photographic presentation of a Blood Tryptose Agar (BTA) plate cross streaked with *Staphylococcus aureus* feeder culture. The mildew drops are the typical characteristic colony morphology *A. paragallinarum*.

Sodium chloride at 1.0% to 1.5% is an essential growth requirement (Blackall, 1989). The required growth media for *Avibacterium* is blood tryptose agar (BTA) plates containing, horse, bovine, sheep, avian or rabbit blood (Yamamoto, 1984). This organism is cross-streaked with a *Staphylococcus* spp., preferably *Staphylococcus epidermidis*, which serves as a feeder culture (Page, 1962), as illustrated in figure 1.4. Haemolysed blood agar has the advantage of storage by means of freezing for a long time (Vargas & Terzolo, 2004). The organism can be maintained on blood agar plates with passages every second day. This organism is microaerophilic, and optimal growth is obtained under enhanced CO_2 concentrations of up to 5%. The optimal growth temperature of this organism is 37°C -38°C (Yamamoto, 1984). These conditions usually are obtained through incubation in a candle jar at 37°C for 18 hrs (Yamamoto, 1984). The colonies are typically tiny (0.3 mm in diameter) and dewdrop shaped when grown on suitable media (Blackall, 1989).

In addition to BTA plates, a liquid medium can also be used to culture the organism. The broth include modified Casman's medium, supplemented with chicken serum (Eaves *et al*., 1989), or a broth version of TM/SN medium. The TM/SN medium consists of oleic albumin complex, chicken serum and NADH (Eaves *et al*., 1989). Liquid medium supplemented with serum does not require increased levels of CO_2 (Eaves *et al*., 1989). The optimal pH for growth ranges from 6.9-7.6 (Yamamoto, 1984).

The organism is commonly grown in an atmosphere of 10% carbon dioxide; however, it is not an essential requirement. The organism is able to grow under reduced oxygen tension or anaerobically (Page, 1962).

1.4. Biochemical Properties

All avian haemophilli produce nitrite via nitrate reduction and glucose fermentation pathways without any gas formation (Blackall, 1989). The following biochemical properties are characteristic of avian haemophili: oxidase activity**,** presence of enzyme alkaline phosphatase, negative for catalase activity, failure to produce indole or H_2S as well as the failure to hydrolyse urea or gelatin (Blackall, 1989).

Carbohydrate fermentation is possible in a general medium that contains phenol red broth enriched with 1% NaCl, 25 µg NADH, 1% chicken serum, and 1% carbohydrates (Blackall, 1989). The organism has the ability to ferment fructose, glucose and mannose but not trehalose or galactose (Yamamoto, 1984).

1.5. Identification by means of molecular techniques

The use of conventional methods for diagnoses of IC in infected chickens involves the isolation and identification by means of biochemical tests (Chen *et al*., 1996). These methods are, however, very demanding and NAD^+ is required as a growth factor. Economic loses that occur due to this disease can be reduced by means of early, rapid and accurate diagnosis (Chen *et al*., 1998a). Additional problems are associated with the use of conventional methods (Chen *et al*., 1996). Firstly, it is difficult to grow this bacterium as pure cultures *in vitro*. The media are also difficult and expensive to make (Chen *et al.,* 1996). Secondly, *A. paragallinarum* is a fastidious, slow growing organism and is easily over grown by other organisms (Chen *et al.,* 1996). Lastly, various haemophilic organisms are present in chickens, e.g. *P. avium* which forms part of the normal microbiota of chickens (Mutters *et al*., 1985).

Chen and co-workers (1996) developed a species specific PCR test (HPG-1-PCR and HPG-2-PCR) that is specific for *A. paragallinarum.* They constructed a genomic library from genomic DNA extracted from the Modesto strain. By the use of southern blots four probes

were identified that reacted specifically to 56 *A. paragallinarum* isolates that were used during the study. In this study, 24 bacterial isolates from closely related genera such as *Pasteurella* and *Actinobacillus* as well as field isolates *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, were included.

In the combinations of F1/R1 (HPG-1) and N1/R1 (HPG-2), as listed in Table 1.1, Chen & co-workers (1996) obtained fragments of about 1.6kb for HPG-1 and 0.5kb for the HPG-2-PCR. The HPG2-PCR can be performed directly from swabs or from culture plates. This is then used to confirm the isolation of this haemophilic organism (Chen *et al*., 1996, 1998a). This technique has probes and primers designed that is specific for *A. paragallinarum* (Chen *et al*., 1996; 1998b).

Table 1.1: Primers designed by Chen and co-workers (1996).

Primer	Sequence
F1	5'-CAA TGT CGAT CCT GGT ACA ATG AG-3'
N1	5'-TGA GGG TAG TCT TGC ACG CGA AT-3'
R1	N1; R1 5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'

Both these PCR's are specific and sensitive and give positive results with NAD^+-dependent and NAD^+-independent isolates (Corney *et al*., 2008), as well as being able to accurately distinguish between *A. paragallinarum* isolates and *Ornithobacterium. rhinotracheale* (Miflin *et al*., 1999).

1.6. Serological classification

1.6.1. Haemagglutination (HA) and Haemagglutination inhibition (HI)

There are two serological classification systems that can be applied to *A. paragallinarum*, the Page and the Kume classification systems (Page 1962; Kume *et al*., 1983). The agglutination test of Page (1962) recognized three serovars namely; A, B and C. A drawback of the Page scheme is that some isolates could not be typed due to non-agglutination (Blackall *et al.,* 1990).

The Haemagglutination (HA) and Haemagglutination-inhibition (HI) test, detecting haemagglutinins, was first described by Kume and co-workers (1983). The method used involved treating bacterial cells with potassium thiocyanate (KSCN), followed by sonication. The result was the detection of an additional antigen, together with haemagglutinins that was able to agglutinate fresh and gluteraldehyde-fixed chicken erythrocytes.

Kume and co-workers (1983) based a scheme on haemagglutination where three serogroups and seven serovars were recognized. The serogroups were grouped I, II and III and the serovars were grouped HA-1 to HA-7. Serovars HA-1 to HA-3 belonged to serogroup I, serovars HA-4 to HA-6 to serogroup II and serovar HA-7 to serogroup III (Kume *et al*., 1983). Eaves and co-workers (1989) discovered an additional serovar, HA-8, and assigned it to serogroup I. An additional serovar was found by Blackall and co-workers (1990), belonging to serogroup II, which was termed HA-9. The identification of the two additional serovars by Eaves and co-workers (1989) and Blackall and co-workers (1990) showed the likelihood for the discovery of new serovars. This prompted Blackall and co-workers (1990) to alter the nomenclature of the Kume scheme. The Kume serogroups I, II, III corresponded to Page serovars A, C and B, which led to the proposal that the Kume scheme be changed to the nine currently recognized serovars (Blackall *et al*.,1990), as listed in Table 1.2.

Table 1.2: Comparison of original and newly proposed nomenclature of the Kume serotyping scheme for *A. paragallinarum* (Adapted from Blackall *et al*., 1990).

Reference isolates	Original scheme (Kume)		New scheme (Blackall)	
	Serogroup	**Serovar**	**Serogroup**	**Serovar**
0083/221	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
H18	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
0222	III	HA-7	B	B-1

The most accepted method for the serological characterization of *A. paragallinarum* is the original scheme by Kume and co-workers (1983) and the modified new scheme by Blackall and co-workers (1990). However, HA/ HI is a time consuming technique and it is difficult to serotype accurately to the serovar level, making a molecular technique the preferred alternative.

1.6.2. Molecular Serotyping techniques

A limited number of molecular serotyping techniques exist (Soriano *et al*., 2004b; Sakamoto *et al*., 2012). One such technique is the Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) (Soriano *et al*., 2004b). Long sequence primers of about 22 base pairs in length are used for sufficient hybridization to the chromosomal DNA sequences at low annealing temperatures (Soriano *et al*., 2004b). ERIC-PCR sequences are highly conserved but their chromosomal locations differ between species and strains. This technique has been successfully used for molecular typing of *Haemophilus somnus, Haemophilus influenza and Haemophilus parasuis (*Soriano *et al*., 2004b). It is a simple and rapid technique that can be performed with small quantities of bacterial cultures (Soriano *et al*., 2004b). The application of ERIC-PCR to subtype *A. paragallinarum* resulted in consistent results being obtained for reference isolates (Bragg 2010-personal communications). However, when the technique was used on South African field isolates inconsistent results

was obtained, as the banding patterns observed for the field isolates did not correlate to the banding patterns of the reference isolates (Bragg, 2010).

During a recent study conducted by Wu and co-workers (2011) a hypervariable region was found in the haemagglutinin protein of serogroups A and C. Based on this hypervariable region a more recent technique developed by Sakamoto and co-workers (2012) was reported in literature, a multiplex PCR and RFLP analysis. The multiplex PCR is based on the amplification of a hypervariable region within the haemagglutinin gene of *A. paragallinarum* A and C-serovars, thus improving on work done by Wu and co-workers (2011). This region encodes an outer-membrane protein, HMTp210, which serves as a major protective antigen of *A. paragallinarum* (Sakamoto *et al*., 2012). The *HMTp210* gene can be divided into three regions based on DNA sequence homology. Regions 1 and 3 are highly conserved between serovars A and C, as reported by Wu and co-workers (2011). The homology of region 2 between serovars A and C is about 50%. Therefore, Sakamoto and co-workers (2012) developed a multiplex and RFLP PCR based on region 2 of the *HMTp210* gene as it seems to be a serovar-specific region. The primer sets that were used in this PCR were designed based on region 2 of the HMTp210 protein. Multiplex PCR resulted in amplified regions of 800 bp (serovar A), 1100 bp (serovar B) and 1600 bp (serovar C). The RFLP PCR makes use of a different set of primers, resulting in a 1600 bp region being amplified. The 1600 bp product was then digested with restriction enzyme *Bgl*II, which resulted in dissimilar banding patterns that allowed separation of serogroups (Sakamoto *et al*; 2012). One drawback is that both the multiplex PCR and RFLP have only given successful results on reference isolates and not on field isolates

This prompted the need for the investigation into a more reliable molecular serotyping technique to serotype reference and field isolates accurately. A molecular technique is less time consuming, which is of benefit to the poultry industry.

1.7. Temperate bacteriophages

1.7.1. Background into the discovery and life cycles of bacteriophages

Bacteriophages were independently discovered in 1915 by Frederick W Twort in England and by Felix d'Herelle at the Pasteur Institute in Paris in 1917 (Duckworth, 1976), there has however been some controversy on who actually discovered bacteriophages first. Bacteriophages are viruses that infect bacteria and the name is derived from the word bacteria and the Greek word phagein which means "to eat" or "to devour" (Duckworth, 1976).

Bacteriophages can replicate either through a lytic or lysogenic lifecycle (Snyder & Champness., 2003), as illustrated in Figure 1.5 (http://bcs.whfreeman.com/thelifewire8e/bcs-pages). Virulent phages always replicate by means of the lytic cycle, this leads to lysis of the host cell and the release of phage progeny (Engelkirk & Burton., 2006). Temperate phages have two life cycles. They can replicate by means of lysing the host cell where their progeny is released into the environment. They can also replicate through the lysogenic life cycle where they establish a stable relationship with the host and their genome becomes integrated into the bacterial chromosome, and it is replicated along with the host DNA (Little, 2005).

During the lysogenic life cycle, the viral genetic material is incorporated into the bacterial chromosome, which is referred to as the prophage, therefore the focus will be on the lysogenic life cycle. The virus is stably maintained within the host genome and replicates with the host DNA. Bacteria harbouring prophages are known as lysogens (Stansfield *et al*., 1996). The expression of lytic genes in temperate phages is prevented by the repression of these genes. This allows the viral genome to be replicated with the host DNA until the virus is under stress and the switch to the lytic stage is possible. The mechanism by which this is achieved varies for different viruses (Little, 2005).

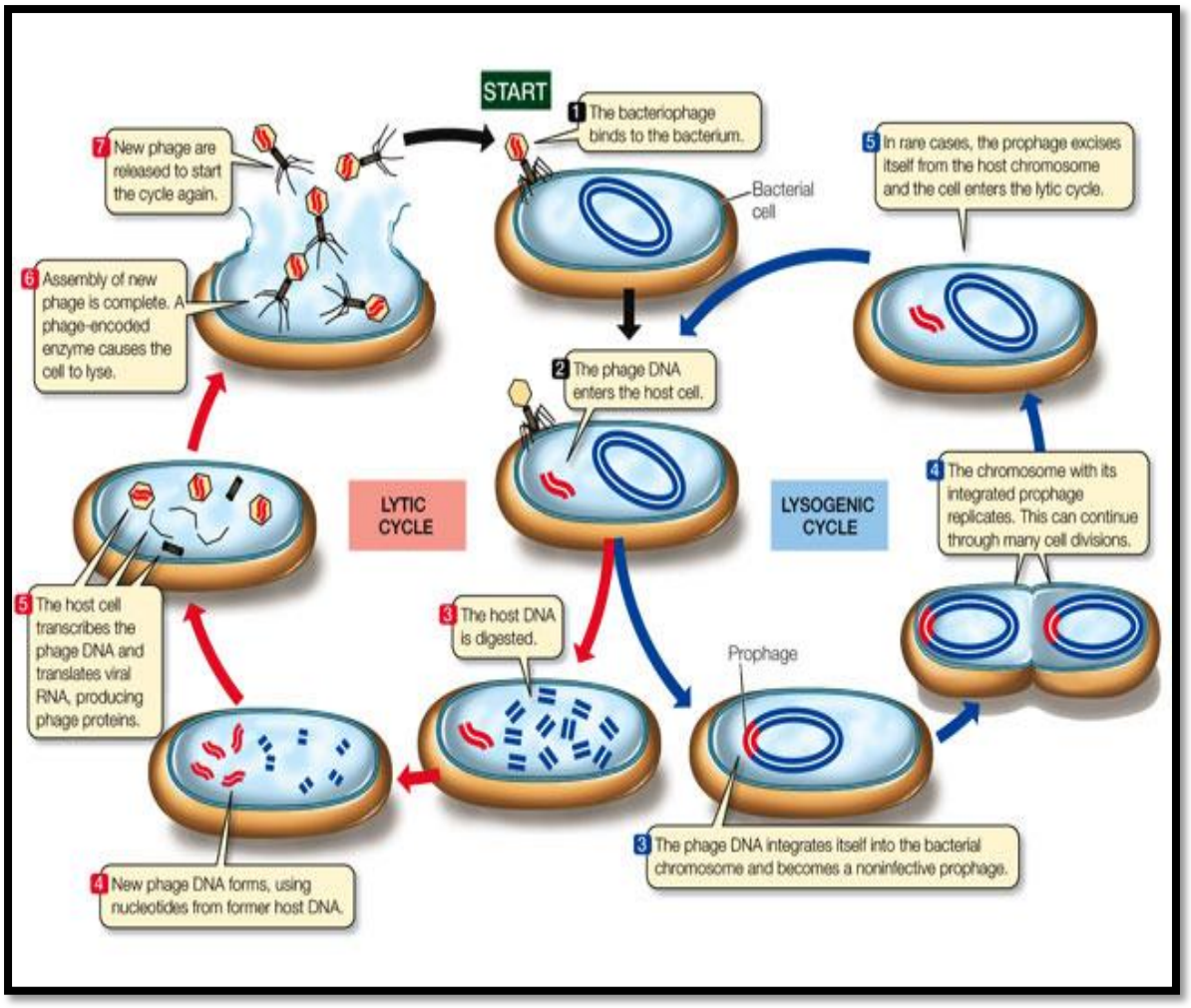

Figure 1.5: Illustration displaying the lytic and lysogenic stages of a typical bacteriophage (http://bcs.whfreeman.com/thelifewire8e/bcs-pages/). The lysogenic life cycle is illustrated by the blue arrows and the lytic cycle is illustrated by the red arrows. Both life cycles starts out the same illustrated by point 1 and 2.

1.7.2. The effect of prophages on the bacterial host

It has long since been established that the presence of prophages has an effect on the virulence and pathogenicity of bacterial species (Wagner & Waldor, 2002). Prophage genes integrated into the host genome can code for different virulence factors like toxins, regulatory factors and enzymes, which all have the ability to alter host bacterial virulence (Wagner & Waldor, 2002). The presence of prophages can play a crucial role in microbial diversity as well as the evolution of bacterial genomes. This is achieved through rearrangement within the bacterial genome which results in interstrain differences within the same bacterial genome (Roodt *et al*.,2012).

One such example is in the case of avirulent strains of *Corynebacterium diphteriae* that were infected with a bacteriophage that yielded virulent lysogens that produced the diphtheria toxin which causes diphtheria in humans (Tinsley *et a*l., 2006). Another example of this is the production of the scarlatina exotoxin by a temperate bacteriophage within the genome of non-toxigenic streptococci (Wagner & Waldor, 2002; Tinsley *et al*., 2006). The presence of prophage gene in the host genome can also cause strains of the same species to be associated with different diseases as is the case with two *Streptococcus pyogenes* strains that belong to different M serotypes, where the differences on a DNA level is as result of prophage sequences (Brüssow *et al*., 2004). Therefore, the presence of prophages can result in the adaptation of existing pathogens to new hosts or even the emergence of new pathogens (Brüssow *et al*., 2004).

Even though prophages can constitute to as much as 20% of a bacterium's genome these prophages can be cryptic, in state of mutational decay or evolutionary remnants (Roodt *et al*., 2012). This means that these phages are not inducible and do not offer any advantage to the host bacterium.

1.7.3. Serotype converting phages

The serotyping of *Shigella flexneri* is based on the structure of the O-antigen lipopolysaccharide. There are 15 known serotypes of *S. flexneri*: 1a, 1b, 1c, 2a, 2b, 3a, 3b, 4a, 4b, 5a, 5b, 6, X, Xv and Y (Sun *et al*., 2011). All of the above mentioned serotypes, except for serotype 6, share a common tetrasaccharide backbone of repeating units of N-acetylglucosamine- rhamnose-rhamnose-rhamnose (Sun *et al*., 2011). Due to the addition of a glucosyl and/or O-acetyl groups to one or more of the sugars on the tetrasaccharide unit, various serotypes are formed. Serotype Y possesses the primary basic O-antigen without any modification of the tetrasaccharide backbone (Sun *et al*., 2011).

Serotype conversion of *S. flexneri* is mediated by temperate bacteriophages, where six different serotype-converting phages, SfI; SfII; Sf6; SfIV; SfV and SfX, have been identified and characterized (Allison *et al*, 2002). The phages can convert serotype Y to serotype 1a, 2a, 3b, 4a, 5a and X respectively (Allison *et al*., 2002). All the phages carry three genes, *gtr*A, *gtr*B, and *gtr* $_{type}$ for O-antigen modification except for Sf6 that only carries a single gene *oac* for the acetylation of the O-antigen (Verma *et al*., 1991). The *gtr*A and *gtr*B genes are highly conserved and interchangeable in function. The gtr_{type} gene encodes a

glucosyltransferase that adds glucosyl molecules to sugar residue(s) on the basic O-antigen repeating unit (Sun *et al*., 2011). These phages integrate into the *S. flexneri* host chromosome. The integrase and O-antigen modification genes are located at the opposite ends of the prophage genome, flanked by an attL sequence on the left and an attR sequence on the right, once it is integrated (Allison & Verma, 2000).

Untypeable or novel serotypes of *S. flexneri* had been recently reported worldwide (Sun *et al*., 2011). In the late 1980s a novel serotype 1c was identified in Bangladesh, this was a predominant serotype in Vietnam and other Asian countries (Sun *et al*., 2011). This serotype occurred due to the modification of serotype 1a, where a glucosyl group was added by a cryptic prophage that carried a *gtr*1C gene cluster (Stagg *et al*., 2009). Such conversions may be due to the susceptibility of a strain to infection by a given serotype-converting bacteriophage. Therefore these findings could suggest the emergence of new *S. flexneri* serotypes in nature (Sun *et al*., 2011). As the emergence of new *S. flexneri* serotypes (Sun *et al*., 2011) has been established, the occurance and/or emergence of different serovars in *A. paragallinarum* could be due to the presence of serotyping converting phages as well.

1.7.4. Prophages present in the *Pasteurellaceae* family

Prophage sequences have been found in members of the *Pasteurellaceae* family. Therefore, it is likely that there might be prophages present in the different *A. paragallinarum* serotypes as well. The family *Pasteurellaceae* includes the *Haemophilus, Actinobacillus, Pasteurella, and Mannheimia* genera of bacteria, which cause a variety of diseases in humans and animals (Highlander *et al*., 2006). At least two prophages have been found in *Mannheimia haemolytica.* Both these prophages encode several Mu ortohologs (Gioia *et al*., 2006). Studies conducted by Froshauer and co-workers (1996), indicate that the antibiotic danofloxacin could induce a prophage in a serotype A1 isolate of *M. haemolytica*.

There are numerous other reports of bacteriophages present in the *Pasteurellaceae* family (Williams *et al*., 2002; Resch *et al*., 2004; Morgan *et al*., 2002; Pontarollo *et al*., 1997, Roodt *et al*., 2012). These prophages include the following; the genomes of two phages, HP1 and HP2, isolated from *H. influenzae* have been sequenced and both are members of the P2 family of temperate bacteriophages (Williams *et al*., 2002). The complete genome of a

lambdoid temperate bacteriophage was reported to be found in *Actinobacillus actinomycetemcomitans* (Resch *et al*., 2004). A Mu-like prophage was identified within the genome of the *H. influenzae* Rd strain (Morgan *et al*., 2002) and HP1-like sequences were also reported in *H. somnus* (Pontarollo *et al*., 1997).

According to work done by Roodt and co-workers (2012) two complete prophages have been assembled from the genome of *A. paragallinarum* Modesto (C-2) serovar. One of these prophages resembles a Mu-phage (ΦAvpmuC-2M) and the other a HP2 phage (ΦAvpC-2M-HP2) that are present in *H. influenzae*. The reports on the presence of prophages within the genome of various organisms and the finding of the complete prophage within *A. paragallinarum*, prompted the need to investigate.

1.8. Introduction into present study

During earlier years the major focus has been on the isolation and identification of IC in chickens. Since then a number of new molecular techniques for isolation, identification and serotyping of *A. paragallinarum* have been developed (Chen *et al*., 1996; Soriano *et al*., 2004b; Sakamoto *et al*., 2012; Mendoza-Espinoza *et al*., 2008). There is still room for improvement for most of these techniques, especially on serotyping of *A. paragallinarum*. In South Africa the C-3 serovars are the most pathogenic and poses the biggest threat to the poultry industry (Bragg *et al*., 1996). Vaccine failures make it difficult to effectively control this disease due to misdiagnosis of the correct strain of *A. paragallinarum* (Bragg *et al*., 1996*).* There is currently little to no cross protection between the different C serovars (Soriano *et al*., 2004a). Therefore the need arises for a reliable and rapid molecular serotyping technique. Better understanding of the virulence and pathogenicity of this bacterium will ultimately lead to better control of this disease.

The findings of prophages in *A. paragallinarum* might contribute to the understanding or explanation of the occurrence of different serotypes in this bacterium. This might also shed light on the existence of NAD^+-independent *A. paragallinarum* strains. Both bacteriophages and plasmids have the capability to enhance the pathogenicity of microorganisms (Prescott *et al*., 2002). Thus, the appropriate mechanisms of the emergence of new and more virulent serovars might be provided by presence of these elements (Prescott *et al*., 2002). Prophages could also be considered as a potential treatment option like vaccines as

recombinant prophage proteins may elicit bactericidal immune responses (Masignani *et al*., 2001).

1.9. Aims of this study

The first objective of this study was to develop a molecular serotyping technique for *A. paragallinarum* reference and field isolates. It is important to test the system on field isolates, as previous techniques developed showed good results for reference isolates but not for field isolates.

The second objective of this study was to screen for the presence of prophage sequences within the genome of *A. paragallinarum* reference isolates. There are currently no reports on any prophages present in all *A. paragallinarum* reference isolates and field isolates. The presence of prophages could account for the occurrence of different serovars and may contribute to the virulence of the different strains.

CHAPTER 2

IDENTIFICATION OF *Avibacterium paragallinarum* REFERENCE AND FIELD ISOLATES

2.1. Introduction

Even though cultivation of *Avibacterium paragallinarum* has long since been established it is still a difficult task, as *A. paragallinarum* needs complex media and growth conditions (Page, 1962; Rimler, 1979; Yamamoto, 1984; Blackall & Yamamoto, 1989; Eaves *et al*, 1989). This bacterium also needs a feeder culture to supply it with NAD^+ to grow, as it is unable to synthesize or recycle NAD^+ on its own (Page, 1962). Due to this, *A. paragallinarum* can easily be overgrown by the feeder culture which may result in contamination. Another disadvantage is that *A. paragallinarum* dies quite rapidly outside its host (Page, 1962) which can make sample collection from romote locations difficult.

To identify the isolates used for this study, a species specific PCR developed by Chen and co-workers (1996) was performed. The 16S rDNA gene is a highly conserved gene (Mendoza-Espinoza *et al*., 2008) and therefore the 16S rDNA PCR was done addionally to ensure the isolates were in fact *A. paragallinarum*. Due to the fact that *A. paragallinarum* can easily become contaminated with the feeder culture accurate identification is very important.

A. paragallinarum is inactivated quite rapidly outside of its host and a reliable storage method is important. Storage by means of lyophilisation (freeze-drying) can be used for *A. paragallinarum* (Christensen *et al*., 2007) where chicken embryos of about 6 days are inoculated with single colonies or broth cultures via the yolk sac of the chicken egg. The yolk from the embryos will contain a large number of organisms which will be frozen at -80ºC or lyophilized. Serial embryo passages should be made at monthly intervals to maintain viable culture as the titers in frozen yolk material may drop about 100 fold (Yamamoto,

1984). This however serves only as a short term storage method. As a long term storage method all of the *A. paragallinarum* reference isolates were stored by means of Lyopilization (freeze-drying).

The aims of this chapter was the cultivation and identification of all the *A. paragallinarum* reference and field isolates used during this study, as well as successful long term storage of all the reference isolates for future work to be conducted.

2.2. Materials and Methods

2.2.1. Enzymes, chemicals, kits and other consumables

The reagents and chemicals used were of molecular biological or analytical grade and were obtained from the following companies:

Amersham Biosciences: GFX™ PCR DNA and Gel Band Purification Kit; Applied Biosystems: BigDye terminator v3.1 Kit; 2720 Thermal Cycler; **Fermentas;** O'GeneRulerTM Express DNA Ladder, O'GeneRulerTM, Orange loading dye, Deoxynucleoside triphosphates (dNTPs); **Inqaba:** All synthesis of primers; **Merck:** Glucose, Myo-inositol, NAD, Nutrient broth, Oleic acid, Peptone, Sodium Chloride and Starch; **New England Biolabs®:** Taq DNA Polymerase with ThermoPol Buffer; **Onderstepoort Biological Products:** Blood Tryptose Agar plates; **Roche:** Bovine Serum Albumin Fraction V; **Sigma Aldrich:** Chicken Serum, Ethidium bromide, Horse Serum and Thiamine Hydrochloride; **Whitehead Scientific (PTY) LTD:** Agarose D1 LE, QIAamp® DNA mini kit.

2.2.2. *Avibacterium paragallinarum* isolates

All of the *A. paragallinarum* strains used for this study are listed in Table 2.1. A full set of the Kume serovar, reference isolates were obtained from Dr. P.J. Blackall at the University of Queensland, Australia. Field isolates from Israel were also used during this study, as well as vaccine strains from India. The South African field isolates used during this study were obtained by Prof. R. R. Bragg, University of the Free State, Bloemfontein.

Table 2.1: List of reference and field isolates used during this study.

Reference Isolate	Kume serovar	Country of origin	Field Isolate	Country of origin
ATCC 29545	A		IC 418	Israel
221	A-1	Japan	IC 462	Israel
0083	A-1	USA	IC 484	Israel
2403	A-2	Germany	Vaccine strain 221	India
E-3C	A-3	Brazil	Vaccine strain Spross	India
HP-14	A-4	Australia	Vaccine strain Modesto	India
0222	B-1	USA	SA-70	South Africa
2671	B-1	Germany	SA-72	South Africa
HP8	C-1	Japan	SA-73	South Africa
Modesto	C-2	USA	SA-74	South Africa
SA-3	C-3	South Africa	155663	Israel
HP-60	C-4	Australia	158125	Israel
			665	Israel
			163396	Israel
			159441	Israel
			484	Israel
			155085	Israel

2.2.3. Cultivation

Blood Tryptose Agar plates were used as a growth media and were obtained from Onderstepoort Biological Products (OBP), Pretoria. TM/SN (Blackall *et al*., 1990) liquid media were also used as growth medium and contained: 0.05% (w/v) peptone, 0.25% (w/v) glucose, 0.5% (w/v) starch and 0.5% (w/v) NaCl. This medium was supplemented with 5% (v/v) oleic albumin complex, 10% (v/v) heat inactivated chicken serum, 0.05% (v/v) NAD, 0.05% (v/v) and thiamine hydrochloride. The liquid media was autoclaved at 121°C for 15 min and the supplements were filter-sterilized before addition to the cooled autoclaved liquid medium.

2.2.4. Genomic DNA Extraction

Genomic DNA was extracted either manually or by making use of the QIAamp® DNA mini kit.

For the manual extraction a protocol for Gram-positive bacteria (Labuschagne & Albertyn, 2007) with slight modifications for Gram-negative bacteria was used. An *A. paragallinarum* culture was prepared in TM/SN liquid medium (37°C for 16h) or cultured on BTA plates (37°C for 16h in a condle jar). The cells were harvested by filling a 1.5 ml microcentrifuge tube and centrifuged at 20 000 *x g* at 4°C for 10 min. The cells were resuspended in 500 µl cell lysis buffer (100 mM tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 1% SDS). This was vortexed for 30 s, followed by 30 s on ice for a total of 5 min. This was followed by the addition of 275 µl ammonium acetate (7M; pH 7.0) to allow for the precipitation of proteins and mixed by vortexing. This mixture was incubated for 5 min at 65°C, followed by 5 min on ice. Chloroform (500 µl) was added, mixed by vortexing and centrifuged at 20 000 x *g* for 2 min. Three clearly visible layers formed; the clear hydrophilic top layer contained the DNA and RNA, the white interphase contained proteins and polysaccharides and the bottom layer contained the chloroform. The top layer was removed and transfered to a clean 1.5 ml microcentrifuge tube. Isopropanol (750 µl) was added and gently mixed by inverting. It was left for 5 min at room temperature to allow the isopropanol to precipitate the DNA out of the solution. This was followed by centrifuging at 20 000 x *g* for 2 min and the supernatant was discarded. The pellet was washed with ice cold 70% ethanol to remove salts and other impurities and centrifuged at 20 000 x *g* for 2 min. The supernatant was discarded and the pellet was air dried. The pellet was dissolved in 50 µl nuclease-free water. Finally, 5 µl RNAse (10 mg/ml^{-1}) was added to remove any residual RNA and then incubated at 37°C for 1 hour. DNA was stored at -20°C until needed.

DNA was also extracted by making use of the QIAamp® DNA mini kit by following the protocol for genomic DNA extraction from bacterial plate cultures or bacterial suspension cultures with no modifications. DNA was extracted from cells that were cultivated in TM/SM liquid media or from BTA culture plates for 16 to 18 hours at 37°C. The cells were hervested (5000 x *g* for 5 min), the supernatant discarded, and the pellet resuspended in 180 µl Buffer ATL (Animal tissue lysis buffer). Subsequently 20 µl Proteinase K (QIAGEN) was added and mixed by vortexing. Samples were incubated at 56°C for at least 1 h until proteins and the membrane had been completely lysed. A volume of 200 µl Buffer AL (containing Qiagen

protease) was added to the sample mixture and incubated at 70°C for 10 min. This was done to ensure proper conditions for DNA binding to the silica membrane and the denaturing of any Proteinase K that could interfere with further upstream applications. The DNA was then precipitated form the extracted material with the addition of 200 µl ethanol (96-100%). This was mixed by using 15 s pulse-vortexing and then transferred to an assembled QIAamp Mini Spin Column and Collection tube. This was centrifuged at 6000 x *g* for 1 min for the DNA to be absorbed on to the silica membrane due to the presence of high concentrations of salts in the buffer. The flow-through from the column was discarded and washed with 500 µl alcohol-based Buffer AW1 and Buffer AW2, respectively. Buffer AW1 contains guanidinium chloride (hydrochloride) that denatures proteins that flows through the column and was discarded. Buffer AW2 contains essentially 70% ethanol that is used to remove salts from the column and aid in purifying DNA. The first wash step with buffer AW1 was carried out at 6000 x *g* for 1 min, while the second wash step with AW2 was carried out at 20 000 x *g* for 3 min. The Mini spin column was transferred to a supplied sterile DNase-free 1.5 ml microcentrifuge tube, 60 µl of nuclease free water was added and incubated at room temperature (RT) for 5 min. The assembled column and microcentrifuge tube was centrifuged at 6000 x *g* for 1 min to elute the purified DNA. DNA was stored at -20°C until needed.

2.2.5. Identification of *A. paragallinarum*

2.2.5.1. HPG 2 PCR

Isolates were identified as *A. paragallinarum* by means of a species-specific PCR termed HPG2-PCR as described by Chen and co-worker (1996). The primers were described by Chen and co-workers (1996), as listed in Table. 2.2. A 50 µl PCR reaction mixture were made up and contained the following: 2 µM of the primer set, 5 µl of 10 x Thermopol buffer, 2U of Taq DNA polymerase, 200 µM of dNTP's and 0.5 µg of template DNA. The PCR conditions consisted of 30 amplification cycles preceded by a "hot start" and were performed in a PCR Thermocycler (Applied Biosystems 2720). The "hot start" entailed an initial 10 min-denaturing step at 94°C followed by the addition of Taq DNA polymerase. The amplification of 30 cycles was performed, which consisted of a denaturing step (94°C for 25 s), annealing step (55°C for 50 s) and elongation step (72°C for 45 s) was performed. This was followed by a final elongation step at 72°C for 7 min.

Table 2.2: Oligonucleotide primers for the 16S rDNA and species-specific PCR.

	Primers	Sequence	Amplicon size (bp)	Author
16S rDNA PCR	**8F**	5'-AGA GTT TGA TCN TGG CTC AG-3'	1500	Mendoza-Espinoza *et al* 2008
	1525R	5'-AAG GAG GTG WTC CAR CC-3'		
Species-specific PCR	**HPG2 Forward**	5'-TGA GGG TAG TCT TGC ACG CGA AT-3'	500	Chen *et al*., 1998
	HPG2 Reverse	5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'		

2.2.5.2. 16S rDNA gene amplification

Universal prokaryote primers 8F and 1525R (Table 2.2) were used to amplify the 16S rDNA, producing a band of approximately 1 500 bp. A PCR was carried out in a total reaction volume of 50 µl consisting of the following: 2 µM of the primer set, 5 µl of 10 x Thermopol buffer, 2U of Taq DNA polymerase, 200 µM of dNTP's and 0.5 µg of template DNA made up to 50 µl with sterile Milli-Q water. The PCR reaction was performed in a PCR Thermocycler (Applied Biosystems 2720), starting with an initial 2 min denaturing step at 95°C. The amplification of 35 cycles was performed, which consisted of a denaturing step (95°C for 30 s), annealing step (52°C for 30 s) and elongation step (72°C for 1 min 30 s) was performed. This was followed by a final elongation step at 72°C for 2 min.

2.2.6. Gel Electrophoresis

DNA was analysed on a 1% (w/v) agarose gel containing 0.3 µg/µl ethidium bromide. The gel was prepared and electrophoresed in TAE buffer [0.1M Tris, 0.05M Na_2EDTA (pH 8.0) and 0.1mM glacial acetic acid]. Gel electrophoresis was conducted for 30 min at 90V/cm. The DNA bands were then visualized under ultraviolet (UV) light by making use of the BIORAD Gel Doc™ EZ Imager. The relative sizes of the DNA fragments were estimated by comparing their electrophoresis mobility with that of the molecular marker, which were run with the samples on each respective gel. A 1 Kb express O'Generuler Fermentas molecular marker or an O'generuler Fermentas DNA Ladder Mix molecular marker was used.

2.2.7. Sequencing of the 16S rDNA PCR products

In order to determine the nucleotide composition of the 16S rDNA PCR products, purified positive templates were used in sequencing reactions. DNA was purified from enzymatic reactions or agarose gels using the Illustria™ DNA and Gel Band Purification Kit (GE Healthcare) according the manufacturer's instructions. A GFX column was placed in a collection tube per purification to be performed. For an enzymatic reaction 500 µl of capture buffer was added to the column to ensure binding of the PCR product to the spin column. When purification was performed from exised agarose gel PCR products, 500 µl of capture buffer was added and tubes were incubated at 60°C until the agarose gel was completely dissolved. The PCR DNA solution was transferred to the column and mixed thoroughly. The column was centrifuged (Eppendorf Centrifuge 5417R) at full speed for 30 s and the flow-through discarded. The column was placed back into the collection tube, 500 µl of wash buffer was added to remove excess dNTPs, enzymes and primers not used during amplification and the column was centrifuged at full speed for 30 s. The collection tube was discarded, the column transferred to a sterile microcentrifuge tube and 50 µl of elution buffer (10mM Tris-HCl, pH 8.0) was added to the column. The column was incubated at RT for 1 min and centrifuged at full speed for 1 min to recover the purified DNA. Purified DNA samples were stored at -20°C until needed.

Sequencing was performed by making use of the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems). Sequencing was performed in 10 µl reactions of appropriate DNA template (see Table 2.4), 3.2 pmoles of the primers in separate reactions, 0.5 µl premix and 2 µl 5 x sequencing buffer. A sequencing PCR was then performed under the following conditions; 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min.

Table 2.3: The amount, in nanograms (ng), of template needed for sequencing, based on the size of the cleaned PCR product to be sequenced.

PCR product size (bp)	Template added (ng)
100-200	1-3
200-500	3-10
500-1000	5-20
1000-2000	10-40

The BigDye EDTA/ethanol precipitation sequencing clean-up protocol was used without modifications. PCR products containing fluorescently labelled DNA were filled up to 20 µl with sterile nuclease-free water. This was transferred to a clean 1.5 ml microcentrifuge tube. DNA was precipitated by adding of 60 µl 100% ethanol and 5 µl ethylenediaminetetraacetic acid (EDTA) (0.125M, pH 8.0) this was done to remove unincorporated BigDye terminators. DNA was pelleted by centrifugation (20 000 x *g* for 15 min) and the supernatant was discarded. The pellet was washed with 60 µl ice-cold 70% (v/v) ethanol and centrifuged at 20 000 x *g* for 5 min. The supernatant was removed and the samples were dried in a Speedvac Concentrator (SAVANT). Sequencing was performed with an Applied Biosystems 3130xl ABI Genetic Analyzer at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State.

Sequence analysis was performed using Geneious Pro v5.4 (Biomatters Ltd). Analyzed sequences were compared with known sequences in GenBank, using a BLAST analysis. The nucleotide sequences were translated and aligned using Geneious Pro v5.4 (Biomatters Ltd).

2.2.8. Storage of *Avibacterium paragallinarum* isolates

All of the reference isolates were isolated in pure culture and stored by means of lyophilisation (freeze-drying). Isolates were cultivated in TM/SN broth for less than 24 h and then centrifuged at 20 000 x *g* for 15 min. The pellet was resuspended in a cryomedia, in this case meso-inositol nutrient broth consisting of 6.67 g meso-inositol, 0.825 g nutrient broth and 33.3 ml distilled water. This was autoclaved at 121°C for 15 min. It was substituted with 100 ml sterile inactivated horse serum. Isolates were then freeze-dried at the University of the Free State, Food Sciences Department.

2.3. Results

2.3.1. PCR results

2.3.1.1. HPG2-PCR

All reference and field isolates were cultivated in TM/SN broth and then cultured on BTA plates by cross-streaking with a feeder culture. Typical dewdrop colonies were selected and DNA was extracted. A species-specific PCR, HPG2-PCR, was performed on all the reference isolates and the field isolates. The expected band size of 500 bp was observed for all 12 reference isolates (Figure 2.1) and all 17 field isolates as (Figures 2.2. and 2.3). An addition band of approximately 100 bp formed for some of the reference (Figure 2.2).

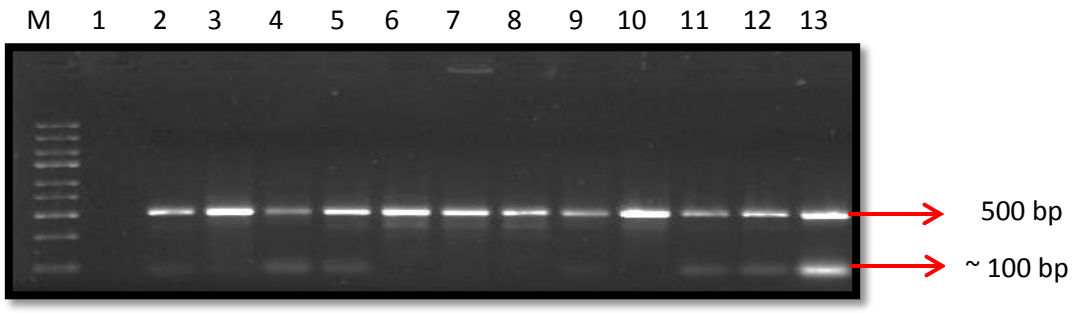

Figure 2.1: HPG2-PCR for all of the reference isolates with the expected band size of 500 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: negative control; Lane 2-13 shows amplification for all twelve reference isolates. Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-1); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). Amplification was observed for all of the isolates. The extra bands are primer dimers.

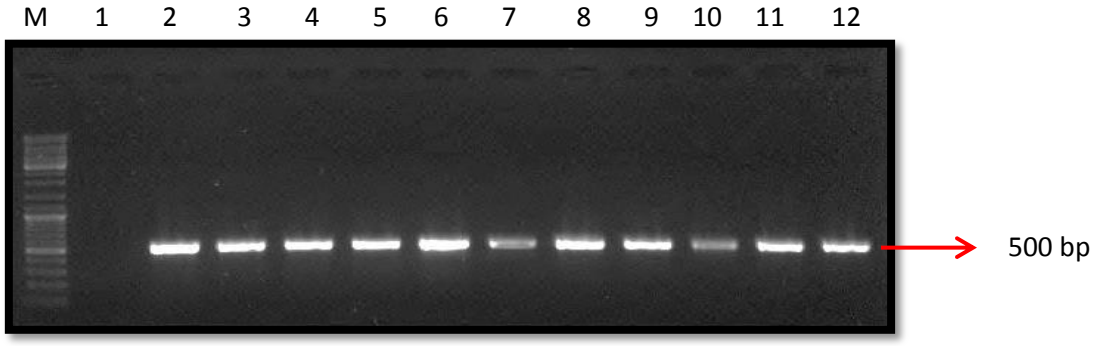

Figure 2.2: HPG2-PCR for all the field isolates with the expected band size of 500 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: negative control; Lane 2: positive control; Lane 3-5 shows amplification for Israeli field isolates. Lane 3: IC 418; Lane 4: IC 462; Lane 5: IC 484; Lane 6-8 shows amplification for Indian vaccine isolates. Lane 6: Vaccine strain 221; Lane 7: Vaccine strain Spross; Lane 8: Vaccine strain Modesto; Lane 9-12 shows amplification for South African field isolates. Lane 9: SA isolate 70; Lane 10: SA isolate 72; Lane 11: SA isolate 73; Lane 12: SA isolate 74. Amplification was observed for all for all of the isolates.

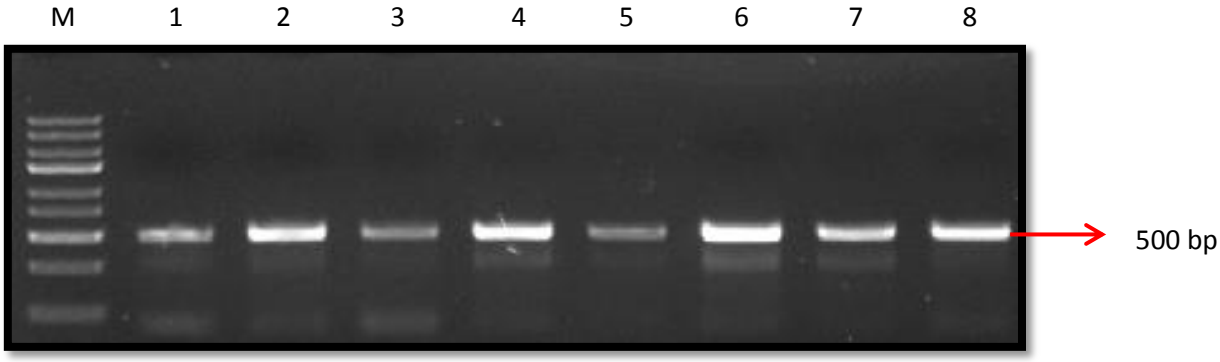

Figure 2.3: HPG2-PCR for all the field isolates with the expected band size of 500 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: positive control; Lane 2: 155663; Lane 3: 158125; Lane 4: 665; Lane 5: 163396; Lane 6: 159441; Lane 7: 484; Lane 8: 155085. Amplification was observed for all of the isolates.

2.3.1.2. 16S rDNA gene amplification

The identity of three of the *A. paragallinarum* isolates was confirmed by amplifying the 16S rDNA gene based on the 8F and 1525 region, as this is one of the highly conserved region across evolutionary lines amongst bacteria. The PCR products of about 1 500 bp were observed for all of the isolates screened (Figure 2.4).

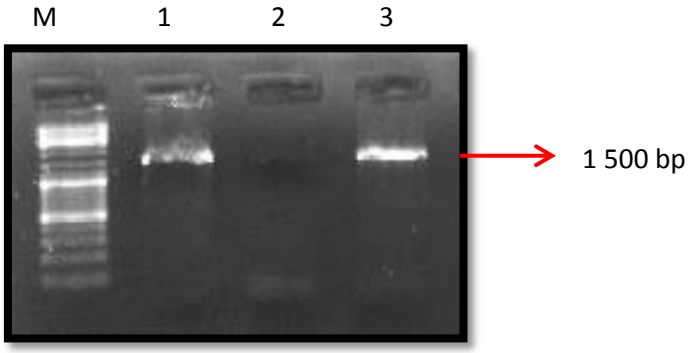

Figure 2.4: 16S rDNA PCR for three reference isolates with the expected band size of 1 500 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: 221 (A-1); Lane 2: 2671 (B-1); Lane 3: HP60 (C-4). Amplification was observed for all three the isolates.

2.3.2. Sequencing results of the 16S rDNA PCR product

All of the samples that were amplified during the 16S rDNA PCR screening were sequenced without subcloning. Sequences were analysed using Geneious Pro v5.4 and compared with known sequences in the GenBank Database using a nucleotide-nucleotide BLAST analysis tool. The sequencing results for the 16S rDNA PCR is outlined in Tables 2.5. The identification of the bacterial strains using the species-specific PCR and the comparisons of the 16S rRNA sequences on the NCBI database revealed similar results and they confirmed the identity of the strains to be *A. paragallinarum.*

Table 2.5: Nucleotide-nucleotide BLAST results for the 16S rDNA PCR products with isolates of highest percentage identity and their GenBank accession numbers.

Sample	Isolate/Species	Accession number	Query coverage	Identity
221	*Heamophilus paragallinarum* strain 0083 16S ribosomal RNA gene, partial sequence	AY498867.1	100%	91%
2671	*Heamophilus paragallinarum* strain 0002 16S ribosomal RNA gene, partial sequence	AY498869.1	100%	91%
HP60	*Avibacterium paragallinarum* strain HP60 16S ribosomal RNA, partial sequence	KC951276.1	69%	86%

2.4. Discussion

The reference isolates that were used during this study is a full set of Kume reference isolates. All of the reference and field isolates of *A. paragallinarum* was successfully cultivated in TM/SN liquid media as well as on BTA plates where the characteristic dewdrop colonies were observed (Sawata & Kume, 1983). All of the reference isolates were then successfully stored by means of freeze-drying. Cells were still viable when later used. Therefore the long term storage method is well established and proved to be successful which will ensure performing experiments on these isolates in the future. This is especially important as this is one of the few labs in the world that has a complete set of Kume reference isolates.

In order to achieve the aims of this study all of the isolates used had to be successfully identified as *A. paragallinarum*. Genomic DNA was extracted from the liquid media and/or culture plates and was successfully identified as *A. paragallinarum* by means of the species specific PCR as the expected band size of 500 bp was observed. For some of the reference isolates an additional band of approximately 100 bp was observed. This was not expected and is most likely due to primer dimers forming.

In order to make sure the strains are in fact *A. pargallinarum* and that the species specific PCR is in fact specific three reference isolates were selected and a 16S rDNA PCR was performed were the expected 1 500 bp band was observed for the selected isolates. These three samples were directly sequenced whithout any subcloning. Sequence analysis for all of these isolates showed the highest homology for *A. paragallinarum*. From the results obtained we can say with certainty that all the isolates are indeed *A. paragallinarum* therefore we were able to conduct further experiments to obtain the aims of the study.

CHAPTER 3

DEVELOPING A MOLECULAR SEROTYPING TECHNIQUE TO DISTINGUISH *Avibacterium paragallinarum* ISOLATES

3.1. Introduction

Avibacterium paragallinarum is mainly serotyped by making use of the modified Kume serotyping scheme (Blackall *et al*., 1990). The main method by which this is achieved is the haemagglutination and haemagglutination inhibition (HA/HI) assays (Kume *et al*., 1983). The Kume serotypying scheme is technically demanding as well as a very complex technique and no labaoratory in the world performs full Kume serotyping (Blackall, 1999). This technique can also be limiting as it is very subjective. This technique is suitable to serotype to the serogroup level, but the HI test is not able to serotype accurately up to serovar level (Blackall, 1999).

Other drawbacks with the HA/HI assays is that *A. paragallinarum* is a difficult organism to cultivate as it is overgrown quite rapidly by other microorganisms and it needs complex media to grow (Chen *et al*., 1996). It is also difficult to acquire samples from remote areas that are still viable upon receiving, as it is an organism that is inactivated quite rapidly outside its host (Bragg *et al*., 2004).

It was also attempted to serotype *A. paragallinarum* by making use of monoclonal antibodies (Mabs) although some success was achieved by this means it is not a viable option due to the fact some of the Mabs produced did not react with the haemagglutinins of the reference strains (Bragg *et al*., 1997). Therefore, there is a need for a molecular serotyping technique that will result in more accurate diagnosis. A molecular serotyping technique will be less

time consuming and will greatly aid in better vaccine production against IC in the poultry industry.

A few molecular serotyping techniques which have been attempted. Examples are the ERIC PCR (Soriano *et al*., 2004b) and a multiplex and RPFL PCR (Sakamoto *et al*., 2012). There are, however a few problems with these techniques. The ERIC PCR works well for the reference isolates but not for the field isolates as was determined during a project within this research group at the University of the Free State (Bragg, 2010). For the multiplex and RFLP PCR you can serotype to serogroup level but not to serovar level (Sakamoto *et al*., 2012) Due to these above mentioned limitations with the currently available techniques there is still room for improvement in the development of a molecular serotyping technique. Blackall and co-workers (2005) reclassified *A. paragallinarum* the similarity of 16S rRNA gene sequences of the strains 0222 and 0083 of *A. paragallinarum* to Modesto strain was 98.8%. Therefore another problem with the development of a better molecular technique is the lack of sequences that is available for all of the different *A. paragllinarum* serovars and the sequences that are available do not show variation between the different serovars.

This chapter focuses on the development of a molecular serotyping technique to accurately differentiate between the field isolates in determining whether these isolates are C-2 or C-3 serovars of *A. paragallinarum*, which are two of the main causes of IC in South Africa. The C-3 serovar is also currently the most prevalent and virulent strain in South Africa (Bragg, 2002). Therefore one of the techniques that were developed was a serotyping PCR that was designed to specifically target the C-3 serovar. The improvement of the multiplex and RFLP PCR developed by Sakamoto and co-workers (2012) was also attempted during this chapter. This was done to enable successful serotyping of all the reference isolates, as well as being able to correlate the results observed for the field isolates to those observed for the reference isolates.

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3.2. Materials and Methods

3.2.1. Enzymes, chemicals, kits and other consumables

The reagents and chemicals used were of molecular, biological or analytical grade and were obtained from the following companies.

Amersham Biosciences: GFX™ PCR DNA and Gel Band Purification Kit; Applied Biosystems: BigDye terminator v3.1 Kit; 2720 Thermal Cycler; **Fermentas;** O'GeneRuler™ Express DNA Ladder, O'GeneRuler™, Orange loading dye, Deoxynucleoside triphosphates (dNTPs), and restriction endonuclease *Bgl*II; **Inqaba:** All synthesis of primers; **New England Biolabs®:** One Taq™Hot start DNA Polymerase with Standard reaction Buffer; **Sigma Aldrich:** Ethidium bromide; **Whitehead Scientific (PTY) LTD:** Agarose D1 LE, QIAamp® DNA mini kit.

3.2.2. *Avibacterium paragallinarum* isolates

The same reference and field isolates were used as stated in Chapter 2, Section 2.2.2, Table 2.1.

3.2.3. Cultivation

Bacterial cultures were cultivated in the same manner as stated in Chapter 2, Section 2.2.3, by making use of BTA plates as well as TM/SN liquid media.

3.2.4. Genomic DNA Extraction

Genomic DNA was extracted either manually or by making use of the QIAamp® DNA mini kit. For the manual extraction a protocol for gram positive bacteria (Labuschagne, & Albertyn, 2007) was used, this protocol was modified for gram negative bacteria; where the use of glass beads were omitted as this is too harsh for gram negative bacteria because of their thin peptidoglycan layer. For the QIAamp® DNA Mini kit from Qiagen genomic DNA was extracted following the protocol for genomic extraction from bacterial plate cultures or

bacterial suspension cultures with no modifications. The methods are outlined in Chapter 2, Section 2.2.4. The genomic DNA was eluted in 60 µl nuclease-free water.

3.2.5. Primer design

The primers that were used for the species-specific PCR (HPG2-PCR) are described by Chen and co-workers (1996) and are listed in Table 2.1. The development of a PCR that specifically targets the C-3 serovar of *A. paragallinarum* was attempted. The haemagglutinins are the major typing and protective antigens of *A. paragallinarum* and therefore the *hag*A gene which forms part of the haemagglutinins was targeted for the C-3 specific PCR (Hobbs *et al*., 2002). The primers were designed based on the *hag*A gene sequences on NCBI accession numbers; AF491820 (0083), AF491826 (221), AF491817 (2403), AF491825 (E-3C), AF491821 (HP14), AF491819 (0222), AF491822 (2671), AF491823 (H-18), AF491824 (SA-3) and AF491818 (HP60) (Hobbs *et al*., 2002). The sequences of all the C-serovars were aligned, as listed in figure 2.1. It was established that there are not significant differences between the serogroup C-serovars. Therefore the primers were designed at regions where there are only 3 bp differences. These differences were incorporated at the 3' ends of the primer sets. The placement of the 3' end of the primer is critical for a successful PCR as the polymerase reads from the 3' end during the PCR amplification reaction. Therefore the 3' ends must anneal to the templates to be elongated by a DNA polymerase (Onodera & Melcher, 2004). Minimal mismatch should occur within the last 5-6 nucleotides at the 3' end of the primer but in certain instances the mispairing can be useful (Dieffenbach *et al*., 1993) as it is in this case. Recommended compositions for of 3' ends are: no T at 3' end, at least one W (A/T) in the 3'end triplets, S(C/G) at 3' end and no GC/CG due to potential formation of hairpins and primer-dimers and low G+C (Onodera & Melcher, 2004). Therefore the primers should theoretically bind more strongly to the C-3 isolates and should result in non-specific binding for all of the other serovars. This will allow you to distinguish C-3 from the rest of the C-serovars. The primer pair is listed in Table 2.2.

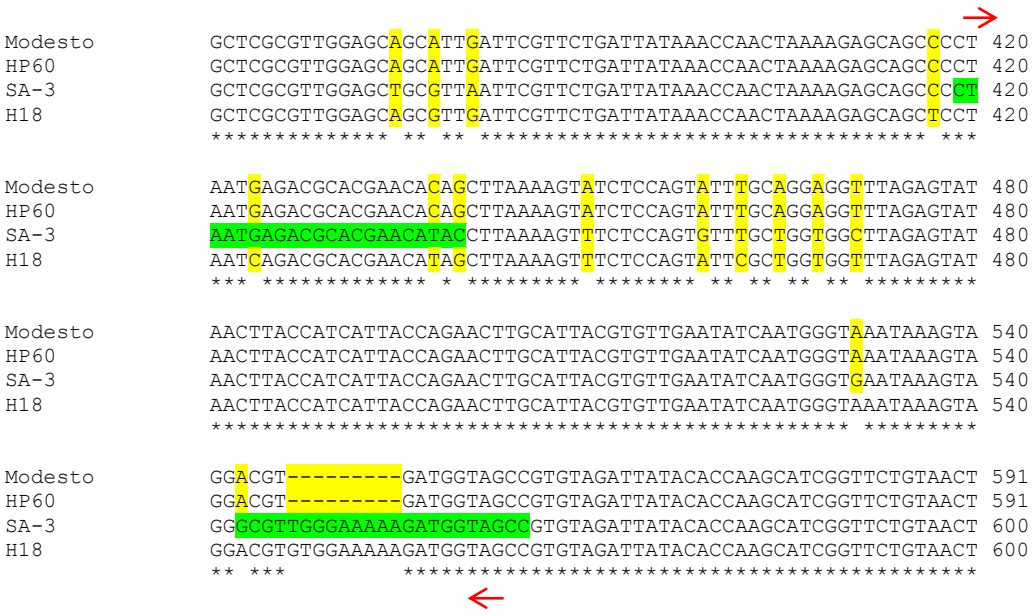

Figure 3.1: This figure illustrates the C-3 primer design. The regions that are highlighted in green illustrates the forward and reverse primers and the regions highlighted in yellow illustrates the sequence differences between the different C-serovars.

The primers that were used for the Multiplex Serotyping PCR are those described by Sakamoto and co-workers (2012) and are listed in Table 3.1.

Table 3.1: Oligonucleotide primers used for C-3 and Serotyping PCR.

	Primers	Sequence	Amplicon size (bp)	Author
C3 Primers	**HRC forward**	5'-CTA ATG AGA CGC ACG AAC ATA C-3'	147	
	HRC reverse	5'-GGC TAC CAT CTT TTT CCC AAC GC-3		
ABC primer	**ABC forward**			
		5'-CGC GGG ATT GTT GAT TTT GTT-3	800	
	A reverse			
		5'-GGC TCA CAG CTT TAT GCA ACG AA-3'		
	ABC forward			
		5'-GGT GAA TTT CAC CAC ACC AC-3'	1100	Sakamoto *et al* 2012
	B reverse			
		5'-GGC TCA CAG CTT TAT GCA ACG AA-3'		
	ABC forward			
		5'-TAA TTT TCT TAT TCC CAG CAT CAA TAC CAT-3'	1600	
	C reverse			

3.2.6. Molecular Serotyping techniques

3.2.6.1. C-3-specific Polymerase Chain Reaction

The 50 µl PCR reaction mixture was made up and contained the following: 2 µM of the primer set, 5 µl of 10 x Thermopol buffer, 2U of Taq DNA polymerase, 200 µM of dNTP's and 0.5 µg of template DNA. The PCR conditions consisted of an initial 2 min denaturation step. This was followed by 30 cycles which consisted of a denaturing step (94°C for 30 s), an annealing step (51°C for 30 s) and an elongation step (72°C for 30 s). This was followed by a final elongation step at 72°C for 5 min, resulting in amplicons of approximately 147 bp. Amplified fragments were observed under UV illumination on a 1% agarose gel stained with ethidium bromide.

3.2.6.2. Serotyping Polymerase Chain Reaction

A multiplex PCR and RFLP were performed based on the work done by Sakamoto and co-workers (2012). The primers that were used for the PCR are the same as those used by Sakamoto and co-workers (2012) and are listed in Table 2.2. A 50 µl PCR reaction mixture was made up and contained the following: 0.2 µM of the primer set (ABC-F, A-R, B-R and C-R), 10 µl of 5 x One Taq™Standard reaction buffer, 2U of One Taq™Hot Start DNA polymerase, 0.2 mM of dNTP's and 0.5 µg of template DNA. The PCR consisted out of the following amplification steps: An initial denaturation step (98°C for 1 min); 30 cycles which consisted of a denaturation step (98°C for 10 s); an annealing step (56°C for 10 s) and elongation step (72°C for 2 min), this was followed be a final elongation step at 72°C for 7 min. The results that were obtained differed from the results obtained by Sakamoto and co-workers (2012). Therefore an attempt was made to optimize the PCR by means of a temperature gradient PCR.

The PCR reaction conditions were the same except for the addition of a gradient step at the annealing step with a temperature rang of between 40°C-60°C. This was done to determine the optimum annealing temperature of 57.1°C. The rest of the PCR conditions stayed the same. This resulted in a band of approximately 1 000 bp for all of the isolates.

3.2.6.3. Restriction digest

A restriction digest was performed with the restriction enzyme *Bgl*II, as reported by Sakamoto and co-workers (2012). The reaction mixture contained 1 µl of the enzyme, 2 µl of buffer O, 10 µl PCR product and was filled up to a final reaction volume of 30 µl by the addition of sterile nuclease free water. The samples were incubated at 37°C for 2 hours in a waterbath. The restriction digest should result in the following banding patterns according to Sakamoto and co-workers (2012): Serovar A (768 bp and 868 bp), Serovar B (1 600 bp) and Serovar C (1 284 bp and 339 bp). Fragments were observed under UV illumination on a 1% agarose gel stained with ethidium bromide.

3.2.7. Sequencing of C-3 PCR products

Sequencing was performed by making use of the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems) and the manufacturers protocol was followed with no modifications as outlined in Section 2.2.7, Chapter 2. Sequence analysis was performed using Geneious Pro v5.4 (Biomatters Ltd). Analyzed sequences were compared with known sequences in the GenBank Database using a nucleotide-nucleotide BLAST analysis tool.

3.3. Results

3.3.1. Molecular serotyping techniques results

3.3.1.1. C-3 PCR

A PCR that specifically targeted the C-3 serovar of *A. paragallinarum* was developed (as discussed in section 3.2.4 and 3.2.5.) and performed for all of the reference and field isolates and the expected 147 bp band was observed for all of the isolates and not just for the C-3 isolates as expected as depicted in Figure 3.2 and 3.3. Additional bands of approximately 100 bp were observed for some of the isolates as depicted in Figure 3.2 and 3.3.

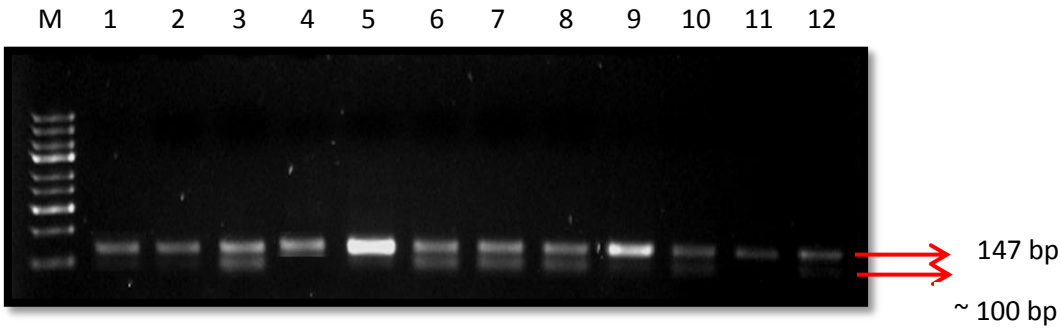

Figure 3.2: C-3 PCR for all of the reference isolates with the expected band size of 147 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1-12 shows amplification for all twelve reference isolates. Lane 1: ATCC 29545 (A); Lane 2: 221 (A-1); Lane 3: 0083 (A-1); Lane 4: 2403 (A-2); Lane 5: E-3C (A-3); Lane 6: HP-14 (A-4); Lane 7: 0222 (B-1); Lane 8: 2671 (B-1); Lane 9: HP 8 (C-1); Lane 10: Modesto (C-2); Lane 11: SA-3 (C-3); Lane 12: HP-60 (C-4). Amplification was observed for all of the isolates. Amplification was observed for all of the isolates.

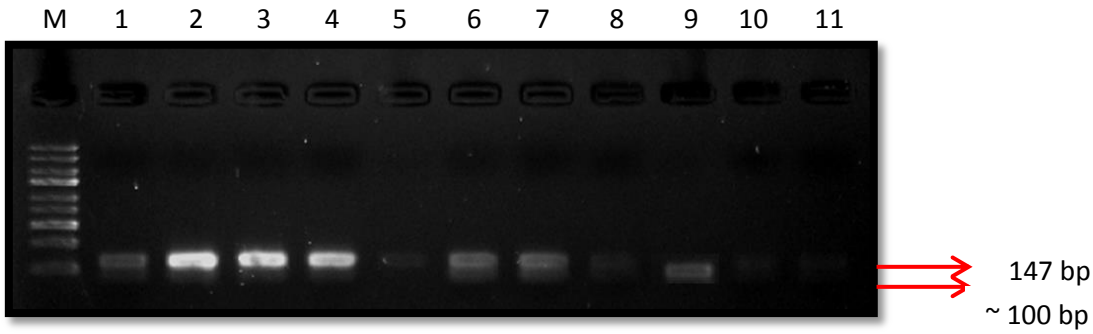

Figure 3.3: C-3 PCR for all the field isolates with the expected band size of 147 bp. Molecular marker: O'GeneRuler™Express DNA ladder, Lane 1: + Control; Lane 2-4 shows amplification for Israeli field isolates. Lane 2: IC 418; Lane 3: IC 462; Lane 4: IC 484; Lane 5-7 shows amplification for Indian vaccine isolates. Lane 5: Vaccine strain 221; Lane 6: Vaccine strain Spross; Lane 7: Vaccine strain Modesto; Lane 8-11 shows amplification for South African field isolates. Lane 8: SA isolate 70; Lane 9: SA isolate 72; Lane 10: SA isolate 73; Lane 11: SA isolate 74. Amplification was observed for all of the isolates. Amplification was observed for all of the isolates.

3.3.1.1.1. Optimization of the C-3 PCR

Further optimization was performed for the C-3 PCR. A temperature gradient PCR was performed within a 44-70°C range to determine the optimum annealing temperature as depicted in Figure 3.4 and Figure 3.5 From the results seen in Figure 3.5 it can be determined that 64°C is the optimum annealing temperature. At annealing temperature of 64°C amplification were observed for the SA-3 (C-3) reference isolate as well as 2403 (A-2), 0222 (B-1) and HP8 (C-1) reference isolates as depicted in figure 3.6, therefore even further optimization is necessary. At an annealing temperature of 64°C amplification was observed for IC 418, IC 462, IC 484, SA-72, SA-74 (Figure 3.7) as well as for all 7 of the other Israeli field isolates as depicted in Figure 3.8.

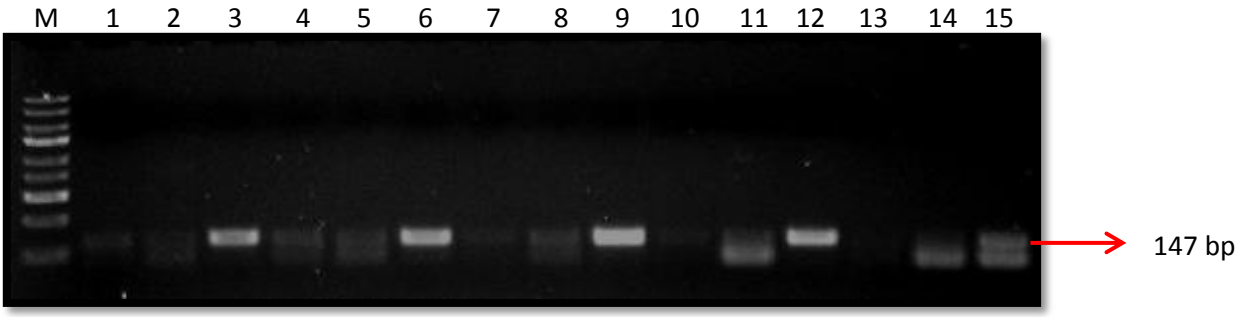

Figure 3.4: Temperature gradient C-3 PCR, with annealing temperature range 47.1-58.6°C. Lane 1-3: 47.1°C; Lane 4-6: 50.2°C; Lane 7-9: 51.4°C; Lane 10-12; 53.5°C; Lane 13-15: 58.6°C. Lane 1,4,7,10,13: ATCC 29545 (A); Lane 2.5,8,11,14: Modesto (C-2); Lane 3,6,9,12,15: SA-3 (C-3). Amplification was observed for all of the isolates.

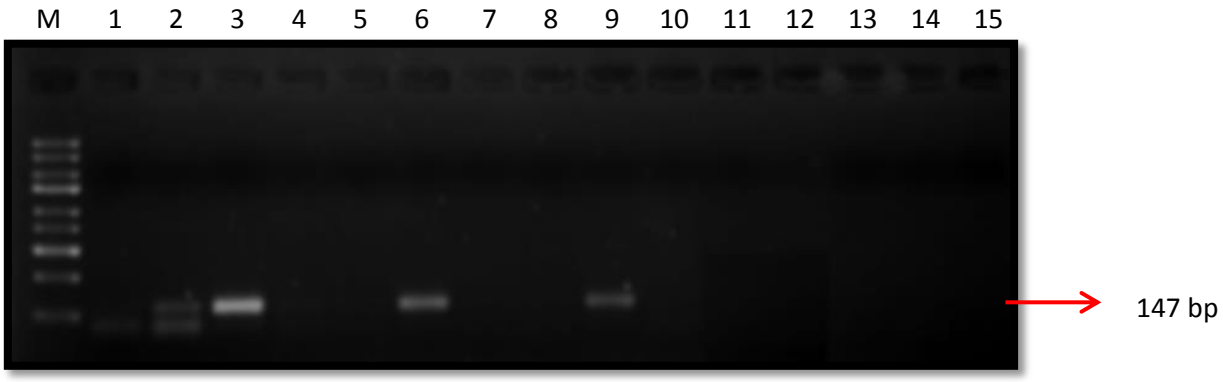

Figure 3.5: Temperature gradient C-3 PCR, with annealing temperature range 59.6-68.4°C. Lane 1-3: 59.6°C; Lane 4-6: 61.9°C; Lane 7-9: 64.6°C; Lane 10-12: 66.3°C; Lane 13-15: 68.4°C. Lane 1,4,7,10,13: ATCC; Lane 2.5,8,11,14: Modesto (C-2); Lane 3,6,9,12,15: SA-3 (C-3). Amplification was observed for all 3 isolates in lanes 1-3. Amplification was observed for SA-3 (C-3) in lanes 6 and 9. A very faint band is observed for 221 (A-1) in lane 4.

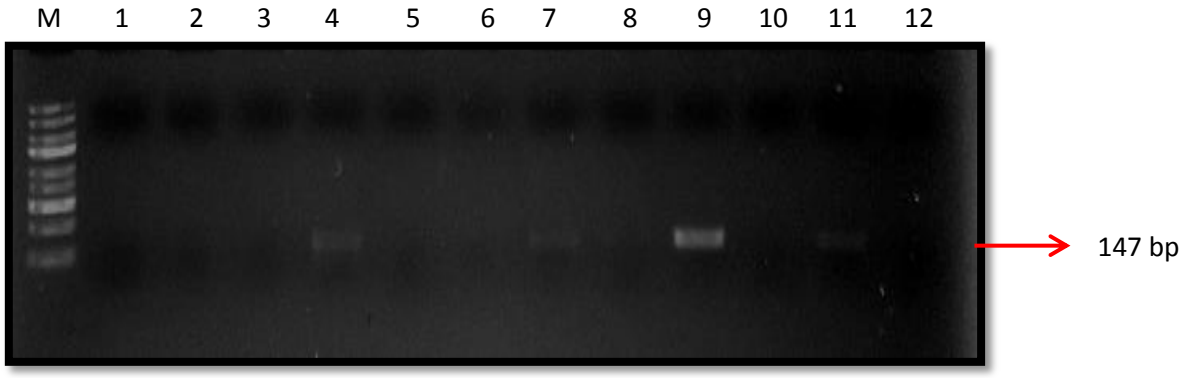

Figure 3.6: C-3 PCR at optimum annealing temperature of 64°C for all of the reference isolates with the expected band size of 147 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1-12 shows amplification for all twelve reference isolates. Lane 1: ATCC 29545 (A); Lane 2: 221 (A-1); Lane 3: 0083 (A-1); Lane 4: 2403 (A-2); Lane 5: E-3C (A-3); Lane 6: HP-14 (A-4); Lane 7: 0222 (B-1); Lane 8: 2671 (B-1); Lane 9: HP 8 (C-1); Lane 10: Modesto (C-2); Lane 11: SA-3 (C-3); Lane 12: HP-60 (C-4). At the optimum temperature of 64°C amplification was observed for lanes 4, 7, 9 and 11.

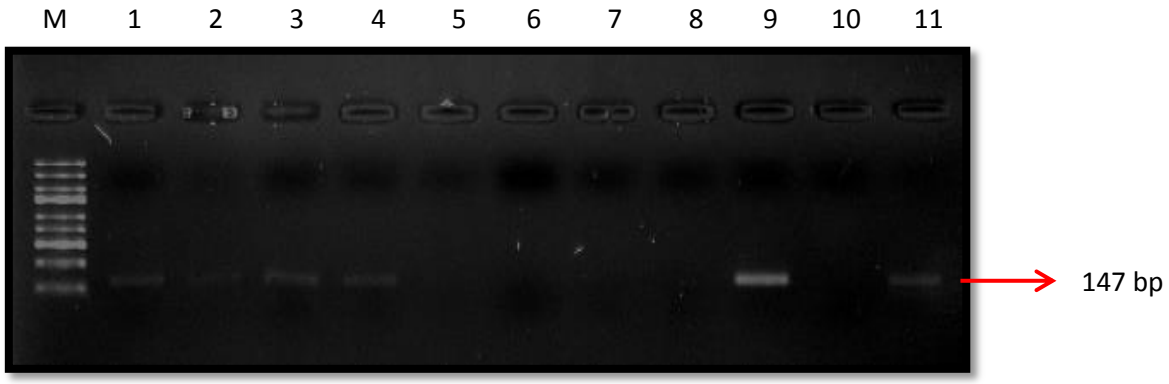

Figure 3.7: C-3 PCR at the optimum annealing temperature of 64°C for all the field isolates with the expected band size of 147 bp. Molecular marker: O'GeneRuler™Express DNA ladder, Lane 1: + Control; Lane 2-4 shows amplification for Israeli field isolates. Lane 2: IC 418; Lane 3: IC 462; Lane 4: IC 484; Lane 5-7 shows amplification for Indian vaccine isolates. Lane 5: Vaccine strain 221; Lane 6: Vaccine strain Spross; Lane 7: Vaccine strain Modesto; Lane 8-11 shows amplification for South African field isolates. Lane 8: SA isolate 70; Lane 9: SA isolate 72; Lane 10: SA isolate 73; Lane 11: SA isolate 74. At the optimum temperature of 64°C amplification was observed for lanes 1, 2, 3, 4, 9 and 11.

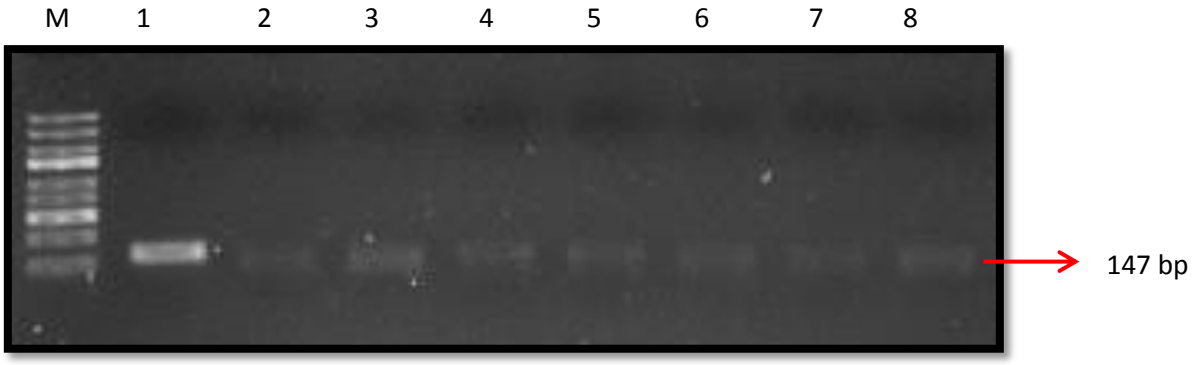

Figure 3.8: C-3 PCR at optimum annealing temperature of 64°C for all of the Israeli isolates with the expected band size of 147 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: SA-3; Lane 2: 155663; Lane 3: 158125; Lane 4: 665; Lane 5: 163396; Lane 6: 159441; Lane 7: 484; Lane 8: 155085. Amplification was observed for all of the isolates.

From Figure 3.6, it seems that the field isolates can be either 2402, 0222, C-1 or C-3. Optimization of this technique has proven to be unsuccessful as seen from above mentioned results. Therefore a different molecular serotyping technique was approached.

3.3.1.2. Serotyping PCR

The serotyping PCR was performed according to Sakamoto and co-workers (2012), where the expected bands were: 800 bp (serovar A), 1 100 bp (serovar B) and 1 600 bp (serovar C). The band sizes hower did not correspond to those of the article as non-specific banding patterns were observed (Figure 3.9). The PCR resulted in bands between 1 500 bp and 1 100 bp for all four isolates tested as depicted in Figure 3.9.

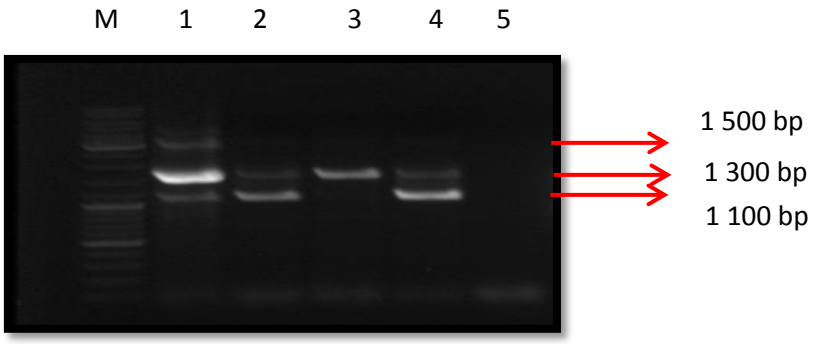

Figure 3.9: Serotyping PCR for serogroups A, B and C. Molecular marker: O'GeneRuler™Mix DNA ladder; Lane 1: 221 (A-1); Lane 2: 0222 (B-1); Lane 3: Modesto (C-2); Lane 4: (SA-3) C-3; Lane 5: Negative control.

A temperature gradient PCR was conducted on serovars 221 (A-1), 0222 (B-1) and SA-3 (C-3). A negative control was included at all of the different annealing temperatures. At low temperatures of about 40°C, no amplification was observed and at the middle temperatures of about 45°C-50°C small bands of about 500 bp was observed as depicted in Figure 3.10A. The gel in Figure 3.10A shows bright bands at all of the temperatures and samples whith the highest concentrations were at a temperature of 57.1°C therefore this was selected as the optimum temperatur. At this temperature a band of approximately 1 000 bp was consistently observed for all the isolates, therefore all further experiments were performed at this temperature.

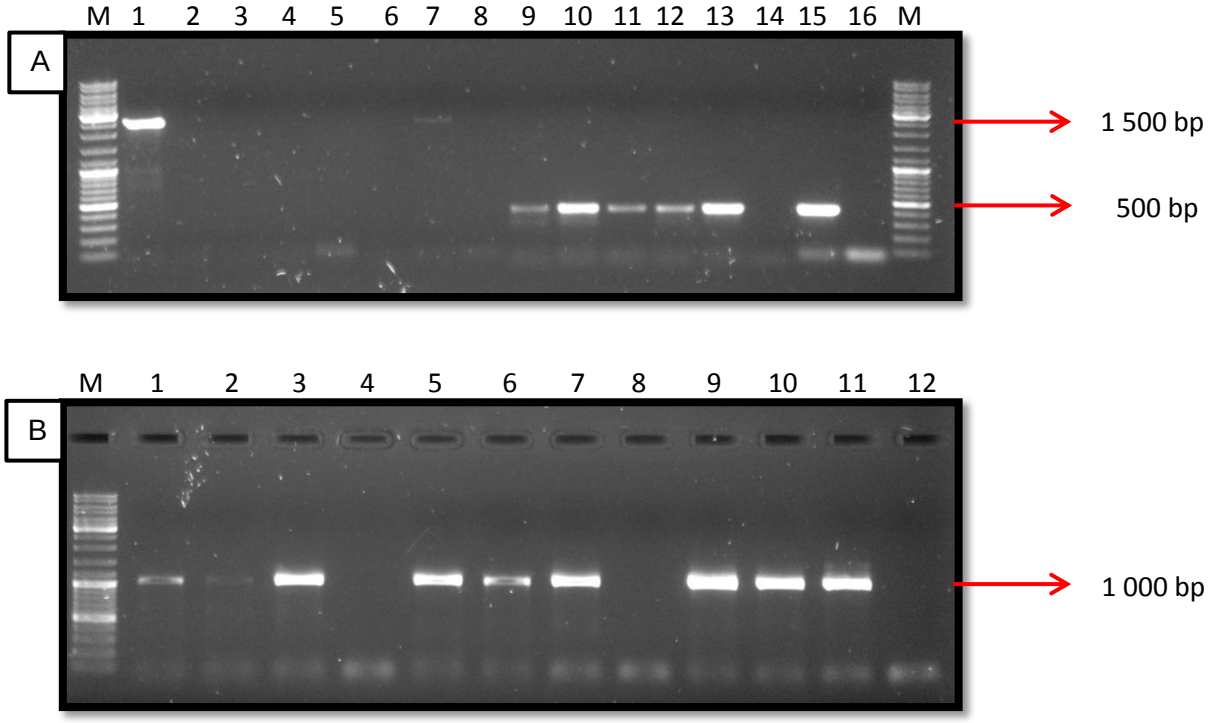

Figure 3.10: Temperature gradient PCR of 3 reference isolates (221 (A-1), 0222 (B-1), SA-3 (C-3)) at different annealing temperatures. A: Annealing temperatures between 40-55°C. Lane 1-4: Annealing temperature of 40°C; Lane 5-8: Annealing temperature of 45°C; Lane 9-12: Annealing temperature of 50°C; Lane 13-16: Annealing temperature of 55°C. Lane 1, 5, 9, 13: 221 (A-1); Lane 2, 6, 10, 14: 0222 (B-1); Lane 3, 7, 11, 14: SA-3 (C-3); Lane 4, 8, 12; 16: - Control B: Annealing temperatures between 56-60°C. Lane 1-4: Annealing temperature of 56°C; Lane 5-8: Annealing temperature of 58°C; Lane 9-12: Annealing temperarure of 60°C. Lane 1, 5, 9: 221 (A-1); Lane 2, 6, 10: 0222 (B-1); Lane 3, 7, 11: SA-3 (C-3); Lane 4, 8, 12: Negative control.

A multiplex serotyping PCR developed by Sakamoto and co-workers (2012) was optimized, modified and performed for all of the reference and field isolates and a band of approximately 1 000-1 100 bp was observed for all of the reference isolates except for HP60 (C-4) where a band of about 500 bp was observed (Figure 3.11 A-E) and for the field isolates except SA 70 and SA 73 where no amplification was observed (Figure 3.12). For the other 7 Israeli isolates a 1 000 bp band was observed as depicted in Figure 3.13. These results that were observed were different than expected as Sakamoto and co-worker reported the following: 800 bp (Serogroup A), 1 100 bp (Serogroup B) and 1 600 bp (Serogroup C).

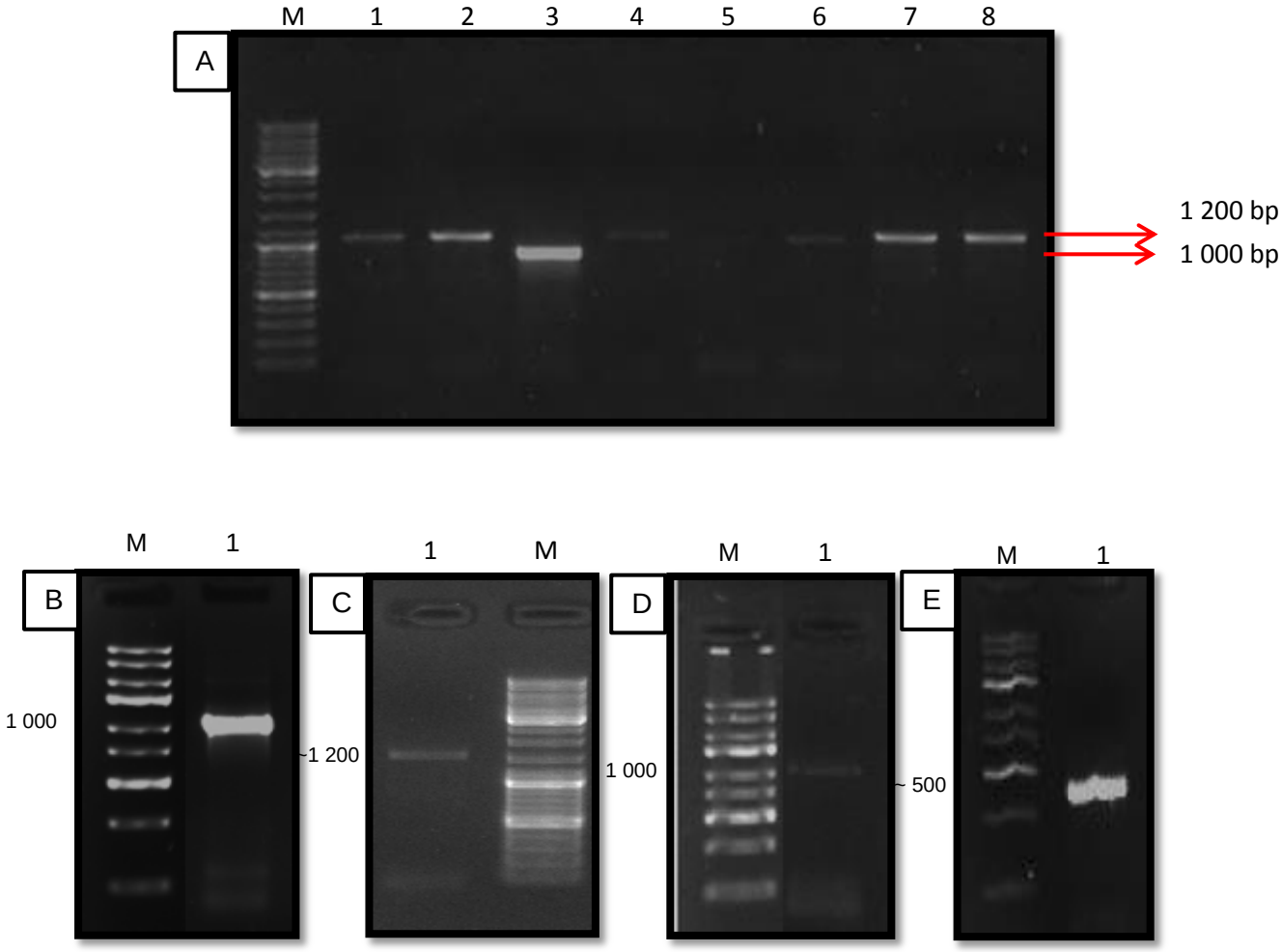

Figure 3.11: Serotyping PCR for all of the reference isolates with the expected band size of ~1 000 bp. A: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1-8 shows amplification for all twelve reference isolates. Lane 1: ATCC 29545 (A); Lane 2: 221 (A-1); Lane 3: 0083 (A-1); Lane 4: 2403 (A-2); Lane 5: E-3C (A-3); Lane 6: HP-14 (A-4); Lane 7: 0222 (B-1); Lane 8: 2671 (B-1); B: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: HP 8 (C-1); C: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: Modesto (C-2); D: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: SA-3 (C-3); E: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: HP-60 (C-4).

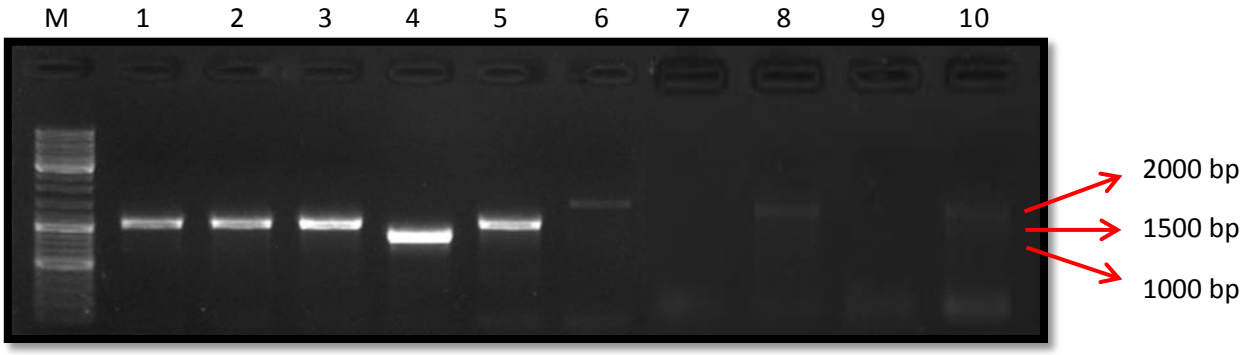

Figure 3.12: Serotyping PCR for all the field isolates with the expected band size of 500 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1-3 shows amplification for Israeli field isolates. Lane 1: IC 418; Lane 2: IC 462; Lane 3: IC 484; Lane 4-6 shows amplification for Indian vaccine isolates. Lane 4: Vaccine strain 221; Lane 5: Vaccine strain Spross; Lane 6: Vaccine strain Modesto; Lane 7-10 shows amplification for South African field isolates. Lane 7: SA isolate 70; Lane 8: SA isolate 72; Lane 9: SA isolate 73; Lane 10: SA isolate 74.

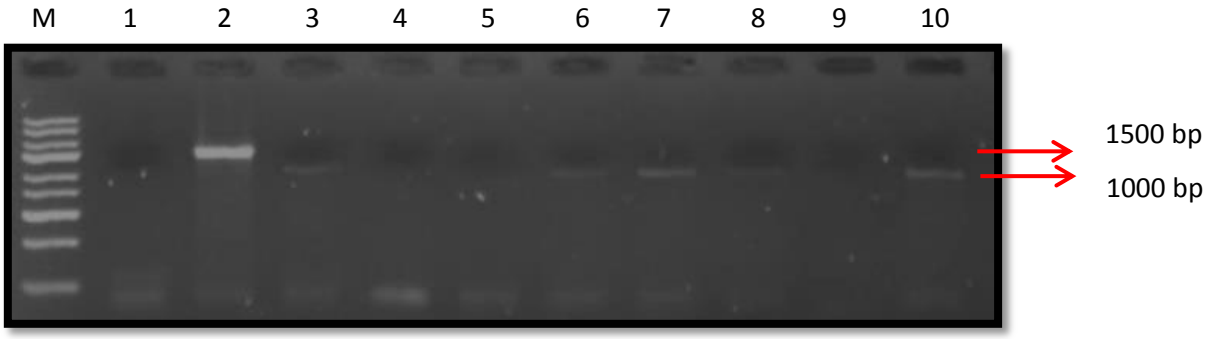

Figure 3.13: Serotyping PCR for all the Israeli field isolates with the expected band size of 1 000-1 100 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: Negative control; Lane 2: Modesto; Lane 3: SA-3. Lane 4-10 shows amplification for Israeli field isolates. Lane 4: 155663; Lane 5: 158125; Lane 6: 665; Lane 7: 163396; Lane 8: 159441; Lane 9: 484; Lane 10: 155085.

The serotyping PCR was repeated for the three Israeli isolates (15563, 158125 and 159441) that did not show any amplification when the PCR was performed the first time. The PCR was performed with different template concentration (2 µl and 10 µl), this was the only aspect that was changed for this PCR reaction. The expected band size of about 1 000 bp was observed for Israeli isolate 155663 when 2 µl of template was used. But no amplification was observed for any of the other isolates.

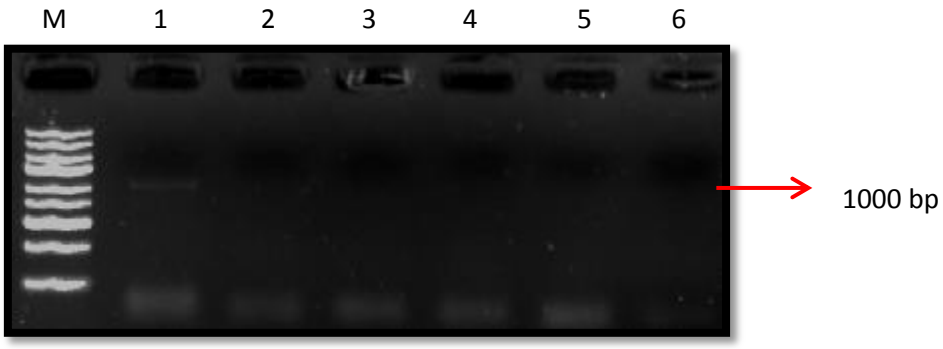

Figure 3.14: Serotyping PCR for the three Israeli isolates that did not show amplification at different template concentration with the expected band size of 1 000 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: 155663; Lane 2: 158125; Lane 3: 159441; Lane 4: 155663; Lane 5: 158125; Lane 6: 159441.

3.3.2. Restriction Digest

A restriction digest was performed on all of the reference and field isolates following the PCR with *Bgl*II. There was no digestion observed for A and B serogroups, as depicted in Figure 3.15A. For the serogroup C-serovars different banding patterns were obtained; for C-1 (1 000 bp); C-2 (400 bp and 1 000 bp); C-3 (400 bp and 700 bp) and C-4 (~ 500 bp) as depicted in Figure 3.15 B-E. These results also were not supported by the results obtained by Sakamoto and co-workers (2012) where the following banding patteren was reported following a restriction digest with *Bgl*II: Serovar A (768 bp and 868 bp), Serovar B (1600 bp) and Serovar C (1 284 bp and 339 bp). From Figure 3.16, it can be proposed that IC 418, 462,484, SA 72 and 74 are C-3 isolates. The vaccine strains banding pattern correspond to those of the 221 (A-1), B-1 and Modesto (C-2) reference isolates respectively and SA 70 and 73 was untypable as no amplification or digestion was observed. From Figure 3.17, it can be proposed that 665, 163396, 155085 and 159441 are C-3 isolates and 155663, 158125 and 484 are untypable.

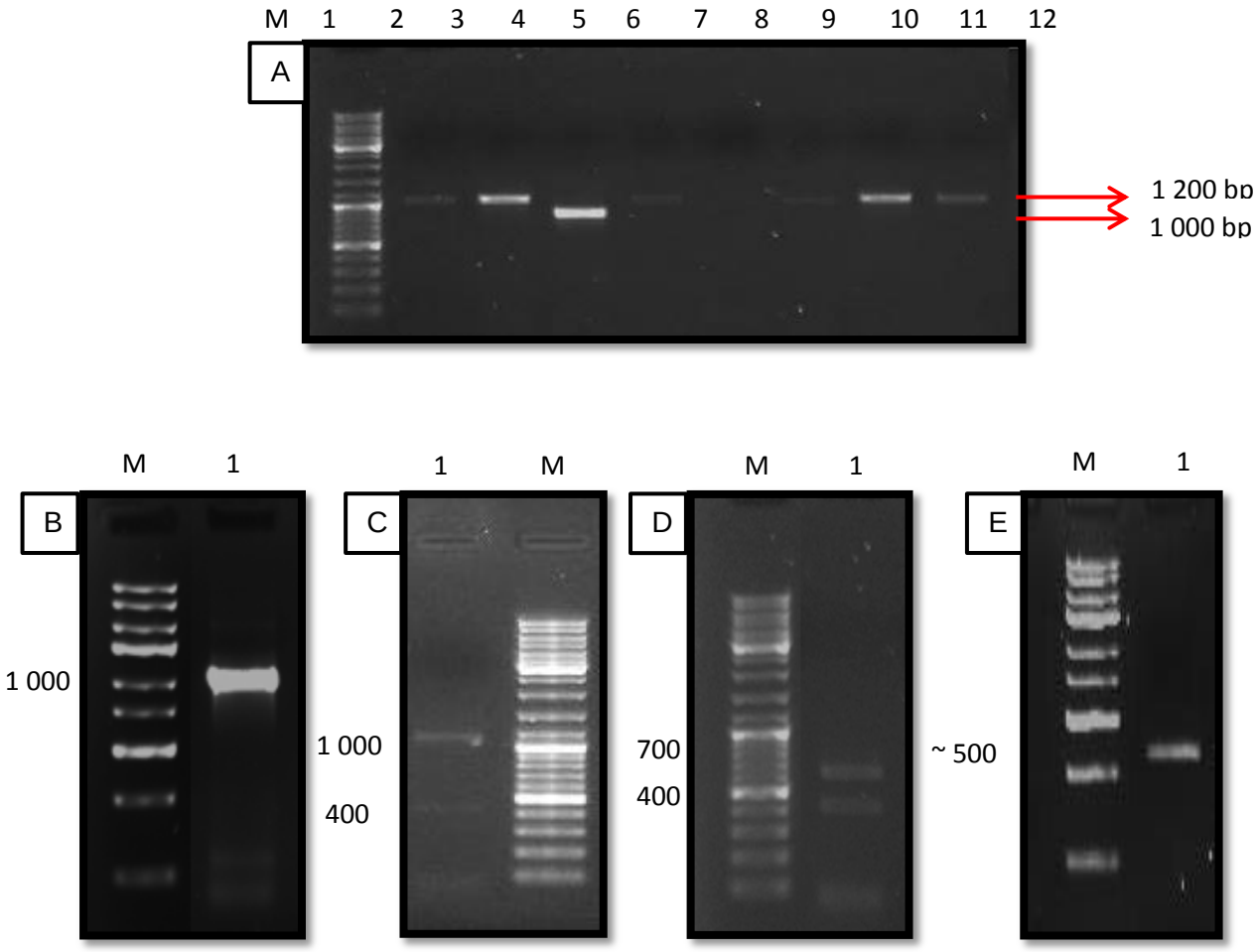

Figure 3.15: *Blg*II restriction digest for all of the reference isolates. A: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1-12 shows amplification for all twelve reference isolates. Lane 1: ATCC 29545 (A); Lane 2: 221 (A-1); Lane 3: 0083 (A-1); Lane 4: 2403 (A-2); Lane 5: E-3C (A-3); Lane 6: HP-14 (A-4); Lane 7: 0222 (B-1); Lane 8: 2671 (B-1); B: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: HP 8 (C-1); C: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: Modesto (C-2); D: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: SA-3 (C-3); E: Molecular marker: O'GeneRuler™Express DNA ladder; Lane 12: HP-60 (C-4).

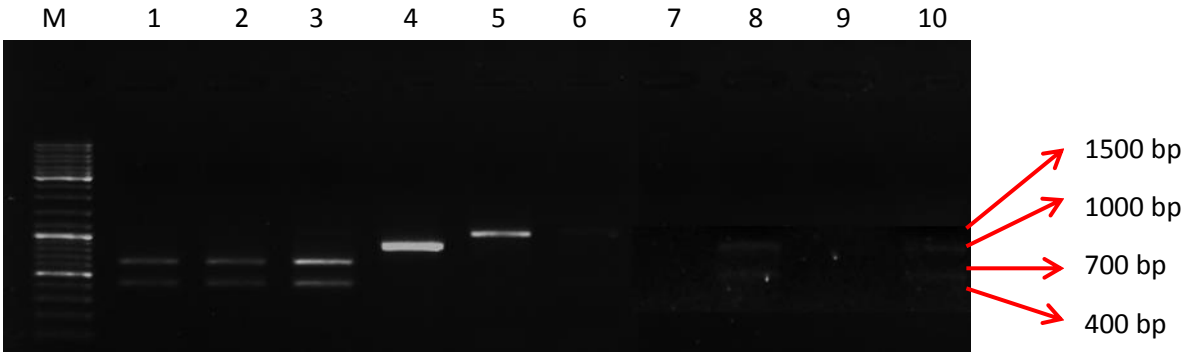

Figure 3.16: *Blg*II restriction digest for all the field isolates. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1-3 shows amplification for Israeli field isolates. Lane 1: IC 418; Lane 2: IC 462; Lane 3: IC 484; Lane 4-6 shows amplification for Indian vaccine isolates. Lane 4: Vaccine strain 221; Lane 5: Vaccine strain Spross; Lane 6: Vaccine strain Modesto; Lane 7-10 shows amplification for South African field isolates. Lane 7: SA isolate 70; Lane 8: SA isolate 72; Lane 9: SA isolate 73; Lane 10: SA isolate 74.

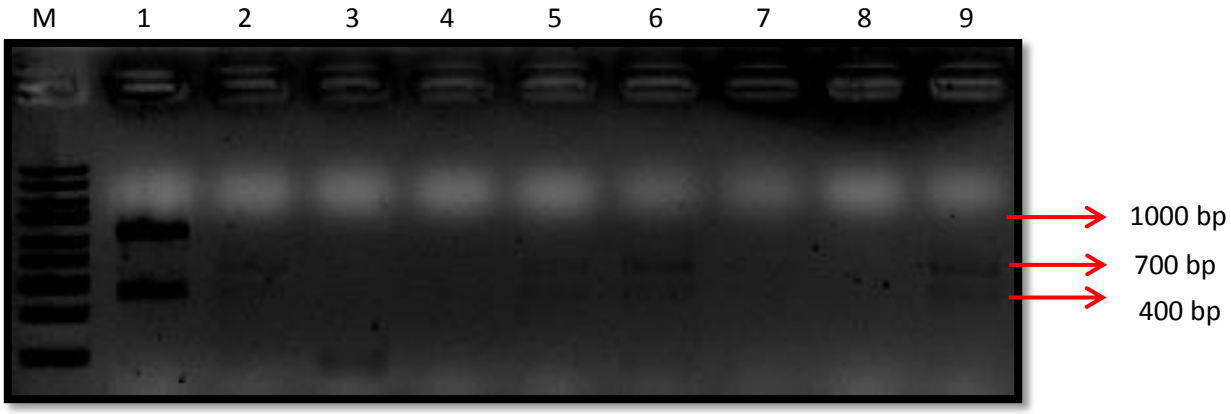

Figure 3.17: *Blg*II restriction digest for all the field isolates. Molecular marker: O'GeneRuler™Express DNA ladder, Lane 1: Modesto, Lane 2: SA-3. Lane 3-10 shows amplification for the Israeli field isolates. Lane 3: SA-3; Lane 4: 155663; Lane 5: 158125; Lane 6: 665; Lane 7: 163396; Lane 8: 159441; Lane 8: 484; Lane 9: 155085.

A restriction digest with *Bgl*II was performed for Israeli isolate 15563 but no bands were observed. This could be due to the fact that the concentrations of the template were too low to be observed on the gel. At this stage it seems like Israeli isolates 155663, 158125 and 159441 are untypable.

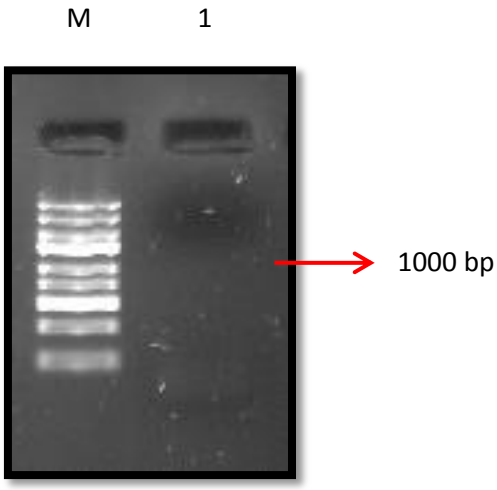

Figure 3.18: *Blg*II restriction digest for Israeli isolate 155663. Molecular marker: O'GeneRuler™Express DNA ladder, Lane 1: 155663.

3.3.3 Sequencing results of the C-3-specific PCR products

All of the samples that were amplified during the C-3 PCR screening were sequenced without subcloning. Sequences were analysed using Geneious Pro v5.4 and compared with known sequences in the GenBank Database using a nucleotide-nucleotide BLAST analysis tool. The sequencing results for the C-3 specific PCR are outlined in Tables 3.2.

Table 3.2: Nucleotide-nucleotide BLAST results for the C-3 PCR for the reference isolates with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	Query coverage	Identity
Reference isolates	ATCC 29545	*Haemophilus paragallinarum* Modesto	AF491827.1	55%	75%
		Haemophilus paragallinarum 221	AF4891826.1	55%	75%
		Haemophilus paragallinarum SA-3	AF4891824.1	55%	75%
	0083	*Haemophilus paragallinarum* Hp8	AY457174.1	81%	77%
		Haemophilus paragallinarum SA-3	AF491824.1	81%	77%
		Haemophilus paragallinarum 0083	AF491820.1	81%	77%
	221	*Haemophilus paragallinarum* Hp8	AY457174.1	72%	89%
		Haemophilus paragallinarum 221	AF4891826.1	72%	89%
		Haemophilus paragallinarum SA-3	AF4891824.1	72%	89%
	2403	*Haemophilus paragallinarum* Hp8	AY457174.1	64%	79%
		Haemophilus paragallinarum SA-3	AF4891824.1	64%	79%
		Haemophilus paragallinarum 0083	AF491820.1	64%	79%
	E-3C	*Haemophilus paragallinarum* H-18	AF491823.1	67%	75%
		Haemophilus paragallinarum SA-3	AF4891824.1	70%	74%
		Haemophilus paragallinarum E-3C	AF4891825.1	70%	74%
	HP-14	*Haemophilus paragallinarum* Hp8	AY457174.1	52%	75%
		Haemophilus paragallinarum SA-3	AF4891824.1	52%	75%
		Haemophilus paragallinarum E-3C	AF4891825.1	52%	75%
	0222	*Haemophilus paragallinarum* Hp8	AY457174.1	56%	89%
		Haemophilus paragallinarum SA-3	AF4891824.1	56%	89%
		Haemophilus paragallinarum 0222	AF4891819.1	56%	89%
	2671	*Haemophilus paragallinarum* Hp8	AY457174.1	37%	87%
		Haemophilus paragallinarum SA-3	AF4891824.1	37%	87%
		Haemophilus paragallinarum 2671	AF4891822.1	37%	89%
	HP-18	*Haemophilus paragallinarum* SA-3	AF491824.1	99%	96%
		Haemophilus paragallinarum Hp8	AY457174.1	99%	96%
		Haemophilus paragallinarum 221	AF4891826.1	99%	96%
	Modesto	*Haemophilus paragallinarum* SA-3	AF491824.1	97%	99%
		Haemophilus paragallinarum Hp8	AY457174.1	97%	98%
		Haemophilus paragallinarum Modesto	AF491827.1	97%	91%
	SA-3	*Haemophilus paragallinarum* SA-3	AF491824.1	96%	94%
		Haemophilus paragallinarum Hp8	AY457174.1	96%	94%
		Haemophilus paragallinarum Modesto	AF491827.1	96%	94%
	HP-60	*Haemophilus paragallinarum* SA-3	AF491824.1	94%	98%
		Haemophilus paragallinarum Hp8	AY457174.1	94%	98%
		Haemophilus paragallinarum Modesto	AF491827.1	94%	98%

Table 3.3: Nucleotide-nucleotide BLAST results for the C-3 PCR for the field isolates with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	Query coverage	Identity
Field isolates	IC 418	*Avibacterium paragallinarum* SA-3	AF491824.1	81%	88%
		Haemophilus paragallinarum Hp8	AY457174.1	81%	88%
		Haemophilus paragallinarum 221	AF4891826.1	81%	88%
	IC 462	*Avibacterium paragallinarum* SA-3	AF491824.1	100%	99%
		Haemophilus paragallinarum Hp8	AY457174.1	100%	97%
		Haemophilus paragallinarum 221	AF4891826.1	100%	97%
	IC 484	*Avibacterium paragallinarum* SA-3	AF491824.1	99%	99%
		Haemophilus paragallinarum Hp8	AY457174.1	99%	98%
		Haemophilus paragallinarum 221	AF4891826.1	99%	98%
	221	*Haemophilus paragallinarum* Hp8	AY457174.1	89%	82%
		Haemophilus paragallinarum SA-3	AF491824.1	89%	82%
		Haemophilus paragallinarum 0083	AF491820.1	89%	82%
	Spross	*Haemophilus paragallinarum* SA-3	AF491824.1	88%	91%
		Haemophilus paragallinarum Hp8	AY457174.1	88%	91%
		Haemophilus paragallinarum 0083	AF491820.1	88%	91%
	Modesto	*Haemophilus paragallinarum* H-18	AF491823.1	97%	77%
		Haemophilus paragallinarum SA-3	AF491824.1	98%	77%
		Haemophilus paragallinarum 0083	AF491820.1	98%	77%
	72	*Haemophilus paragallinarum* Hp8	AY457174.1	73%	92%
		Haemophilus paragallinarum SA-3	AF491824.1	73%	92%
		Haemophilus paragallinarum 0083	AF491820.1	73%	92%
	74	*Haemophilus paragallinarum* Hp8	AY457174.1	71%	88%
		Haemophilus paragallinarum SA-3	AF491824.1	71%	88%
		Haemophilus paragallinarum 0083	AF491820.1	71%	88%
	155663	*Haemophilus paragallinarum* Hp8	AY457174.1	62%	76%
		Haemophilus paragallinarum SA-3	AF491824.1	62%	76%
		Haemophilus paragallinarum 0083	AF491820.1	62%	76%
	158125	*Haemophilus paragallinarum* Hp8	AY457174.1	63%	84%
		Haemophilus paragallinarum SA-3	AF491824.1	63%	84%
		Haemophilus paragallinarum 0083	AF491820.1	63%	84%
	665	*Haemophilus paragallinarum* Hp8	AY457174.1	75%	88%
		Haemophilus paragallinarum SA-3	AF491824.1	75%	88%
		Haemophilus paragallinarum 0083	AF491820.1	75%	88%
	163396	*Haemophilus paragallinarum* Hp8	AY457174.1	66%	83%
		Haemophilus paragallinarum SA-3	AF491824.1	66%	83%
		Haemophilus paragallinarum 0083	AF491820.1	66%	83%
	159441	*Haemophilus paragallinarum* Hp8	AY457174.1	67%	82%
		Haemophilus paragallinarum SA-3	AF491824.1	67%	82%
		Haemophilus paragallinarum 0083	AF491820.1	67%	82%
	484	*Haemophilus paragallinarum* Hp8	AY457174.1	73%	90%
		Haemophilus paragallinarum SA-3	AF491824.1	73%	90%
		Haemophilus paragallinarum 0083	AF491820.1	73%	90%
	155085	*Haemophilus paragallinarum* Hp8	AY457174.1	76%	83%
		Haemophilus paragallinarum SA-3	AF491824.1	76%	83%
		Haemophilus paragallinarum 0083	AF491820.1	76%	83%

3.4. Discussion

Vaccine failures against IC in chickens have increased in the poultry industry. This can be due to the fact that there are increased incidences in the C-3 isolate in SA and the current vaccines not containing the C-3 isolate (Bragg, 2005). Due to poor or no cross protection between the C serovars the inclusion of the C-3 isolate in the vaccines used in South Africa is of vital importance (Bragg, 2005; Soriano *et al*., 2004a). Due to HA/HI being a subjective technique the wrong serovars are often included into the vaccines (Bragg, 2005), which is the main reason for vaccine failures to occur. Therefore there is a need for an accurate molecular serotyping technique. The aim of this chapter was to develop a molecular serotyping technique and two different approaches were followed.

The first approach was the development of a C-3 specific PCR based on the *hag*A gene (Hobbs *et al*., 2002), forms part of haemaggluttinin gene. This gene plays an important role in virulence of the disease causing organism (Hobbs *et al*., 2002). The C-3 PCR was performed on all of the strains and the expected band of 147 bp was observed for all of the isolates and not just for the C-3 serovar as expected (Figure 3.2 and 3.3). Further optimization was performed to try and overcome this problem. It was determined that the optimum annealing temperature for the C-3 PCR was 64°C but amplification was still observed for 4 of the reference isolates (2403, 0222, C-1 and C-3) (Figure 3.6). Therefore this technique was not as specific as anticipated as amplification was only expected for the SA-3 (C-3) isolates. For the reference isolates an addition band of approximately 100 bp was formed which is most likely due to the formation of primer dimers. When the C-3 PCR was performed on the field isolates (Figure 3.7), at the optimum annealing temperature, for the Israeli, Indian and South African isolates from Figure 3.7 it seems that the field isolates can be 2403, 0222, C-1 or C-3. However when the serotyping PCR was performed on the same isolates (Figure 3.11) it was determined that these isolates were serovar C-3. Therefore IC 418, 462,484, SA-72 and 74 were most likely the C-3 serovar of *A. paragallinarum*. When the C-3 PCR was conducted for the other 7 Israeli isolates (Figure 3.8) amplification was observed for all of the isolates but when the serotyping PCR (Figure 3.13) was conducted amplification was not observed for 155663, 158125 and 484. This however does not correspond to the results that were observed during the serotyping PCR. The *hag*A gene was chosen for this PCR as it was the only gene of *A. paragallinarum* that had sequences available on GenBank for all of the reference isolates and it forms part of the haemaggluttinin gene which plays a major role in the virulence of the bacterium (Hobbs *et al*., 2002). Sequencing was performed on the reference isolates (Table 3.2), as well as the

field isolates (Table 3.3) and from the results it was determined that all of the isolates tested were *A. paragallinarum* but it could not distinguish between the different serovars. The reason for this is the fact that the sequences for the reference isolates is too similar and it has proved to be difficult to detect only a 1 base pair change between the different isolates.

In a study performed by Kyger and co-workers (1998) they combined clamping with real-time PCR and were able to successfully detect a single base pair mutation in their targeted gene. Therefore designing probes with clamps for the use in melt curve analysis by means of real-time PCR could be a viable solution to consider in future research.

Due to the C-3 PCR being unsuccessful at this stage a different approach was taken. A multiplex/RFLP PCR was developed by Sakamoto and co-workers (2012) which were also performed. Sakamoto and co-workers (2012) observed the following bands when they performed the multiplex PCR: Serogroup A (800 bp); Serogroup B (1 100 bp) and Serogroup C (1 600 bp). When the multiplex PCR was performed we only observed multiple bands. At the optimum annealing temperature of 57.1°C a 1 000-1 100bp band for all of the reference as well as field isolates were observed. This band corresponds to the 1 100 bp observed for the Serotype B that was observed by Sakamoto and co-workers (2012).

A temperature gradient PCR was performed and the PCR was optimized for our experiments. The restriction digest was performed, with restriction enzyme *Bgl*II and the following banding patterns were observed for the C serovars: C-1 (1 000 bp); C-2 (400 bp and 1 000 bp); C-3 (400 bp and 700 bp) and C-4 (~ 500 bp) as depicted in Figure 3.15 B-E. No digestion was observed for any of the other reference isolates as depicted in Figure 3.15 a. When the restriction digest was performed for the field isolates IC 418, 462, 484, SA-72, SA-74 and Israeli isolates 665, 163396, 159441 and 155085 showed the same banding pattern as the C-3 serovar as depicted in figure 3.16. and 3.17. Therefore, these isolates are most likely the C-3 serovar of *A. paragallinarum*. For 155662, 158125, 484, 70 and 73 the PCR was redone with lower and higher template concentration (Figure 3.14) this was done because something in the PCR reaction might have inhibited the PCR reaction. The expected 1 000 bp band was only observed for isolate 155662 (Figure 3.14) but when the restriction digest (Figure 3.18) was performed no bands was observed this can be due to the

fact that the concentrations was too low to observe it on a gel. Therefore we determined that those isolates were untypable.

The multiplex serotyping PCR resulted in different results than those observed by Sakamoto and co-workers (2012). They observed different sized bands for the serogroups: 800 bp (Serogroup A), 1 100 bp (Serogroup B) and 1 600 bp (Serogroup C). A band of approximately 1 000 -1 100 bp band were observed for all the reference isolates when the multiplex PCR was performed. From our results it seems as if all of the isolates serotyped are B serovars as all isolates has a band of approximately 1 000 bp which corresponds to the multiplex PCR by Sakamoto and co-workers (2012). For our experiments a full set of the Kume serotype reference isolates were used, these are the same isolates that the Kume serotyping scheme are based on. In the article published by Sakamoto and co-workers (2012) the following vaccine strains were used: Serogroup A (W, Georgia, Germany), Serogroup B (Spross), Serogroup C (53-47; HK-1), as well as 221 (A-1), 0083 (A-1), 0222 (B-1) and Modesto (C-2), but nowhere the history of these isolates are stated and it is therefore assumed that these strains are also vaccine strains. This could be the reason for the observed different results than Sakamoto and co-workers (2012). This is the first report where different results were obtained with the multiplex PCR developed by Sakamoto and co-workers (2012), using a full set of reference strains.

The restriction digests resulted in faint bands. This can be a problem as seen for Israeli isolate 155663 where no product for the restriction digest was observed on the gel. This can be due to the fact that the DNA concentrations were too low. The bands observed for the reference isolates were brighter than those observed for the field isolates. Therefore it seems that the DNA concentration is an important factor to consider in the future.

One of the major problems with the ERIC PCR developed by Soriano and co-workers (2004b) was that the banding patterns observed for the reference isolates could not be correlated to the field isolates, which made serotyping very difficult. The serotyping PCR can accurately distinguish between the C-2 and C-3 isolates which is already a step in the right direction as these are the major causes of IC in South Africa (Bragg, 2005). Future research for the serotyping PCR will include the sequencing of the 1 000 bp bands as well as performing a restriction enzyme analysis. A double or triple digest will then be performed

and hopefully we will be able to distinguish between all of the different serovars by observing different banding patterns.

3.5. Concluding remarks

The C-3 PCR shows a lot of promise as a diagnostic technique but further optimization is necessary. A huge step forward was already accomplished with the serotyping PCR as this test is able to accurately distinguish between C-2 and C-3 serovars of *A. paragallinarum*. As the same banding pattern was observed for the field isolates as well as the reference isolates this can already serve as a viable diagnostic test in South Africa where C-2 and C-3 strains are the major cause for IC infection in South Africa. This technique still needs further optimization nd although we were able to accurately distinguish between the C-2 and C-3 serovars this assay cannot be used as a replacement for conventional serotyping.

CHAPTER 4

SCREENING FOR PROPHAGE GENES WITHIN THE GENOME OF *Avibacterium paragallinaurm* REFERENCE AND FIELD ISOLATES

4.1. Introduction

The presence of bacteriophages in the *Pasteurellaceae* family has been determined in numerous cases, which includes the HP2 phage in *Haemophilus influenza*, as well as Mu-like phages in *H. influenza* and *Mannheimia haemolytica* (Williams *et al*., 2002; Resch *et al*., 2004; Morgan *et al*., 2002; Pontarollo *et al*., 1997, Roodt *et al*., 2012). Roodt and co-workers (2012) published the first report on a complete HP2-like and Mu-like prophage, as well as lamdoid phage genes in the *A. paragallinarum* C-2 (Modesto) strain.

Prophages that gets incorporated into the host genome can alter the virulence and pathogenicity of the host bacterium by transfering new functions to the host examples include, resistance to antibiotics, detoxification of heavy metals, acquisition and utilization of certain nutrients, evasion of predators, and production of toxins or colonization of specific environments (Tinsley *et al*., 2006). It has also been seen that in certain cases the presence of prophages could play a role in serotype conversion (Sun *et al*., 2011).

To understand how these detected phages might affect *A. pargallinarum,* it must first be determined whether there are phage genes present in all of the reference isolates. Therefore, this chapter was aimed at the screening for different HP2-like and Mu-like phage genes within the genome of *A. paragallinarum* reference as well as field isolates. The genes that were selected are genes present in the phages found by Roodt and co-workers (2012) and were selected based on their roles during lysogeny. The evolutionary relationship was also determined between the positive screened samples and the Modesto (C-2) strain used

by Roodt and co-workers (2012) and the positive isolates screened by compiling phylogenetic trees.

4.2. Materials and Methods

4.2.1. Enzymes, chemicals, kits and other consumables

The reagents and chemicals used were of molecular, biological or analytical grade and were obtained from the following companies.

Applied Biosystems: BigDye terminator v3.1 Sequencing Kit and 2720 Thermocycler; **Fermentas:** O'GeneRuler™Express DNA ladder, O'GeneRuler™ DNA ladder mix, O'GeneRuler™orange loading dye and deoxynucleoside Triphosphates (dNTP's); **Inqaba:** All synthesis of primers; **Merck:** Glucose, Myo-inositol, NAD, Nutrient broth, Oleic acid, Peptone, Sodium Chloride and Starch; **New England Biolabs®:** Taq Polymerase with Thermopol buffer; **Onderstepoort Biological Products:** Blood Tryptose Agar plates; **Roche:** Bovine Serum albumin; **Sigma Alddrich:** Chicken Serum, Ethidium bromide, Horse Serum and Thiamine Hydrochloride; **Whitehead Scientific (PTY) LTD:** Agarose D1 LE, QIAamp® DNA mini kit.

4.2.2. *Avibacterium paragallinarum* isolates

Bacterial strains used are the same as listed in Chapter 2, Table 2.1. The C-2 Modesto strain that Roodt (2009) used during a PhD study at the University of the Free State was also included into this study as a positive control for the phage screening. The field isolates used during this study were serotyped as discussed in Chapter 3 and is listed in Table 4.1.

Table 4.1: List of field and vaccine isolates and the serotyping results.

Field isolate	Serovar	Vaccine strains	Serovar
IC 418	C -3	Vaccine strain 221	A-1
IC 462	C-3	Vaccine strain Spross	B-1
IC 484	C-3	Vaccine strain Modesto	C-2
SA-70	NT*		
SA-72	C-3		
SA-73	NT*		
SA-74	C-3		
155663	NT*		
158125	NT*		
665	C-3		
163396	C-3		
159441	C-3		
484	NT*		
155085	C-3		

* NT=non-typeable

4.2.3. Cultivation

Bacterial cultures were cultivated in the same manner as stated in Chapter 2, Section 2.2.3, by making use of BTA plates as well as TM/SN liquid media.

4.2.4. Genomic DNA extractions

Genomic DNA was extracted either manually or by making use of the QIAamp® DNA mini kit. For the manual extraction a protocol for gram positive bacteria (Labuschagne, & Albertyn, 2007) was used. This protocol was modified for gram negative bacteria; the use of glass beads were omitted as this is too harsh for gram negative bacteria because of their thin peptidoglycan layer. For the QIAamp® DNA Mini kit from Qiagen genomic DNA was extracted following the protocol for genomic extraction from bacterial plate cultures or bacterial suspension cultures with no modifications. The methods are outlined in Chapter 2, Section 2.2.4. The genomic DNA was eluted in 60 µl nuclease-free water.

4.2.5. Primer design

The primer pairs listed in table 4.2 was designed for the screening of possible phage genes within the genome of *A. paragallinarum*. All of the reference isolates as well as the field isolates were screened. The screening of phage genes was prompted from a PhD study conducted within the research group. Roodt and co-workers (2012) completely sequenced the genome of *Avibacterium paragallinarum* serogroup Modesto (C-2) and upon annotation and assembly of the genome; the presence of a complete HP2-like phage genome and Mu-like phage genome were detected. This posed the question whether similar results will be obtained within the remaining serogroups of *A. paragallinarum*.

4.2.5.1. Gene targets

For the phage screening, genes were selected that play an important role in lysogeny and are therefore most likely to still be intact and functional. The genes selected: HP2-like phage [Tail sheath gene (TSG), C-Repressor gene (CRG), Tail tube gene (HTT), and rep gene (HDP)] and Mu-like phage [Transposase gene (TRP), Major tail subunit gene (MTS), Tail fiber gene (MTF), and Major head subunit gene (MHS)] genes. The tail and head genes plays an important structural role (Williams *et al*., 2002), the C-repressor gene is an important switch to lysogeny (Williams *et al*., 2002), the DNA polymerase gene is important for replication (Williams et al., 2002) and the transposase gene is important for transposition, which is the manner through which Mu phages replicate (Morgan *et al*., 2002). The selected genes of HP2-like phage found in *Haemophilus influenza* (accession number: AY027935) were aligned to selected genes HP2 phage ΦAvpC-2M-HP2 (accession number: JN627908). The genes selected for Mu-like phage (accession number: AF083977) were aligned to selected genes of Mu-like phage ΦAvpmuC-2M (accession number: JN627905) (Morgan *et al*., 2002, Williams *et al*., 2002 Roodt *et al*., 2012). A conserved region was selected for each gene and the primers pairs as listed in Table 4.2 were designed based on this region.

Table 4.2: Different phage screening primer sets as well as the accession numbers of the sequences the primer design is based on.

	Primer sets	Primers	Sequence	Accession numbers
HP2-like phage primers	**Tail sheath gene**	**TSG Forward**	5’-CGC CCT TAA TCA ATT AAG TGG C-3’	AY027935
		TSG Reverse	5’-CGG TGG CAT ACA TTC TCC AGG-3’	JN627908
	Tail tube gene	**HTT Forward**	5’-GCT TAT CCA TTT CCG ACA ACA G-3’	AY027935
		HTT Reverse	5’-GCC GAT TAA ATC GCG GGT GTC-3’	JN627908
	C-repressor gene	**CRG Forward**	5’-GTT AAT ACA GCG GTA TCG CTG-3’	AY027935
		CRG Reverse	5’-GGA AGT GTA TGC CAA AGC G-3’	JN627908
	Rep gene	**HDP Forward**	5’-GAA CAT ATC GCT ATT GCG TGC GG-3’	AY027935
		HDP Reverse	5’-CGC CGT AGA ATT GGA ATT GAC G-3’	JN627908
Mu-like phage primers	**Transposase gene**	**TRP Forward**	5'-GGC AGT TTC TGA TTG CAG -3'	AF083977
		TRP Reverse	5’- CTT ACG CAC AAT CGT TCT GGC -3'	JN627905
	Major Head subunit gene	**MHS Forward**	5'- GCT ATG GCT TCT GGC AAA TGG C -3'	AF083977
		MHS Reverse	5'- CGA TAC CAG CAC GGC GAT AG -3'	JN627905
	Major tail subunit	**MTS Forward**	5'- AGA CCA TTG CGA CAT TAA GT -3'	AF083977
		MTS Reverse	5'- GCG GAA TTG ATT AAC CAA ATC -3'	JN627905
	Tail fiber gene	**MTF Forward**	5'- GTA TAT TGA GAC CAT TGC GAC -3'	AF083977
		MTF Reverse	5'- GGG ATA GTG ATA TTG CCA ATG CC-3’	JN627905

4.2.6. Amplification of phage gene targets by Polymerase Chain Reactions

The reaction mixture contained 5 µl of the 10 x Thermopol buffer, 200 µM of the dNTPs, 2 µM of the of the forward and reverse primers, 2U of Taq DNA polymerase 5 µl of DNA template and the final reaction volume was filled up to 50 µl using sterile nuclease free water. The PCR reaction mixture was the same for all of the different primer sets that were used.

Standard PCR conditions consisting of an initial denaturation (94°C for 2 min) step and 30 cycles of denaturation (94°C for 30 s), annealing (Tm of primer sets for 30 s), elongation (72°C, time depends on amplicon size), and a final elongation cycle of 5 min for 72°C was used throughout. The annealing temperatures and elongation times as well as the amplicon sizes for the different primer sets that were used are shown in Table 4.2. A control was included into each PCR reaction which were the Modesto (C-2) strain that were used during the study conducted by Roodt and co-workers (2012) which Amplified fragments were observed under UV illumination on a 1% agarose gel stained with ethidium bromide.

Table 4.3: The annealing temperatures and the elongation times for the different primer sets, as well as the expected amplicon size for target gene.

Primer set	Annealing temperature (°C)	Elongation time (min)	Amplicon size (bp)
TSG	51	1	986
CRG	49	1	360
HTT	52	1	385
HDP	53	1	791
TRP	50	1	989
MTS	51	1	299
MTF	51	2	1403
MHS	55	1	403

4.2.7. Sequencing

Sequencing was performed by making use of the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems) and the manufacturers protocol was followed with no modifications as outlined in Chapter 2, Section 2.2.7. Sequence analysis was performed using Geneious Pro v5.4 (Biomatters Ltd). Analyzed sequences were compared with known sequences in the GenBank Database using a nucleotide-nucleotide BLAST analysis tool.

4.2.8. Phylogenetic tree constructs

Phage sequences were phylogenetically analyzed. Reconstruction and bootstrapping were conducted using MEGA version 5 (Tamura *et al*., 2011). A Neighbour-Joining (NJ) phylogenetic tree algorithm selected to construct the phylogenetic trees. This method is a simplified version of the minimum evolution (ME) method, where it produces an unrooted tree that does not require the assumption of a constant rate of evolution. However, MEGA displays NJ trees like rooted trees for the ease of inspection (Saitou & Nei., 1987). Isolates from this study were used in construction of these trees.

4.3. Results

4.3.1. Phage PCR results

4.3.1.1. HP2-like phage screening results

Prophage screening PCRs were performed for the following HP2-like phage genes: HTT, TSG, CRG and HDP. The PCRs were performed on both the reference and field isolates.

In the reference isolates various HP2-like phage genes were amplified. The results can be seen in the following figures: HTT (Figure 4.3); TSG (Figure 4.4); CRG (Figure 4.5) and HDP (Figure 4.6).

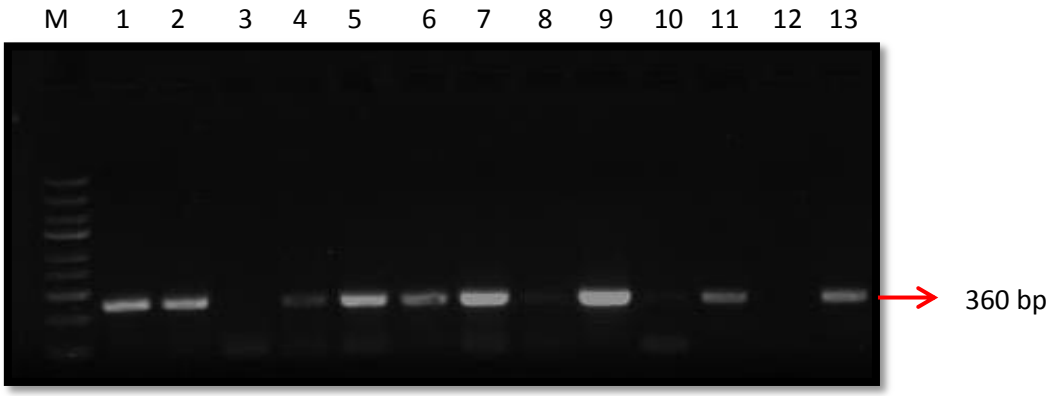

Figure 4.3: Phage screening for tail tube gene (HTT) of the HP2-like phage with an expected band size of 360 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: Control; Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-1); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). No amplification was observed for lanes 3, 10 and 12. The HP 8 (C-1) concentration was too low to sequence.

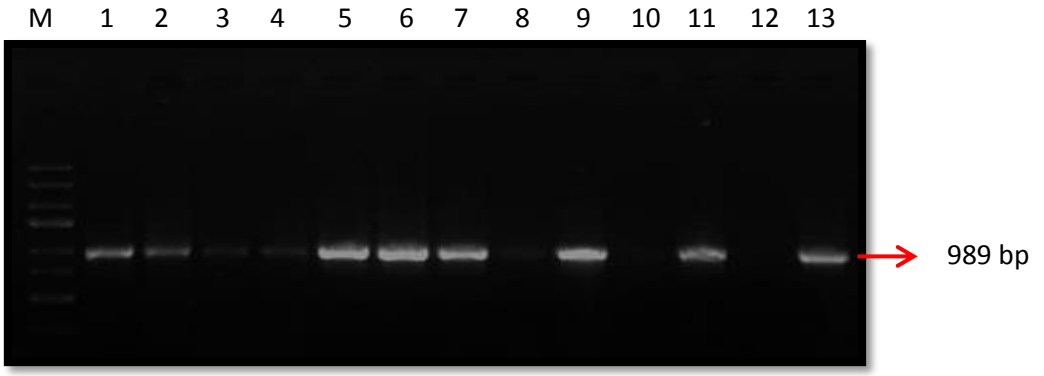

Figure 4.4: Phage screening for tail sheath gene (TSG) of the HP2-like phage with an expected band size of 989 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1; Control; Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-1); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). No amplification was observed for lane 10 and 12.

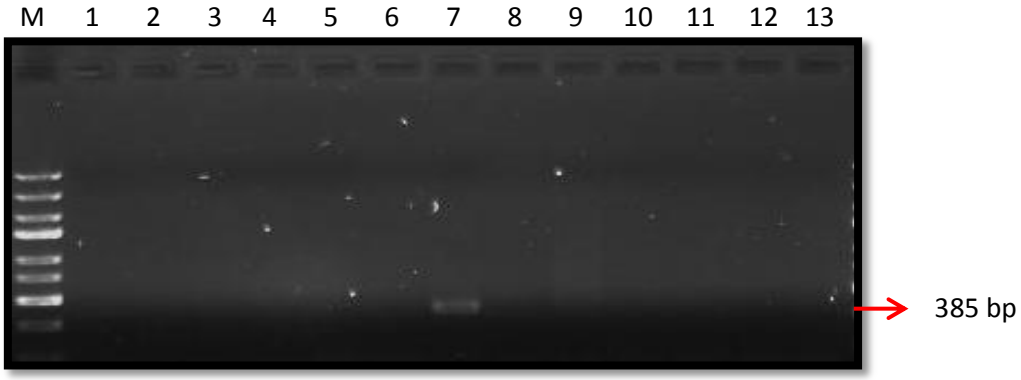

Figure 4.5: Phage screening for C-repressor gene (CRG) of the HP2-like phage with an expected band size of 385 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1; Control; Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-1); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). Amplification was observed for lane 7.

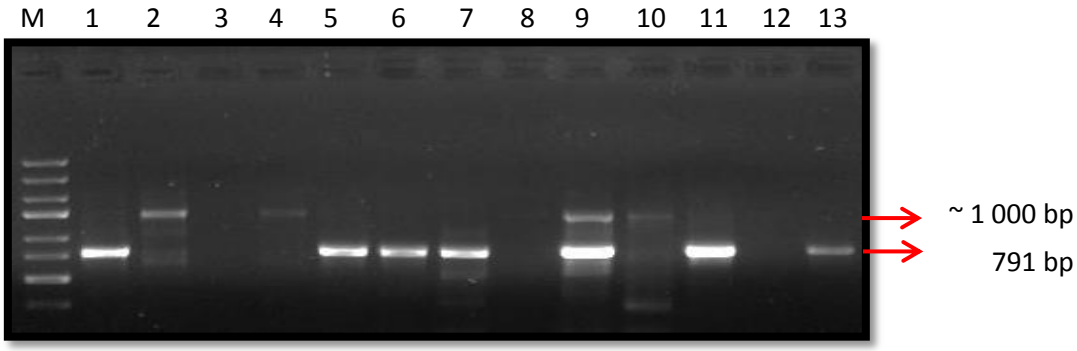

Figure 4.6: Phage screening for rep gene (HDP) of the HP2-like phage with an expected band size of 791 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1; Control; Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-1); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). No amplification was observed for lanes 3, 8, 10 and 12. Non specific binding was observed for lanes 2, 4, 7, 9 and 10.

HP2-like prophage genes were also amplified for the field isolates, the following results are depicted in the following figures: HTT (Figure 4.7 and Figure 4.8); TSG (Figure 4.9 and

Figure 4.10); CRG (Figure 4.11 and Figure 4.12) and HDP (Figure 4.13 and Figure 4.14). These isolates were all serotyped using the molecular serotyping system set up in Chapter 2 and the results of the serotyping are included with the strain name in this chapter.

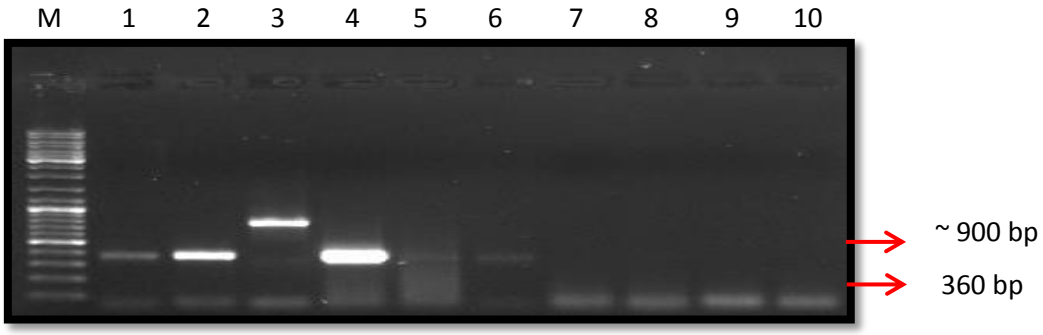

Figure 4.7: Phage screening for tail tube gene (HTT) of HP2-like phage with an expected band size of 360 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). No amplification was observed for lanes 7-10. Non specific binding was observed for lane 3.

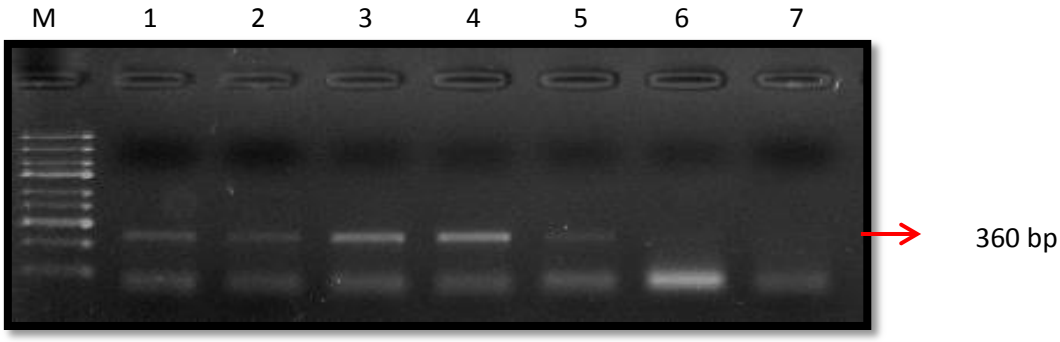

Figure 4.8: Phage screening for tail tube gene (HTT) of HP2-like phage with an expected band size of 360 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). No amplification was observed for lanes 6 and 7.

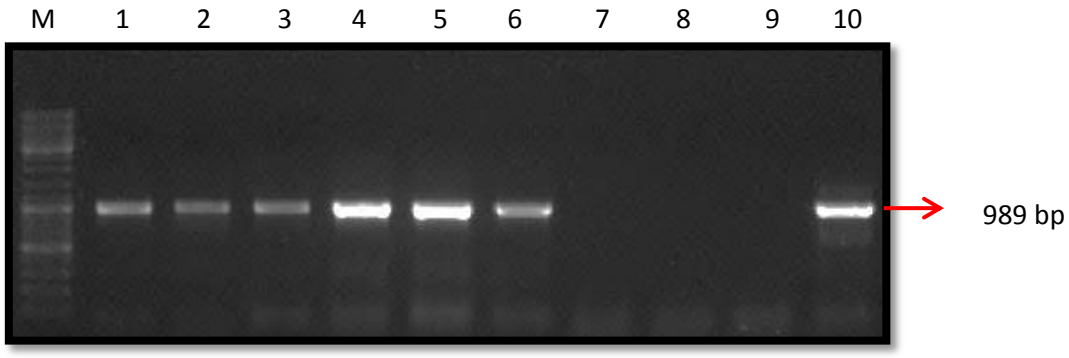

Figure 4.9: Phage screening for tail sheath gene (TSG) of HP2-like phage with an expected band size of 989 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). No amplification was observed for lanes 7, 8 and 9.

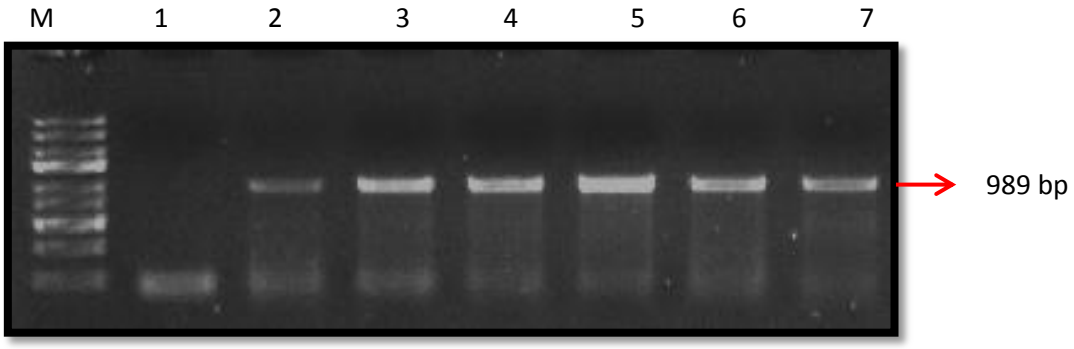

Figure 4.10: Phage screening for tail sheath gene (TSG) of HP2-like phage with an expected band size of 989 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). No amplification was observed for lane 1.

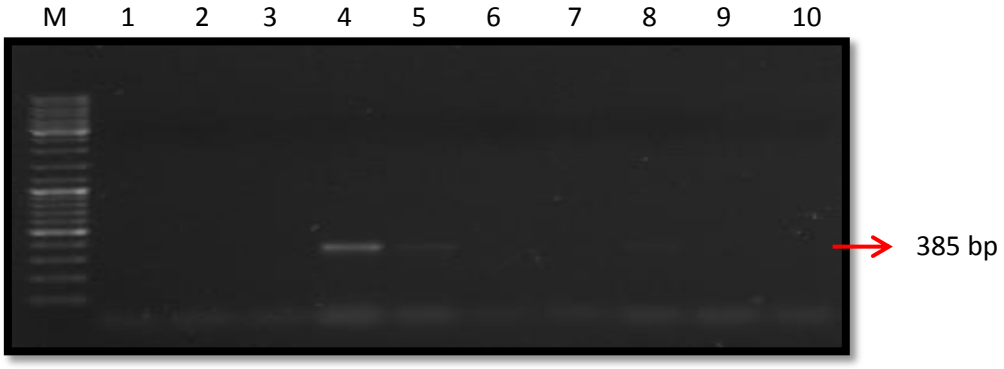

Figure 4.11: Phage screening for C-repressor gene (CRG) of HP2-like phage with an expected band size of 385 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). Amplification was only observed for lanes 4, 5 and 8.

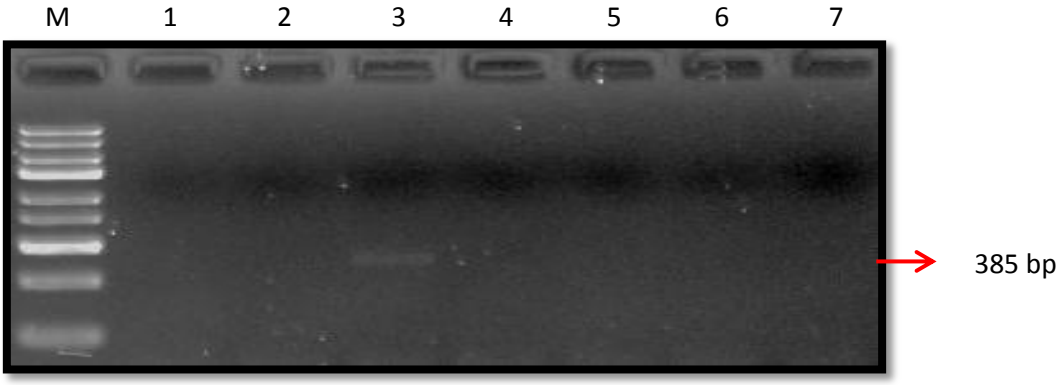

Figure 4.12: Phage screening for C-repressor gene (CRG) of HP2-like phage with an expected band size of 385 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). Amplification was only observed for lane 3.

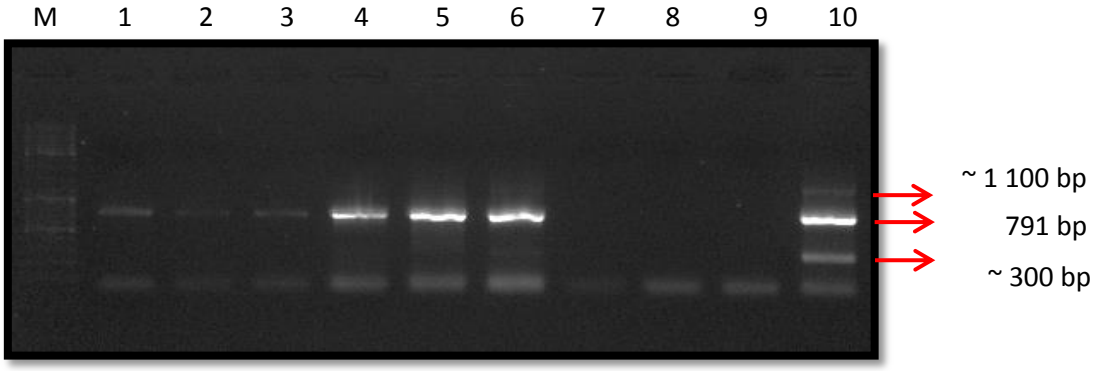

Figure 4.13: Phage screening for rep gene (HDP) of HP2-like phage with an expected band size of 791 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). No amplification was observed for lanes 7, 8 and 9. Non specific binding was observed for lanes 4, 5, 6 and 10.

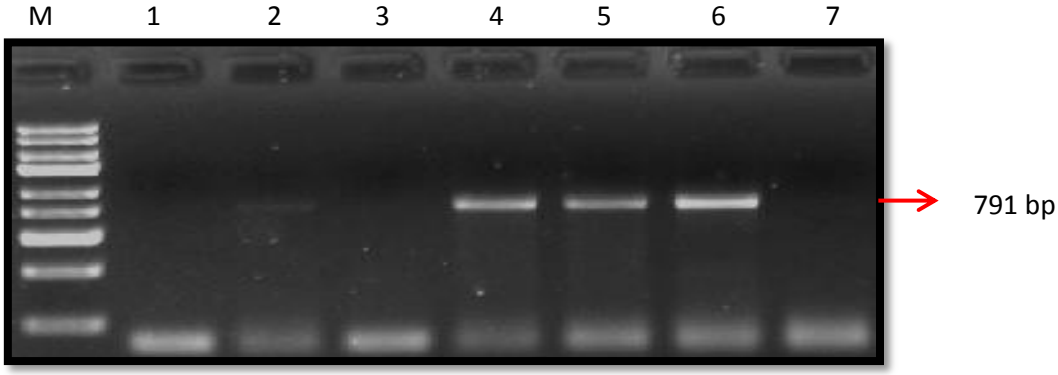

Figure 4.14: Phage screening for rep gene (HDP) of HP2-like phage with an expected band size of 791 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). No amplification was observed for lanes 1, 3 and 7.

From the above observed results it can be seen that not all of the HP2-like phage genes are present in all of the isolates. Thus is can be hypothesized that these genes could therefore be evolutionary remnants present in the bacteria. The HP2-like phage screening results are

summarized below in Table 4.5. All the extra bands that were observed during amplification were also sequenced if the DNA concentration was high enough.

Table 4.4: Summary of HP2-like phage genes present in reference and field isolates.

	Isolate	HTT	TSG	CRG	HDP	Serovar
Reference isolates	ATCC 29545	+	+	-	+	A
	0083	+	+	-	+	A-1
	221	-	+	-	-	A-1
	2403	+	+	-	+	A-2
	E-3C	+	+	-	+	A-3
	HP-14	+	+	+	+	A-4
	0222	-	+	-	-	B-1
	2671	+	+	-	+	B-1
	HP8	-	-	-	-	C-1
	Modesto	+	+	-	+	C-2
	SA-3	-	-	-	-	C-3
	HP-60	+	+	-	+	C-4
Israeli field isolates	IC 418	+	+	-	+	C-3
	IC 462	+	+	-	+	C-3
	IC 484	+	+	-	+	C-3
	155663	-	-	+	-	NT
	158125	+	-	+	+	NT
	665	+	+	+	-	C-3
	163396	+	-	+	+	C-3
	159441	+	-	+	+	C-3
	484	+	-	-	+	NT
	155085	+	-	-	-	C-3
Indian Vaccine Strains	V-221	+	+	+	+	A-1
	V-Spross	+	+	+	+	B-1
	V-Modesto	+	+	-	+	C-2
SA Field Isolates	SA 70	-	-	-	-	NT
	SA-72	-	-	+	-	C-3
	SA 73	-	-	-	-	NT
	SA 74	-	+	-	+	C-3
Control	Y-C2	+	-	-	+	C-2

+: samples where a PCR product was observed; -: samples where no PCR profuct was observed; HTT: Tail tube gene; TSG: Tail sheath gene; HDP: DNA polymerase gene/rep protein gene; CRG: C-repressor gene; NT: Non-typable

4.3.1.2. Mu-like phage screening results

Prophage screening PCRs were performed for Mu-like phage genes. PCRs were performed on both the reference and field isolates. The following results, as depicted in figures outlined were observed for the reference isolates: MTS (Figure 4.15); MHS (Figure 4.16); MTF (Figure 4.17) and TRP (Figure 4.18).

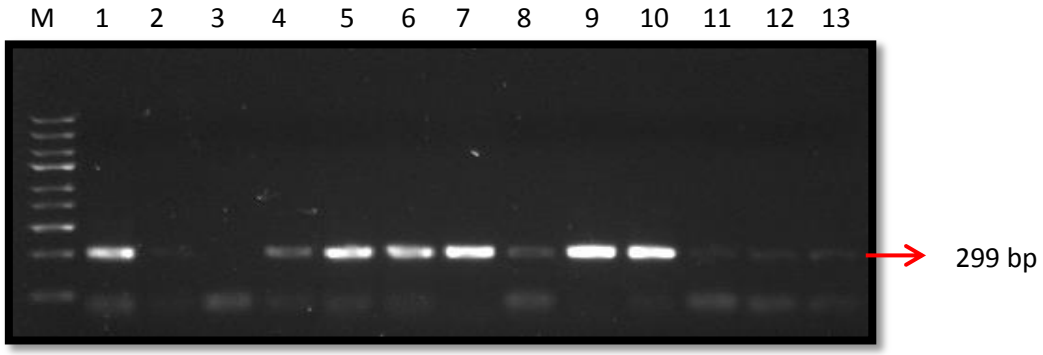

Figure 4.15: Phage screening for major tail subunit gene (MTS) of the Mu-like phage with an expected band size of 299 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1; Control; Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-1); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). No amplification was observed for lane 3.

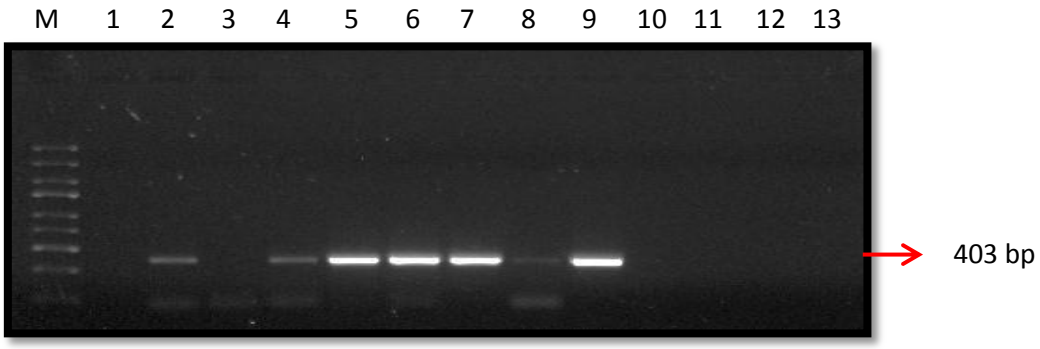

Figure 4.16: Phage screening for major head subunit gene (MHS) of the Mu-like phage with an expected band size of 403 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: Control; Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-1); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). No amplification was observed for lanes 1, 3,10,11,12 and 13.

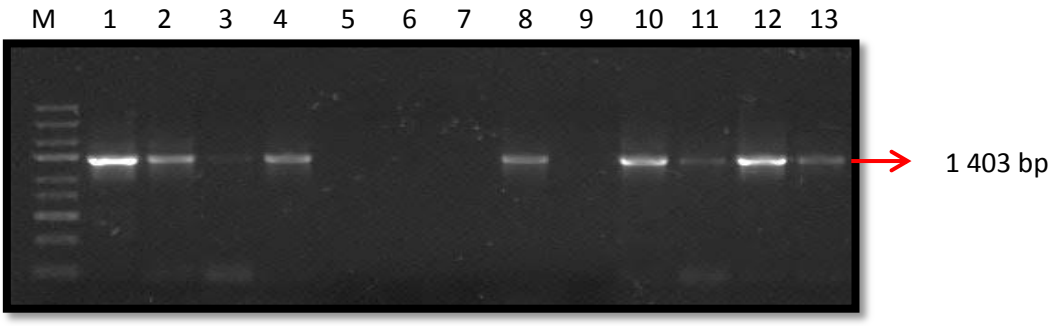

Figure 4.17: Phage screening for tail fiber gene (MTF) of the Mu-like phage with an expected band size of 1 403 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: Control; Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-2); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). No amplification was observed for lanes 5, 6, 7 and 9.

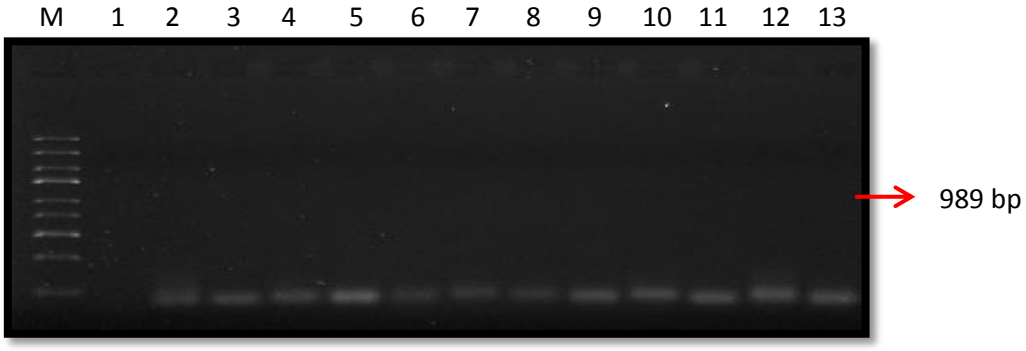

Figure 4.18: Phage screening for transposase gene (TRP) of the Mu-like phage with an expected band size of 989 bp. Molecular marker: O'GeneRuler™Express DNA ladder; Lane 1: Control; Lane 2: ATCC 29545 (A); Lane 3: 221 (A-1); Lane 4: 0083 (A-1); Lane 5: 2403 (A-2); Lane 6: E-3C (A-3); Lane 7: HP-14 (A-4); Lane 8: 0222 (B-1); Lane 9: 2671 (B-1); Lane 10: HP 8 (C-1); Lane 11: Modesto (C-2); Lane 12: SA-3 (C-3); Lane 13: HP-60 (C-4). No amplification was observed for any of the isolates.

For the field isolates the following results, as depicted in figures outlined were observed: MTS (Figure 4.19 and Figure 4.20); MHS (Figure 4.21 and Figure 4.22); MTF (Figure 4.23 and Figure 4.24) and TRP (Figure 4.25 and Figure 4.26).

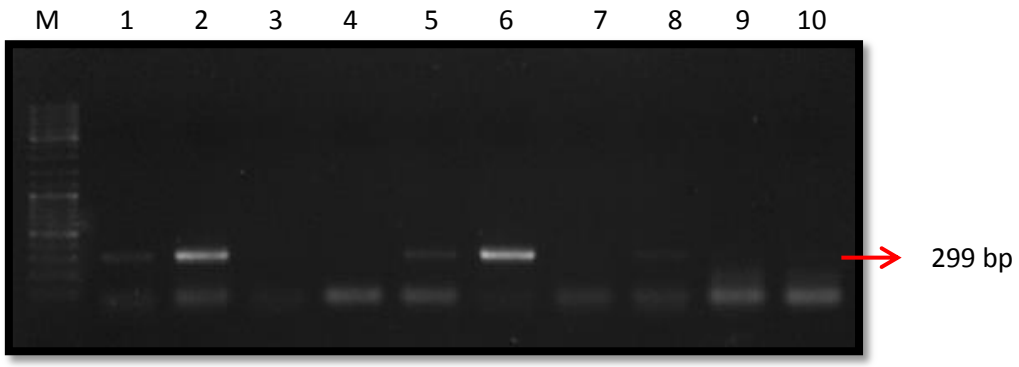

Figure 4.19: Phage screening for major tail subunit gene (MTS) of Mu-like phage with an expected band size of 299 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). Amplification was only observed for lanes 1, 2, 5, 6 and 8.

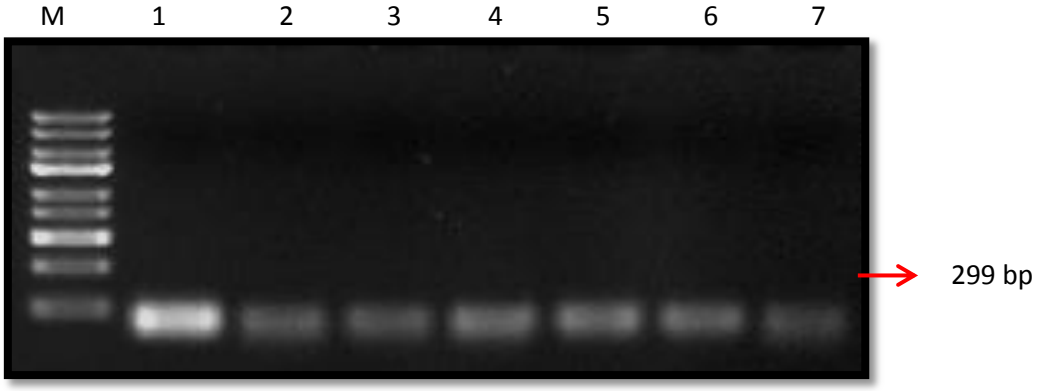

Figure 4.20: Phage screening for major tail subunit gene (MTS) of Mu-like phage with an expected band size of 299 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). No amplification was observed for any of the isolates.

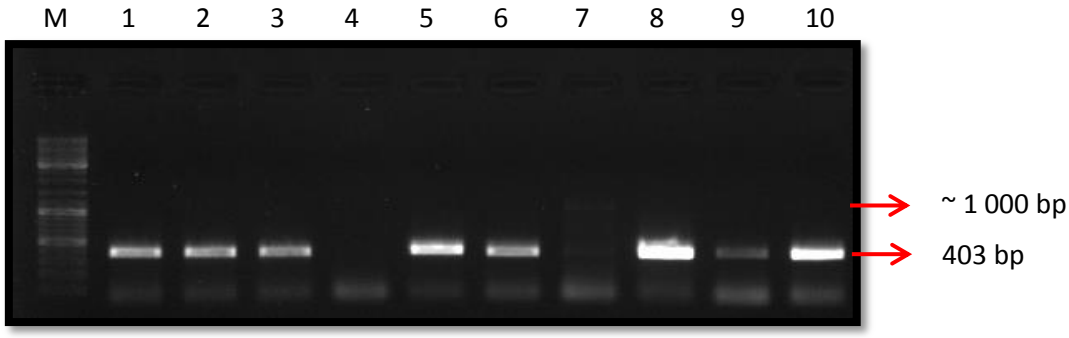

Figure 4.21: Phage screening for Major head subunit gene (MHS) of Mu-like phage with an expected band size of 403 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). No amplification was observed for lane 4. Non specific binding was observed for lane 7.

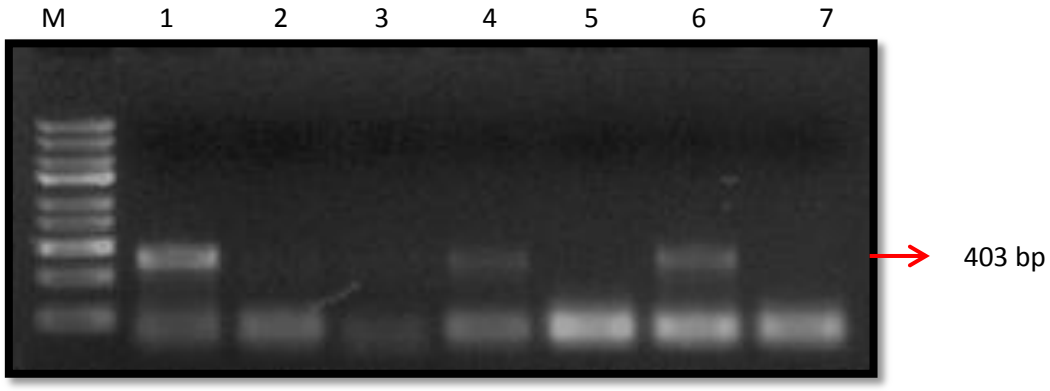

Figure 4.22: Phage screening for major head subunit gene (MHS) of Mu-like phage with an expected band size of 403 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (NT); Lane 6: 484 (NT); Lane 7: 155085 (C-3). Amplification was observed for lanes 1, 4 and 6.

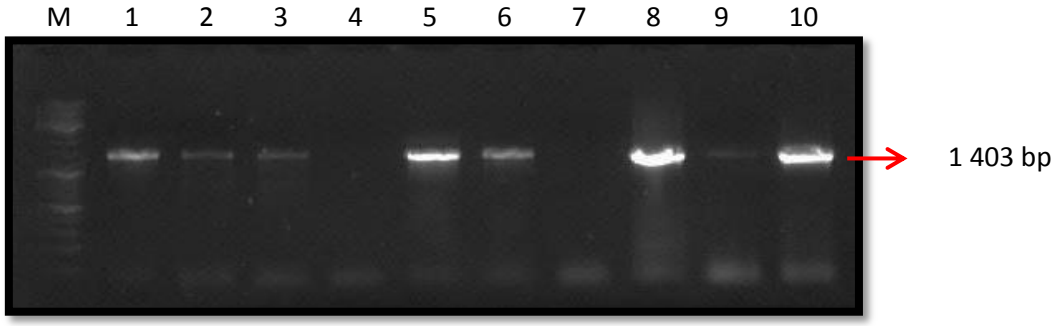

Figure 4.23: Phage screening for tail fiber gene (MTF) of Mu-like phage with an expected band size of 1 403 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). No amplification was observed for lanes 4 and 7. Non specific binding was observed for lane 8 and 10.

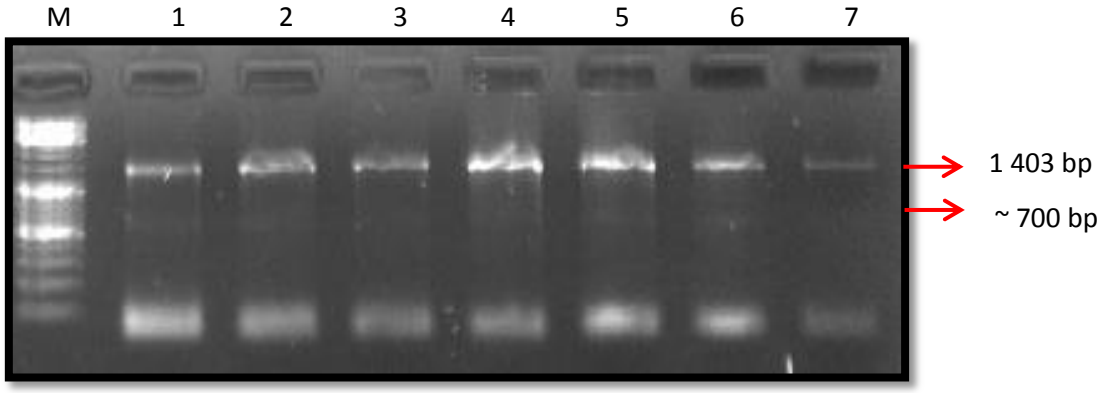

Figure 4.24: Phage screening for tail fiber gene (MTF) of Mu-like phage with an expected band size of 1 403 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). Amplification was observed for all of the isolates. Concentration was too low to sequence non specific bands.

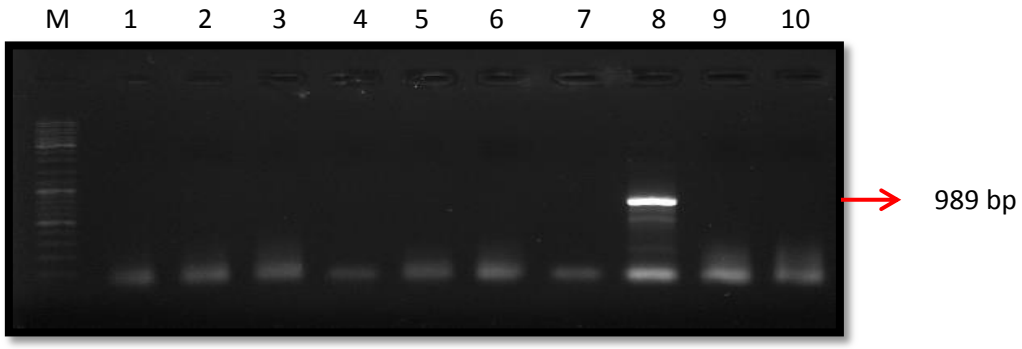

Figure 4.25: Phage screening for transposase gene (TRP) of Mu-like phage with an expected band size of 989 bp. Molecular marker: O'GeneRuler™ DNA ladder Mix; Lane 1: IC 418 (C-3); Lane 2: IC 462 (C-3); Lane 3: IC 484 (C-3); Lane 4: Vaccine strain 221 (A-1); Lane 5: Vaccine strain Spross (B-1); Lane 6: Vaccine strain Modesto (C-2); Lane 7: SA isolate 70 (NT); Lane 8: SA isolate 72 (C-3); Lane 9: SA isolate 73 (NT); Lane 10: SA isolate 74 (C-3). Amplification was observed for lane 8. Non specific binding was observed for lane 8.

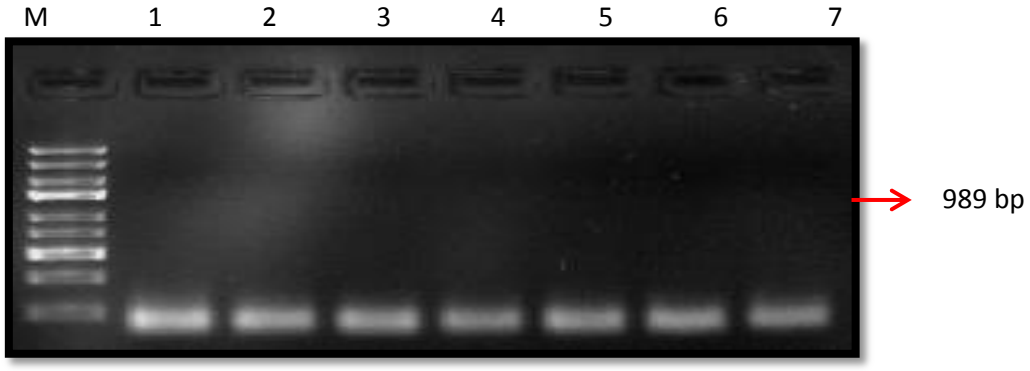

Figure 4.26: Phage screening for transposase gene (TRP) of Mu-like phage with an expected band size of 989 bp. Molecular marker: O'GeneRuler™ Express DNA ladder; Lane 1: 155663 (NT); Lane 2: 158125 (NT); Lane 3: 665 (C-3); Lane 4: 163396 (C-3); Lane 5: 159441 (C-3); Lane 6: 484 (NT); Lane 7: 155085 (C-3). No amplification was observed for any of the isolates.

From the above observed results it can be seen that not all of the Mu-like phage genes are present in all of the isolates. As hypothesized for the HP2-like phages, the presence of these genes could be evolutionary remnants that are present in the isolates of *A. paragallinarum*. The Mu-like phage screening results are summarized in Table 4.6. During prophage screening extra bands were observed in some cases and were also sequenced if the DNA concentration was high enough.

Table 4.5: Summary of all the Mu-like phage genes present in reference and field isolates.

	Isolate	MTS	MHS	MTf	TRP	Serovar
Reference isolates	ATCC 29545	+	+	+	-	A
	0083	+	+	+	-	A-1
	221	-	-	-	-	A-1
	2403	+	+	-	-	A-2
	E-3C	+	+	-	-	A-3
	HP-14	+	+	-	-	A-4
	0222	+	+	+	-	B-1
	2671	+	+	-	-	B-1
	HP8	+	-	+	-	C-1
	Modesto	+	-	+	-	C-2
	SA-3	+	-	+	-	C-3
	HP-60	+	-	+	-	C-4
Israeli field isolates	IC 418	+	+	+	-	C-3
	IC 462	+	+	+	-	C-3
	IC 484	-	+	+	-	C-3
	155663	-	-	+	-	NT
	158125	-	+	+	-	NT
	665	-	+	+	-	C-3
	163396	-	-	+	-	C-3
	159441	-	+	+	-	C-3
	484	-	+	+	-	NT
	155085	-	+	+	-	C-3
Indian VaccineStrains	V-221	-	-	-	-	A-1
	V-Spross	+	+	+	-	B-1
	V-Modesto	+	+	+	-	C-2
South African Field Isolates	SA 70	-	+	-	-	NT
	SA-72	+	+	+	+	C-3
	SA 73	-	+	+	-	NT
	SA 74	-	+	+	-	C-3
Control	Y-C2	+	-	+	-	C-2

+: samples where a PCR product was observed; -: samples where no PCR product was observed; MTS: Major tail subunit gene; MHS: Major head subunit gene; MTF: Tail fiber gene; TRP: Transposase gene; NT: Non-typable

4.3.2. Sequencing results

All of the samples that were amplified during the PCR screening were sequenced without subcloning. For some of the isolates multiple bands were observed that were also sequenced. Sequences were analysed using Geneious Pro v5.4 and compared with known sequences in the GenBank Database using a nucleotide-nucleotide BLAST analysis tool. Sequencing results for the HP2-like phage gene screening is outlined observed in Tables 4.7-4.10 and sequencing results for the Mu-like phage gene screening is in Tables 4.11-4.14.

4.3.2.1. HP2-like phage sequencing results

Table 4.6: Nucleotide-nucleotide BLAST results obtained for the HTT gene for HP2-like phage for both the reference and field isolates, with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	QC	Identity
Reference isolates	ATCC 29545	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	54%	84%
	0083	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	92%	80%
	2403	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	100%	93%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	97%	71%
	E-3C	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	98%	70%
	HP-14	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	100%	97%
	0222	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	100%	70%
	2671	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	100%	83%
	Modesto	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	68%	80%
	HP-60	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	86%	81%
		Haemophilus influenza HP2 complete genome	AY027935.1	52%	68%
	Control	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	100%	88%
Field isolates	IC 462	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	97%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	90%	70%
	IC 418	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	97%	70%
	IC 484	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	99%
	221	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	91%	89%
	Spross	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	83%
	Modesto	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	99%
	155663	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	98%	100%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	92%	71%
	158125	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	70%	75%
	665	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	84%	87%
	163396	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	89%	87%
	159441	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	84%	74%

Table 4.7: Nucleotide-nucleotide BLAST results obtained for the TSG gene for HP2-like phage for both the reference and field isolates, with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	QC	Identity
HP8Reference isolates	ATCC 29545	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	32 %	79 %
	0083	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	71%	76%
	221	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	96%	82%
	2403	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100%	97%
		Haemophilus influenza HP2 complete genome	AY027935.1	96%	70%
	E-3C	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100%	97%
		Haemophilus influenza HP2 complete genome	AY027935.1	98%	70%
	HP-14	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	99%	79%
	0222	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	92%	83%
	2671	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	70 %	84 %
	Modesto	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100%	98%
		Haemophilus influenza HP2 complete genome	AY027935.1	99%	68%
	HP-60	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza HP2 complete genome	AY027935.1	99%	72%
	Control	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza HP2 complete genome	AY027935.1	100%	72%
Field isolates	IC 418	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	95%	69%
	IC 462	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	95%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	93%	67%
	IC 484	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	98%
	221	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	79%	82%
	Spross	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	97%	88%
	Modesto	*Haemophilus influenza* phage HP2 complete genome	AY027935.1	79%	100%
	72	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	75%	81%
	74	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	76%	71%
	158125	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	61%	77%
	665	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	78%
	163396	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	60%	78%
	159441	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	85%	82%
	484	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	99%	81%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	22%	68%
	155085	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	67%	85%

Table 4.8: Nucleotide-nucleotide BLAST results obtained for the CRG gene for HP2-like phage for both the reference and field isolates, with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	QC	Identity
Reference Isolates	HP-14	*Avibacterium paragallinarum* strain modesto clone contig C	JN627908.1	99%	98%
Field isolates	221	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100 %	99%
	Spross	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	95%	98%
	72	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	99%	95%
	665	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100 %	89%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	43%	71%

Table 4.10: Nucleotide-nucleotide BLAST results obtained for the HDP gene for HP2-like phage for both the reference and field isolates, with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	QC	Identity
Reference isolates	ATCC 29545 bottom	*Haemophilus somnus* 2336	CP000947.1	99%	73%
		Haemophilus somnus 129PT	CP000436.1	99%	72%
	ATCC 29545 top	*Pasteurella multocida* 36950 complete genome	CP003022.1	24%	84%
	0083 bottom	*Haemophilus influenza* HP2 genome	AY027935.1	92%	74%
	0083 top	*Pasteurella multocida* 36950 complete genome	CP003022.1	24%	84%
	2403	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	96 %	95 %
	E-3C	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100 %	97 %
	HP-14	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	96 %	82 %
	2671 bottom	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	98 %	65 %
	2671 top	*Pasteurella multocida* 36950 complete genome	CP003022.1	24%	84%
	HP8 top	*Pasteurella multocida* 36950 complete genome	CP003022.1	24%	84%
	Modesto	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	90%	89%
		Haemophilus influenza HP2 complete genome	AY027935.1	90%	73%
	HP-60	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100%	98%
	Reference	*Avibacterium paragallinarum* Modesto clone contig C	JN627908.1	100%	88%
		Haemophilus influenza HP2 genome	AY027935.1	43%	71%
Field isolates	IC 418	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	67%	88%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	35%	71%
	IC 462	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	97%	76%
	IC 484	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	94%	77%
	221	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	72%	79%
	Spross	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	96%	88%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	37%	67%
	Modesto	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	99%	88%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	31%	74%
	72	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	99%	99%
		Haemophilus influenza HP2 genome	AY027935.1	98%	68%
	74 bottom	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	98%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	95%	68%
	74 top	*Pasteurella multocida* 36950 complete genome	CP003022.1	24%	84%
	158125	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	76%
	163396	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	51%	68%
	159441	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza phage HP2 complete genome	AY027935.1	90%	68%
	484	*Avibacterium paragallinarum* strain Modesto clone contig C	JN627908.1	100%	99%
		Haemophilus influenza HP2 genome	AY027935.1	90%	68%

4.3.2.2. Mu-like phage sequencing results

Table 4.11: Nucleotide-nucleotide BLAST results obtained for the MTS gene for HP2-like phage for both the reference and field isolates, with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	QC	Identity
Reference isolates	ATCC 29545	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	94%
	0083	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	94%
	2403	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	80%	74%
	E-3C	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	78%	85%
	HP-14	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	75%	89%
	0222	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	99%
	2671	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	94%
	HP8	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	77%	89%
	Modesto	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	80%	84%
	SA-3	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	92%
	HP60	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	77%	89%
	Control	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	99%
Field isolates	IC 418	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	85%
	IC 462	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	99%
	Spross	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	99%
	Modesto	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	87%	89%
	72	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	93%	85%

Table 4.12: Nucleotide-nucleotide BLAST results obtained for the MHS gene for HP2-like phage for both the reference and field isolates, with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	QC	Identity
Reference isolates	ATCC 29545	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	94%
	0083	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	94%
	2403	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	80%	74%
	E-3C	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	78%	85%
	HP-14	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	75%	89%
	0222	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	99%
	2671	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	94%
Field isolates	IC 418	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	95%
	IC 462	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	96%
	IC 484	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	95%
	Spross	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	83%
	Modesto	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	99%
	70	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	98%	97%
	72	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	98%	97%
	73	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	96%
	74	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	96%
	155663	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	80%	89%
	163396	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	80%	89%
	484	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	80%	89%

Table 4.13: Nucleotide-nucleotide BLAST results obtained for the MTF gene for HP2-like phage for both the reference and field isolates, with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	QC	Identity
Reference isolates	ATCC 29545	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	97%	94%
	0083	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	91%
	0222	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	97%
	HP-8	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	77%	99%
	Modesto	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	83%	100%
	Reference	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	99%
	SA-3	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	85%
	HP-60	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	99%
Field isolates	IC 418	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	83%
	IC 462	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	85%
	IC 484	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	85%
	Spross	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	91%
	Modesto	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	87%
	72	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	85%
	73	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	77%
	74	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	85%
	155663	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	86%
	158125	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	84%
	665	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	84%
	163396	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	84%
	159441	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	100%	84%
	155085	*Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M	JN627905.1	99%	83%

Table 4.14: Nucleotide-nucleotide BLAST results obtained for the TRP gene for HP2-like phage for both the reference and field isolates, with isolates of highest percentage identity and their GenBank accession numbers.

	Sample	Isolate/Species	Accession number	QC	Identity
Reference isolates	72	*Mannheimia haemolytica* M42548 complete genome	CP005383.1	86%	73%
		Haemophilus influenza F3031 complete genome	FQ 670178.1	92%	69%

4.3.3. Phylogenetic tree constructs

Phylogenetic trees were constructed for all of the amplified samples for the HP2-like phage (Figure 4.27-Figure 4.30) and the Mu-like phage (Figure 4.31- Figure 4.34) genes by means of MEGA version 5 (Tamura *et al*., 2011).

4.3.3.1. HP2-like phage genes

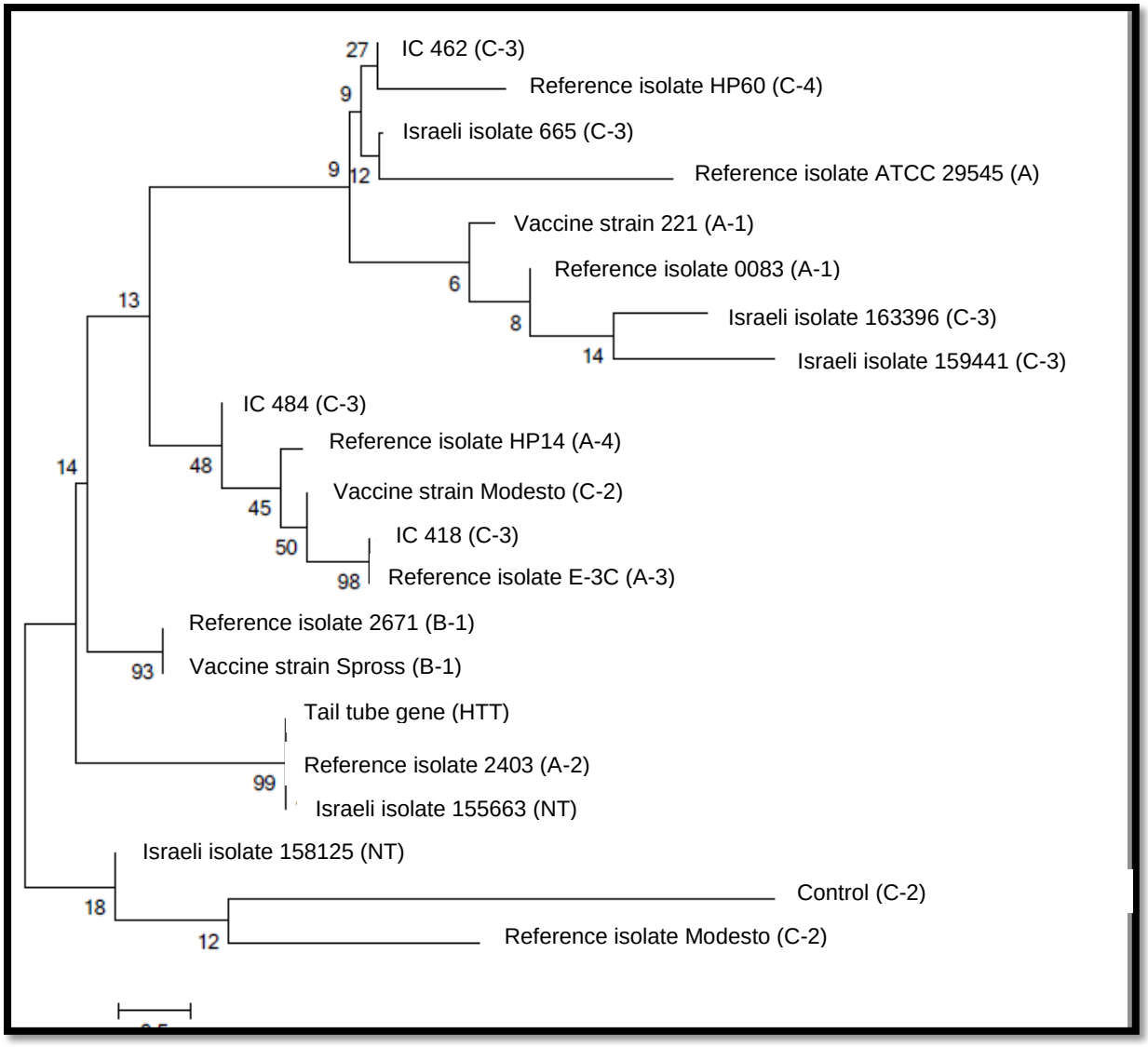

Figure 3.27: A neighbour-joining tree depicting the phylogenetic relationship of the Tail tube gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the HP2-like phage genes posted on GenBank (Accession number: JN627908.1).

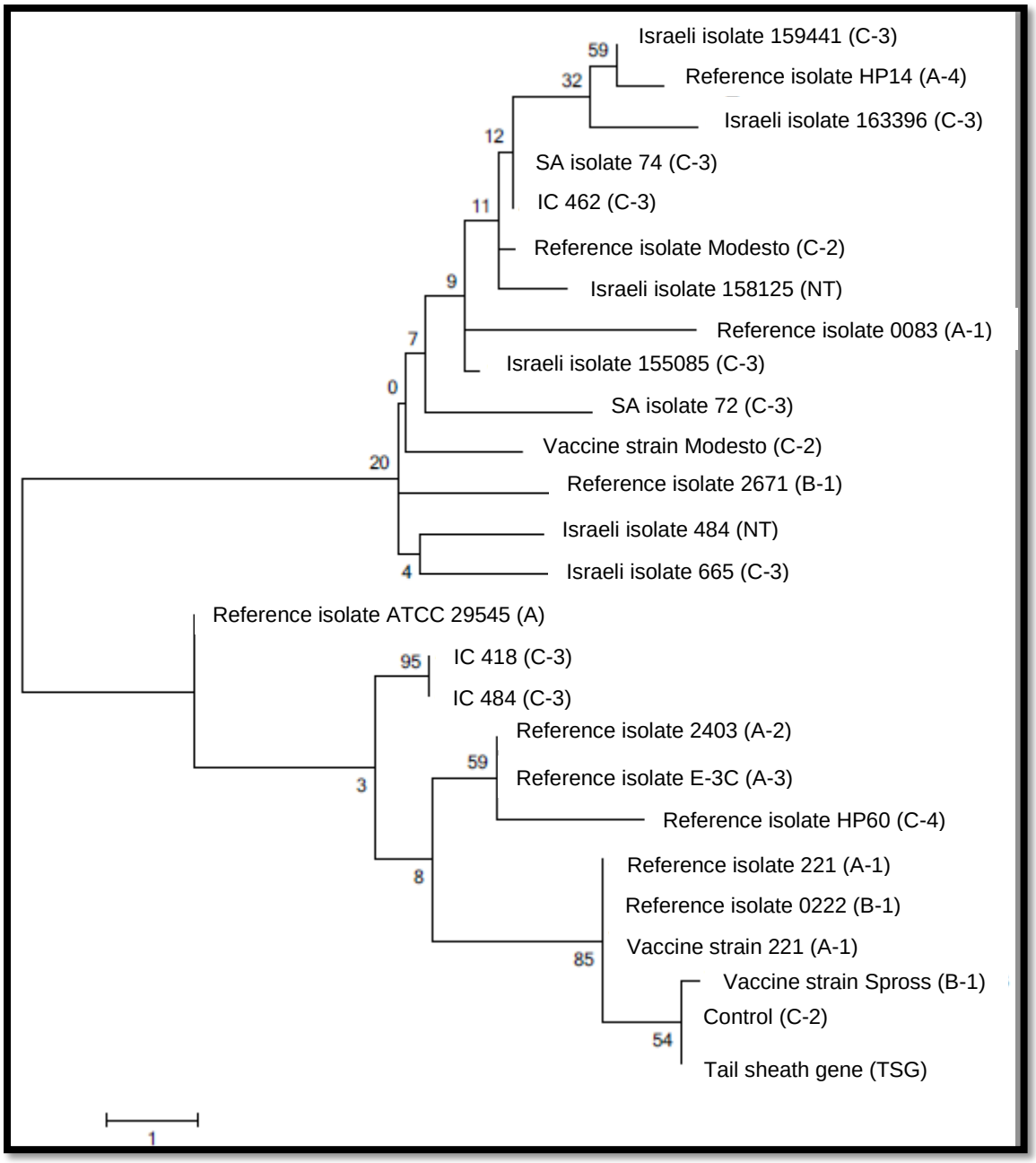

Figure 4.28: A neighbour-joining tree depicting the phylogenetic relationship of the Tail sheath gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the HP2-like phage genes posted on GenBank (Accession number: JN627908.1).

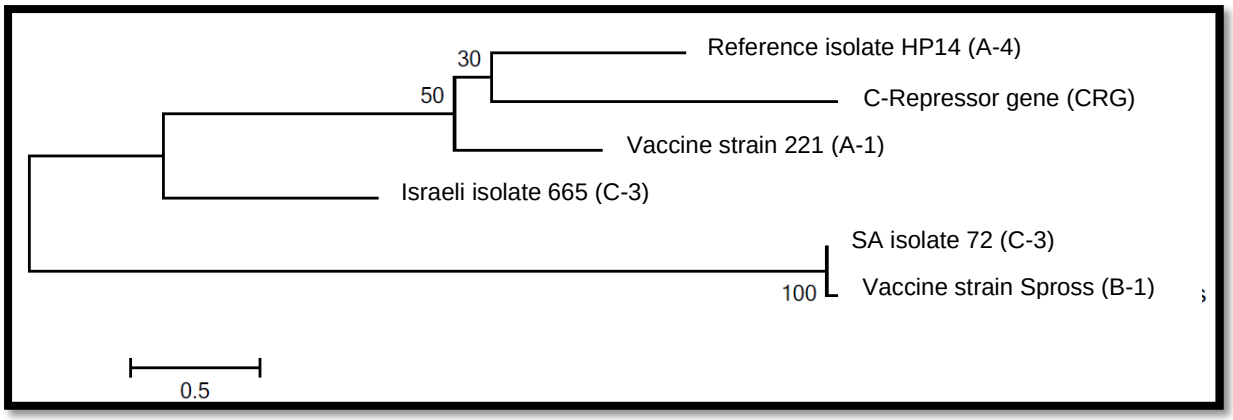

Figure 4.29: A neighbour-joining tree depicting the phylogenetic relationship of the C-Repressor gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the HP2-like phage genes posted on GenBank (Accession number: JN627908.1).

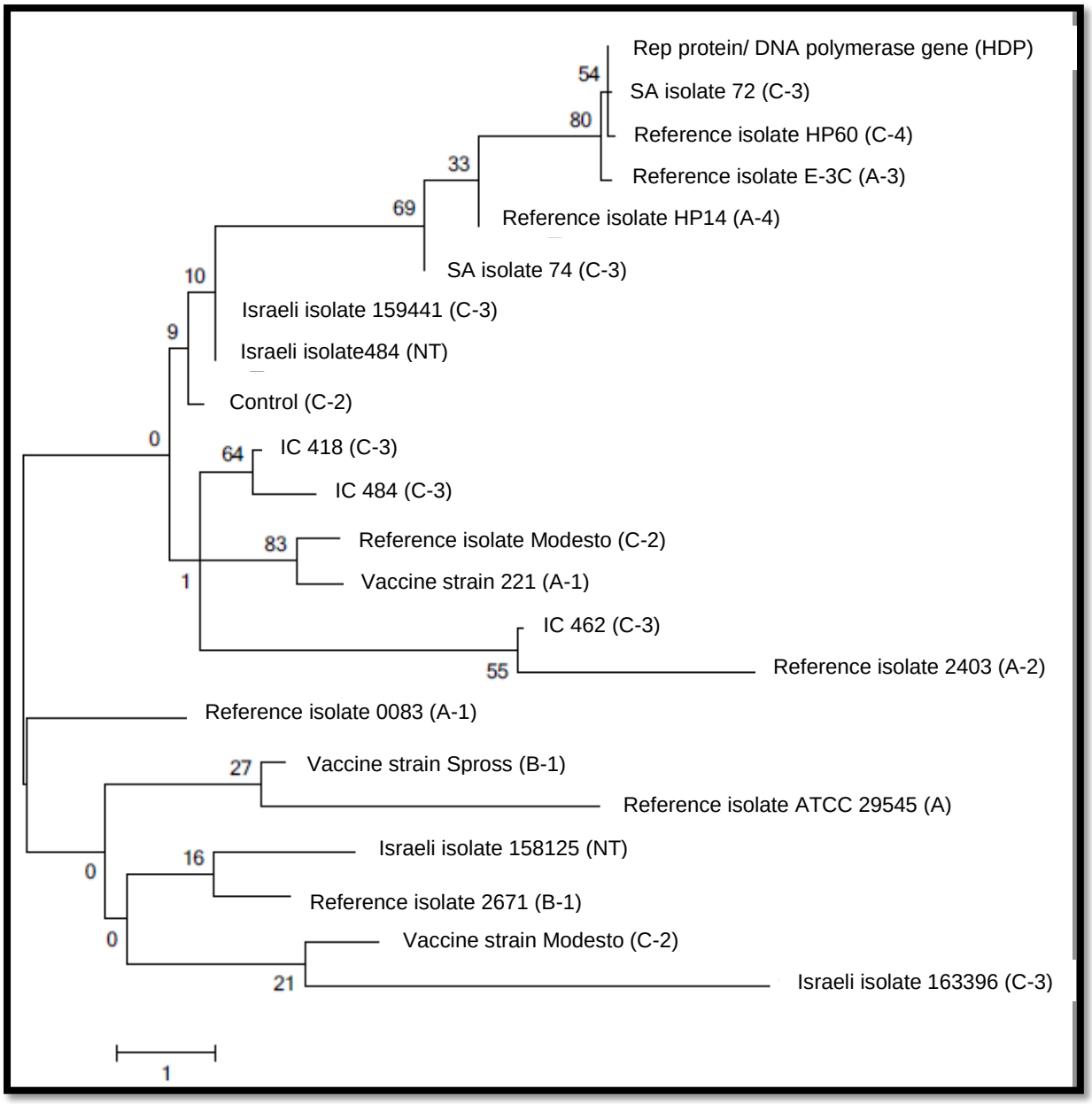

Figure 4.30: A neighbour-joining tree depicting the phylogenetic relationship of the Rep protein/DNA polymerase gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the HP2-like phage genes posted on GenBank (Accession number: JN627908.1).

4.3.3.2. Mu-like phage genes

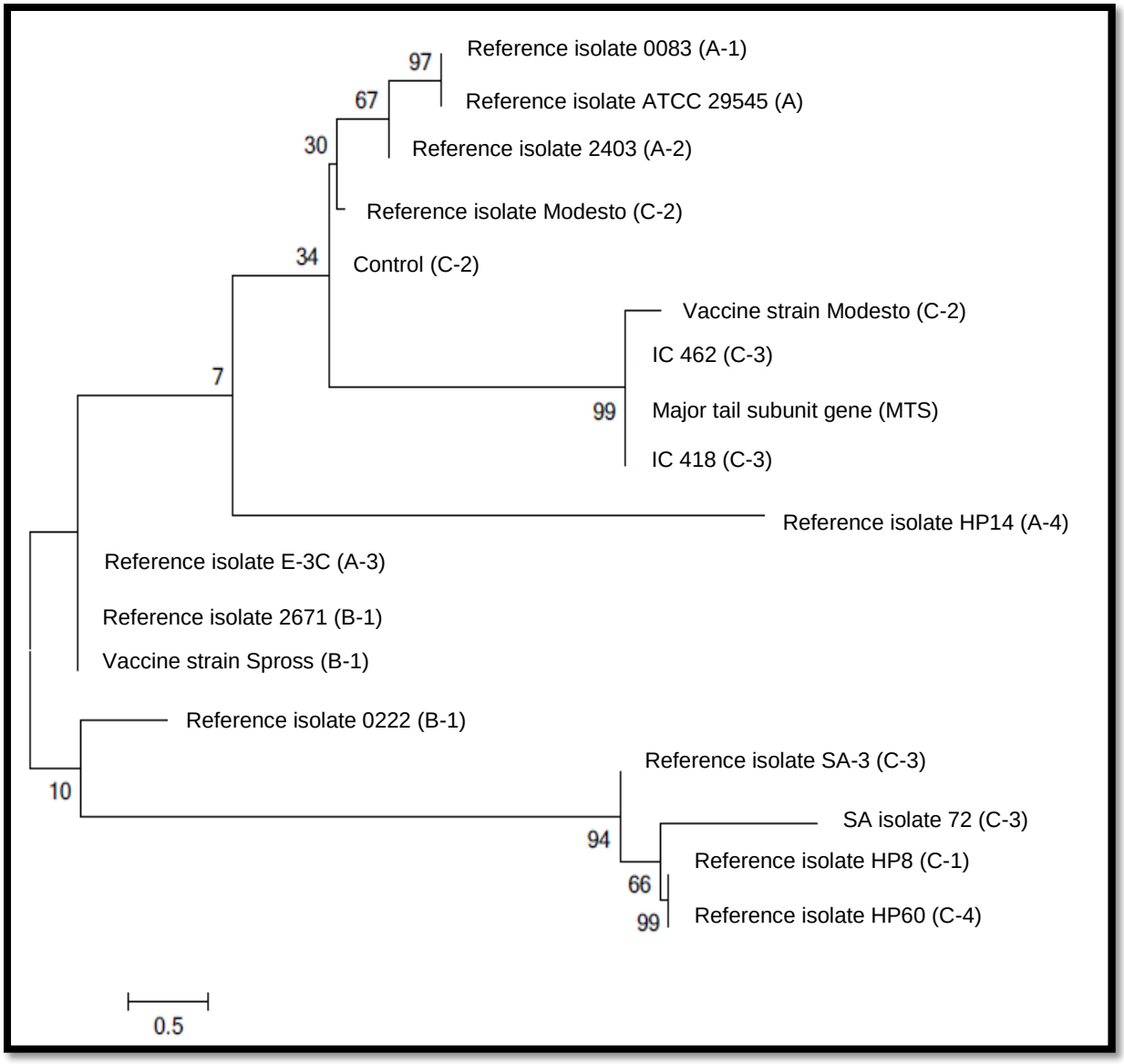

Figure 4.31: A neighbour-joining tree depicting the phylogenetic relationship of the Major tail subunit gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the Mu-like phage genes posted on GenBank (Accession number: JN627905.1).

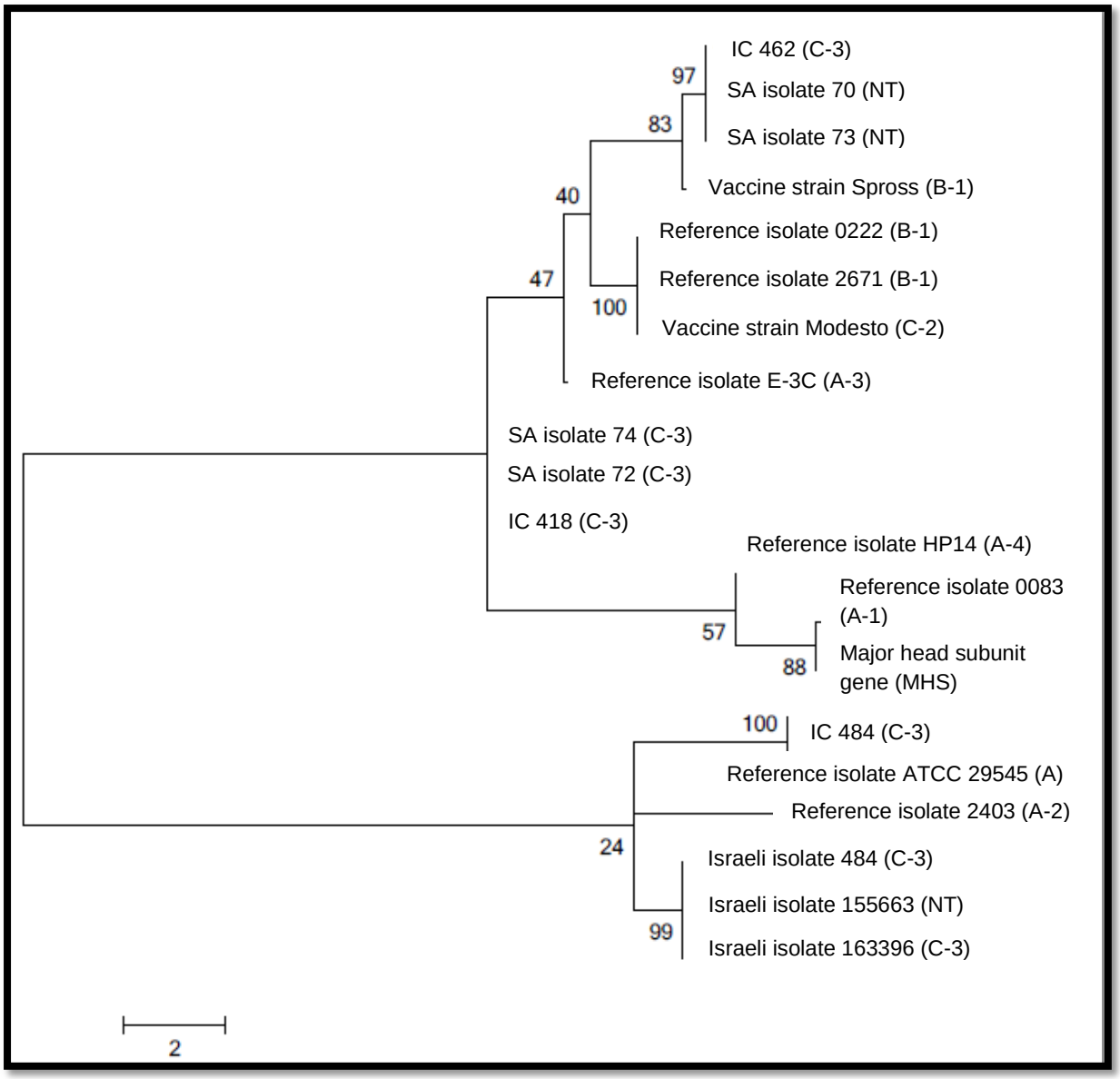

Figure 4.32: A neighbour-joining tree depicting the phylogenetic relationship of the Major head subunit gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the Mu-like phage genes posted on GenBank (Accession number: JN627905.1).

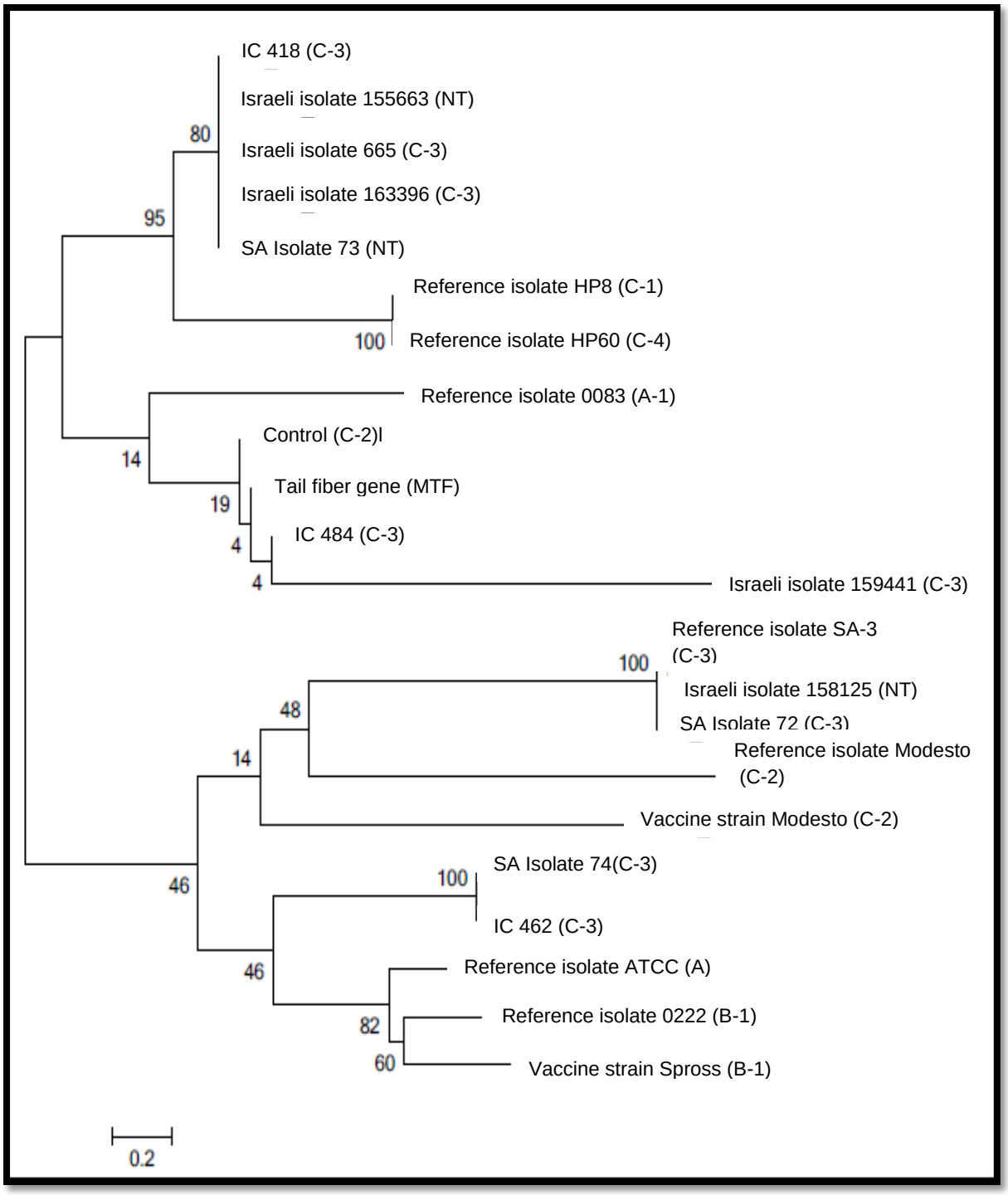

Figure 4.33: A neighbour-joining tree depicting the phylogenetic relationship of the Tail fiber gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the Mu-like phage genes posted on GenBank (Accession number: JN627905.1).

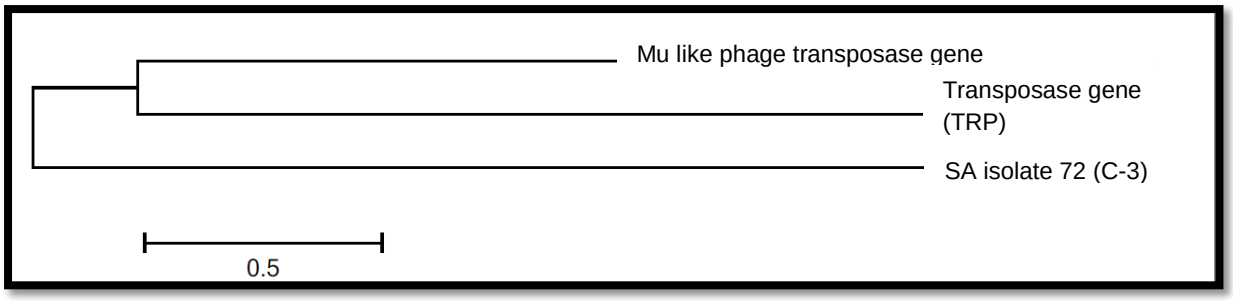

Figure 4.34: A neighbour-joining tree depicting the phylogenetic relationship of the Transposase gene sequences of *A. paragallinarum* reference, field and vaccine isolates with the Mu-like phage genes posted on GenBank(Accession number: JN627905.1).

4.4. Discussion

A. paragallinarum reference, field and vaccine strains were screened for the presence of HP2-like and Mu-like prophage genes within their genome by means of PCR. The genes were selected based on the results reported by Roodt and co-workers (2012). The authors found genes belonging to the HP2-like and Mu-like prophages and genes that are important during lysogeny upon whole genome sequencing of *A. paragallinarum* Modesto (C-2) isolate. To achieve the aim of this chapter all of the isolates which were used for the serotyping experiment described in the previous chapter were used for the detection of prophage genes. In addition the Modesto (C-2) strain used during the full genome sequencing project conducted by Roodt and co-workers (2012) was also included as a control. The PCR screening results are summarized for the HP2-like phage genes in Table 4.4 and for the mu-like phage genes in Table 4.5. This is also the first report that shows the presence of phage genes within the genome of all of the reference isolates as well as in a few field and vaccine isolates.

Different patterns were observed when comparing the HP2-like and Mu-like prophage genes present in all of the reference, field and vaccine isolates. Three (HTT, TSG, HDP) of the four HP2-like phage genes were present as seen in Table 4.4. for all three Modesto (C-2) isolates used during this study i.e. the reference isolate, the vaccine strain used by Roodt and co-workers in 2012 and the Indian vaccine strain. This was expected as this is three strains that are C-3 serovars. For the 221 (A-1) vaccine strain all four HP2-like phage genes were observed but for the 221 reference isolate only 2 of the HP2-like phage genes were

present as depicted in Table 4.4, this result was however not expected. When comparing the screening results for the different Modesto (C-2) isolates none of the isolates had the same or the same amount of Mu-like phage genes present as depicted in Table 4.5. None of the Mu-like phage genes were present in strain 221 (A-1) reference isolate as well as for the vaccine strain 221 (A-1) as depicted in Table 4.5. Therefore it seems that the same serovars does not necessarily have the same phage genes present within their genomes. The fact that the same serovars does not have the same genes present could explain why different results were observed when the multiplex PCR and restriction digest was performed than those observed by Sakamoto and co-workers (2012). This can be due to evolutionary remnant present within the genome of the different serovars, which could have occurred due to horizontal gene transfer (Tinsley *et al*., 2006).

According to the serotyping conducted in Chapter 2 the following isolates were all found to be serovar C-3: IC 418; IC 462; IC 484; SA-72; SA-74; 665; 163396; 159441 and 484. Based on the phage screening result the SA-3 (C-3) reference isolate have none of the HP2-like phage genes present and all of the field isolates have at least one of the genes present as depicted in Table 4.4. It has also been observed that the SA-3 (C-3) reference isolate has 2 Mu-like phage genes present and only 3 of the above mentioned isolates have 2 genes present the rest have more or less genes present as seen in Table 4.5. Therefore it seems as if these HP2-like phage as well as the Mu-like phage genes cannot be used to aid in serotyping.

All four of the HP2-like phage genes are present (Table 4.4) in the following isolates: Reference isolate HP14 (A-4), Vaccine strain 221 (A-1) and Vaccine strain Spross (B-1). For the Mu-like phage screening, South African field isolate 72 have all four of the genes present (Table 4.5). Therefore these strains will be selected when attempting phage induction experiments in the future as these strains most likely have a complete HP2-like phage or Mu-like phage present within their genome. The rest of the isolates do not have all of the genes present and it is therefore most likely that any HP2-like genes or Mu-like genes present might be evolutionary remnants. Also observed are that the C-repressor gene are not present in all of the isolates, as this gene plays an important role in the switch from the lysogenic to the lytic stage (Williams *et al*., 2002) it might mean that some of these isolates have lost the gene over time and are therefore unable to make the switch to the lysogenic stage.

During the phage screening a control was added for all of the genes. The control was the same same sample used by Roodt and co-workers (2012) during the full genome sequencing project. This sample was a Modesto (C-2) isolate of *A. paragalllinrum*. From the results obtained it can observed that amplification did not occur in all of the cases. This result was however not expected as during the genome sequencing project these genes were present. This could mean that the bacteria has lost these genes over time.

The presence of prophages can play a role in virulence of bacteria (Tinsley *et al*., 2006). From these results obtained in this study, no clear linkage could be made between phage genes and the role these genes might have to the virulence of the bacterium. The most virulent strain in South Africa, which is the SA-3 (C-3) (Bragg, 2002), isolate has none of the HP2 phage genes present (Table 4.4) and 1 of the Mu-like phage genes present (Table 4.5) and 0222 (B-1) which is the less virulent strain (Bragg, 2002) in South African and has 1 of the HP2 phage genes present (Table 4.4) and 2 of the Mu-like phage genes present (Table 4.5).

For the HP2-like phage gene screening the sequencing results showed that *Avibacterium paragallinarum* Modesto clone contig had the highest sequence similarity for the HP2-like phage genes which is the sequences posted on GenBank by Roodt and co-workers (2012). *Haemophilus influenza* HP2 complete genome in most cases showed the second highest homology. For the Mu-like phage gene screening the sequencing results showed that *Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M shows the highest sequence similarity for the Mu-like phage genes. It is important to state that prophages gets inserted into their host genomes and the fact that *A. paragallinarum* shows the highest sequence similarity means these genes are present within those host genomes. Phages are very host specific and therefore this results is expected, as those gene observed in *A. paragallinarum* is similar to *H. influenza* as they are both similar in structure to HP2-like phages, as was the case with the Mu-like phage genes. In some of the isolates screened for HP2-like and Mu-like phage genes different *Pasteurallaceae* species also come up during the BLAST search, which is also expected as these phages might have some of the same genes and/or it might be the the same type of bacteriophage. Sequencing results for the screening of the transposase gene (TRP) for South African isolate 72 showed the highest sequence similarity for *Mannheimia haemolytica* M42548 genome for the Glucosamine-fructose-6-phosphate aminotransferase and *Haemophilus influenza* F3031 comlpete genome for the Glucosamine-fructose-6-phosphate aminotransferase. These species are also part of the *Pasteurallaceae*

family and it could mean that the primers were just not specific enough; it could also mean that this transposase gene was not present within South African isolate 72.

During the phage screening experimens extra bands were observed for some of the samples screened. For the DNA polymerase gene (HDP) extra bands were observed for the following reference isolates (figure 4.6): ATCC 29545 (A), 0083 (A-1), HP14 (A-4), 2671 (B-1) and HP8 (C-1) and the following field isolates (figure 4.13): Vaccine strain 221, Vaccine strain Spross, Vaccine strain Modesto and South African isolate 74. The extra bands were sequenced for ATCC, 0083, 2671 and 74 and showed the highest sequence similarity for *Pasteurella* 36950 complete genome. The DNA concentration was too low for the faint extra bands to be sequenced. The PCR was repeated but the DNA concentration continued to be too low to sequenced. Due to the fact that the other bands showed the highest homology for *P. multocida* 36950 complete genome it is likely that the other bands would also show the highest homology for *Pasteurella multocida* 36950 complete genome. These extra bands are most likely due to non specific binding of the primers. Extra bands were also observed for MHS 70 (figure 4.21), MTF (figure 4.24) and TRP 72 (figure 4.25) but could not be successfully sequenced as the DNA concentration was too low. The PCRs were repeated for all of these isolates but the DNA concentration continued to be too low to sequence. From figure 4.7 it can be seen that Israeli isolate 418 had a band bigger than the expected band of 385 bp when screened for the tail tube gene (HTT), this band was sequenced and showed the highest homology for *Haemophilus influenza* HP2 complete genome. The bigger band size could be due to evolutionary changes over time. A faint band of the right size was also observed but could not be sequenced due to the DNA concentration being too low.

In order to have a more meaningful interpretation of the genetic diversity between the HP2-like phage genes present in all of the *A. paragallinarum* isolates screened phylogenetic analyses was perfomed. Reconstruction and bootstrapping were conducted using MEGA version 5 (Tamura *et al.*, 2011). Neighbour-joining (NJ) phylogenetic trees for HTT (fig 4.27), TSG (fig 4.28), CRG (figure 4.39) and HDP (fig 4.30) were constructed. Neighbour-joining (N-J) phylogenetic trees were also constructed for the Mu-like phage genes, for MTS (fig 4.31), MHS (fig 4.32), MTF (figure 4.33) and TRP (fig 4.34). No clear pattern was observed from the phylogenetic trees, as in none of the cases the genes screened for did any of the expected isolates cluster together i.e. the isolates belonging to the same serogroups. This could mean that these genes were most likely obtained through horizontal

gene transfer and have evolved substantially over time; therefore all of the genes present in all of the same isolates like the C-3 isolates, are not necessarily the same. It can however be said that these genes are genetically related to each other. The low bootstrap values that were observed can be due to recombination, gene flow between different branches, low sequence diversity or homoplasy.

Future research will include phage induction experiments; if the phage induction experiments prove successful screening for different virulence genes will be attempted. These results will help to determine the effect these phages might have on the virulence of *A. paragallinarum* isolates. Screening for other types of phages for example lamdoid phage could also be attempted as Roodt and co-workers (2012) found lamdoid phage genes present within the genome of *A. paragallinarum*. Screening for phage conversion genes can also be conducted in the future as this might be the reason for the occurrence of different *A. paragallinarum* serovars.

4.5. Concluding remarks

No correlation were observed between the presences or absence of prophages genes, the serotyping results or known virulence. More in depth studies needs to be conducted to effectively determine the role the presence these phages have on the different *A. paragallinarum* isolates.

The overall conclusion is that all of the strains of *A. paragallinarum* which were used in this study showed evidence of prophage genes. No correlation to either serotyping or virulence could be found from these two prophages. This work indicates that these phage sequences appear to be evolutionary reminates. It would also appear that in most cases, only parts of the phages remain and it is highly unlikely that intact HP-2, or Mu-like phages are present in all but a few isolates.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Vaccine failures are a major problem in the effective control of IC in the poultry industry. This is mainly due to the incorrect strains of *A. paragallinarum* being used in vaccines. Vaccines failures can also be due to the fact that international vaccines are not effective against local strains of *A. paragallinarum* it is therefore important that local strains be included into vaccines (Bragg *et al*., 1996). This is especially a problem in the serogroup C strains as little to no cross protection occurs between the different C-serovars of *A. paragallinarum* (Soriano *et al*., 2004a). Therefore an accurate serotyping method is of great importance, where a molecular technique will be a lot faster, cheaper and accurate to perform. Another advantage of a molecular serotyping technique is the use of FTA cards for the collection of samples, as it is often difficult to get live cultures from some romote locations.

Therefore the first aim of this project was to develop a molecular serotyping technique to distinguish between the most prevalent strains in South Afirca which has been found to be serovar A1, B-1, C-2 and C-3 (Bragg *et al*., 1996). The C-3 serovar is also the most virulent strain in South Africa (Bragg, 2002), therefore a PCR was developed that specifically target a part of the *hag*A gene of the C-3 serovar. Due to the sequences being too similar and it being difficult to distinguish the C-3 serovar from the other serovars by just a 1 bp difference, this technique proved unsuccessful and a different approach was taken. The multiplex and RFLP PCR that was developed by Sakamoto and co-workers (2012), was optimized as different results were observed by Sakamoto and co-workers (2012) when the multiplex PCR was performed. A band of approximately 1 100 bp was observed for all of the reference isolates, where Sakamoto and co-workers (2012) observed the following bands: serogroup A (800 bp), serogroup B (1 100 bp) and serogroup C (1 600 bp). From the multiplex PCR it would seem as if all of the reference and field isolates are the B serogroup. This is however not the case as a full set of Kume reference isolates were used to conduct this study where these isolates were serotyped by means of the HA/HI assay. In order to get more accurate

results a restriction digest was performed with the restriction enzyme *Bgl*II for all of the reference isolates and the following banding patterns were observed for, serovar C-2 (400 bp and 1 000 bp) and serovar C-3 (400 bp and 700 bp) and no digestion for any of the other reference isolates. The results that were observed for the reference isolates correlated with the results obtained for the field isolates that were also used during this study. Field isolates used were obtained from South Africa as well as Israel and India. From the serotyping results obtained of the Israeli and Indian field isolates most of the samples were serotyped as belonging to the C-3 serovar. Therefore, this suggests that the C-3 serovar has recently become a major problem in Israel and India as well. Most of the field isolates were serotyped as being the C-3 serovar. It was possible to accurately distinguish between the C-2 and C-3 serovar of *A. paragallinarum* as two different banding patterms were observed for the serovars following the restriction digest. Therefore some of the field isolates could accurately be serotyped as the C-2 or C-3 serovar. In other molecular techniques like the ERIC PCR it is difficult to serotype as the reference and field isolates show different profiles when run on a gel (Bragg 2010-personal communications). Therefore an accurate and easy molecular technique is of immense importance in the accurate diagnosis of IC in chickens. In our research group various samples have been tested with the serotyping PCR and the same results have consistantly been observed every time. Therefore it is already a major advantage to be able to distinguish between the C-2 and C-3 serovars that is the predominant cause of IC infections in South Africa.

The first report on the presence of HP2-like and Mu-like prophages within the genome of *A. paragallinarum* Modesto (C-2) strain was published by Roodt and co-workers (2012). Therefore the second aim of this study was to screen for different HP2-like and Mu-like phage genes in all of the reference isolates of *A. paragallinarum*, to establish if these phages play a role in serotyping. This is also the first report of prophage genes present in all of the reference isolates as well as field isolates of *A. paragallinarum*.

HP2 and Mu-like phage genes were observed in all of the reference, field and vaccine isolates and are summarized in Table 3.3 and Table 3.4. The genes of the isolates that tested positive for a phage gene, was sequenced in order to confirm that it was the right gene. These sequences were also used for the construction of the phylogenetic trees. From the sequencing results for all of the HP2-like genes screened it can be seen that *A. paragallinarum* Modesto clone contig C showed the highest homology for the HP2-like phage genes which is the sequences posted on GenBank by Roodt and co-workers (2012).

From the sequencing results for all of the Mu-like genes screened it can be seen that *Avibacterium paragallinarum* strain Modesto clone AvpmuC-2M shows the highest homology for the Mu-like phage genes. The sequences posted on GenBank by Roodt and co-workers (2012) are currently the only *A. paragallinarum* phage genes posted which makes interpretation of the results difficult. Phylogenetic trees were also constructed for all of the HP2 and Mu-like genes. From these results it was determined that that the different genes were genetically related. Sequence differences can be due to random mutations over time which also supports the theory that these genes most likely evolutionary remnants. It could also be that these isolates were infected with closely related phages.

It has long since been established that the presence of bacteriophages can transfer new functions to a bacterial cell like the alteration of its virulence (Tinsley *et al*., 2006). An example of this is where avirulent strains of *Corynebacterium diphtheria* that is infected with virulent lysogens resulted in the production of the diphtheria toxin (Tinsley *et al*., 2006). Therefore the presence of prophage sequences could explain the differences in virulence between the different serovars. Therefore screening of phage genes that play an important role in lysogeny was conducted. From the results observed it was determined that prophage genes are present in all of the reference isolates of *A. paragallinarum*. In South Africa It has been established that the C-3 strain is the most virulent but from our results it can be seen that C-3 has none of the phage genes present. Therefore the specific genes screened for during this project do not seem to have any effect on the virulence of the strain. For future research, screening for different types of prophages will be conducted as well as screening for known virulence genes in these phages. In doing so a clearer picture will be obtained on the effect the presence of these phages might have on the virulence of the different *A. paragallinarum* isolates.

Some phages show high host strain specificity and this might be used as a phage typing scheme. Phage typing schemes have been commonly used to differentiate between different strain of bacteria such as *Listeria* and *Salmonella* (Loessner & Busse, 1991; Ward *et al.* 1987). Therefore the presence of prophages can also be used as a possible means to serotype *A. paragallinarum*. At this stage it is not possible to use the phage genes screened for during this study as a possible serotyping scheme. During the experiment conducted for the 221, Modesto and SA-3 strains, the reference isolate as well as a field and/or vaccine strain was used. No useable pattern was observed to aid in the serotyping of

A. paragallinarum as in none of the above mentioned strains the same genes were amplified during the PCR screening. For future research screening for the lamdoid genes that were also found to be present in the *A. paragallinarum* Modesto (C-2) strain by Roodt and co-workers (2012), as well as other phage genes can be conducted. Once this is done possible patterns can be determined that might aid in the serotyping of *A. paragallinarum*.

There have also been reports on serotype-converting phages in *Shigella flexneri* (Sun *et al*., 2011), therefore the occurrence of different serovars in *A. paragallinarum* can be due to the presence of prophage sequences. Screening for serotype converting genes can also be done in the future to determine whether this is the reason for the occurrence of different serovars in *A. paragallinarum* and to determine if there might be any new serovars present.

Phage induction experiments can also be conducted by attempting to induce the phages with the use of mitomycin C. If this proves successful screening for phage virulence genes can be conducted in order to determine whether the presence of these phages has an effect on the virulence of *A. paragallinarum* serovars.

During this study successful serotyping of the C-2 and C-3 serovars of *A. paragallinaurm* was established. For future research accurate serotyping for all of the different serovars will be attempted by making use of the serotyping PCR. Seeing as the C-2 and C-3 serovars poses the biggest problem in South Africa as well as in countries such as India and Israel, this is already a huge step forward for the serotyping of *A. paragallinarum*. From this study it is evident that there are prophage genes present within the genome of all the reference as well as field isolates, there are however still a lot of unanswered questions that needs to be addressed during future research i.e. effect phages have on virulence, whether it be used for serotyping, whether there are serotyping converting genes present. This will lead to more accurate diagnosis, which will result in better vaccines being produced, which will ultimately lead to the control of IC in the poultry industry.

Future Research

For future research it will be attempted to accurately distinguish between all of the serovars by means of the serotyping PCR. Restriction enzyme analysis will be performed on all of the serovars to achieve this. This will then aid in more accurate diagnosis of *A. paragallinarum*, which will lead to better vaccine development and ultimately the control of IC in chickens.

Phage induction experiments and screening for virulence genes once the phages have been induced will also be attempted. Screening for different types of phage genes for example lamdoid phage genes will also be conducted in order to establish what other phages might be present in *A. paragallinarum*. From all of this it might be possible to get a better understanding of the role phages might play in virulence between the different *A. paragallinarum* serovars.

SUMMARY

Avibacterium paragallinarum is an avian pathogen that causes the upper respiratory disease Infectious coryza (IC) in chickens. This disease has the ability to cause vast economic losses due to a decrease in egg production. To date the factors contributing to pathogenicity, immunogenicity and serotyping are still not clearly understood.

Vaccine failures are a major problem that occurs due to no or poor cross-protection occurring especially between the C-serovars of *A. paragallinarum*. This problem will be overcome by having a more accurate serotyping technique available for the diagnosis of IC. Therefore one of the aims of this study was the development of a molecular serotyping technique, where a serotyping PCR was developed which distinguished between the Modesto (C-2) and SA-3 (C-3) *A. paragallinarum* isolates which is the major cause of IC in South Africa.

Reported in a recently published article was the presence of a HP2-like and Mu-like phage within the genome of the Modesto (C-2) strain of *A. paragallinarum*. Therefore another major question addressed during this study was whether there are prophage genes present in all of the reference isolates as well as in field isolates of *A. paragallinarum* and what the effect of these phages might be on the virulence and pathogenicity of these isolates. Phage genes that are important during lysogeny were selected and screened for by means of PCR. From the results it was able to determine that some of these genes are present in some of these isolates but no discernible patterns were detected in terms of the effect on pathogenicity. Therefore future studies will be conducted to mainly focus on the effect these phages might have on the virulence and pathogenicity as well as whether these phages are responsible for the occurrence of different *A. paragallinarum* serovars.

Key words: Serotyping PCR; HP2-like phage; Mu-like phage; *Avibacterium paragallinarum*; vaccine failures; Infectious Coryza; cross-protection

OPSOMMING

Avibacterium paragallinarum is 'n voëlspesiepatogeen wat akuute lugweginfeksie, aansteeklike sinusitis in hoenders veroorsaak. Hierdie siekte het die vermoë om groot ekonomiese verliese te veroorsaak as gevolg van 'n afname in eierproduksie. Tot dusver is die faktore wat bydra tot patogenisiteit, immuunresponsreaksie en serotipering nog onduidelik.

Entstof mislukkings is 'n groot problem wat ontstaan as gevolg van, swak of geen kruis-beskerming wat voorkom, veral tussen die C-serovars van *A. paragallinarum*. Hierdie probleem kan voorkom word deur 'n meer akkurate serotipering tegniek vir die diagnose van IC. Dus een van die doelwitte van hierdie studie was die ontwikkeling van 'n molekulêre serotipering tegniek, waar 'n serotipering PKR ontwikkel is wat kan onderskei tussen die Modesto (C-2) en SA-3 (C-3) *A. paragallinarum* isolate wat die hoof oorsaak is van IC in Suid-Afrika.

In 'n onlangs gepubliseerde artikel was die teenwoordigheid van 'n HP2-tipe en Mu-tipe fage in die genoom van die Modesto (C-2) stam van *A. paragallinarum* gerapporteer. Nog 'n groot vraag wat geadresseer is tydens hierdie studie was of daar profage gene teenwoordig in al die verwysings isolate sowel as in die veld isolate van *A. paragallinarum* en watter effek hierdie fage kan hê op die virulensie en patogenisiteit van hierdie isolate. Profaag gene wat belangrik is tydens lisogenie was geselekteer en gekeur voor deur middel van PKR. Vanuit die resultate was ons daartoe in staat om vas te stel dat sommige van hierdie gene teenwoordig is in sommige van hierdie isolate, maar geen merkbare patrone is waargeneem in terme van die effek op patogenisiteit nie. Daarom sal toekomstige studies gedoen word wat hoofsaaklik fokus op die effek wat hierdie fage kan hê op die virulensie en patogenisiteit asook of hierdie fage verantwoordelik is vir die teenwoordigheid van verskillende *A. paragallinarum* serotipes.

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