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Evidence that the 36 kb plasmid of *Brachyspira hyodysenteriae* contributes to virulence

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Short title: Virulence of *Brachyspira hyodysenteriae*

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ABSTRACT

Swine dysentery (SD) results from infection of the porcine large intestine with the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae*. Recently the genome of virulent Australian *B. hyodysenteriae* strain WA1 was sequenced, and a 36 kilobase (kb) circular plasmid was identified. The plasmid contained 31 genes including six *rfb* genes that were predicted to be involved with rhamnose biosynthesis, and others associated with glycosylation. In the current study a set of PCRs was developed to amplify portions of nine of the plasmid genes. When used with DNA extracted from virulent strain B204, PCR products were generated, but no products were generated with DNA from avirulent strain A1. Analysis of the DNA using pulsed field gel electrophoresis (PFGE) identified a plasmid band in strains WA1 and B204, but not in strain A1. These results demonstrate that strain A1 does not contain the plasmid, and suggests that lack of the plasmid may explain why this strain is avirulent. To determine how commonly strains lacking plasmids occur, DNA was extracted from 264 Australian field isolates of *B. hyodysenteriae* and subjected to PCRs for three of the plasmid genes. Only one isolate (WA400) that lacked the plasmid was identified, and this absence was confirmed by PFGE analysis of DNA from the isolate and further PCR testing. To assess its virulence, 24 pigs were experimentally challenged with cultures of WA400, and 12 control pigs were challenged with virulent strain WA1 under the same conditions. Significantly fewer (P = 0.03) of the pigs challenged with WA400 became colonised and developed SD (13/24; 54%) compared to the pigs infected with WA1 (11/12; 92%). Gross lesions in the pigs colonised with

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WA400 tended to be less extensive than those in pigs colonised with WA1, although there were no obvious differences at the microscopic level. The results support the likelihood that plasmid-encoded genes of *B. hyodysenteriae* are involved in colonisation and/or disease expression.

Keywords: Brachyspira hyodysenteriae; plasmid; virulence; *rfb* genes; swine dysentery

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1. Introduction

Swine dysentery (SD) is an important endemic disease of pigs that results from infection of the large intestine with the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae* (Hampson, 2011). Typically SD manifests as severe mucohaemorrhagic colitis in growing and finishing pigs, with the animals having dysentery and reduced growth rate. In the field, the more obvious signs of SD in infected herds may be masked by the use of in-feed antimicrobials. Dietary ingredients also may influence the occurrence and severity of disease in infected pigs by creating conditions within the large intestine that do not favour colonisation (Pluske et al., 1996; Hansen et al., 2010). Differences in virulence amongst *B. hyodysenteriae* isolates also may account for variations in clinical outcomes. For example, Lysons et al. (1982) reported isolating *B. hyodysenteriae* from pigs in herds that were free of clinical SD. Three isolates did not produce disease when given orally to conventional pigs, although they colonised some individuals. Later, Achacha et al. (1996) challenged groups of weaner pigs with different strains of *B. hyodysenteriae*, and, based on whether or not pigs developed diarrhoea, they categorised them as varying from virulent to avirulent.

The basis of virulence in *B. hyodysenteriae* is incompletely understood (Hampson, 2011). Recently, analysis of the genome sequence of virulent *B. hyodysenteriae* strain WA1 identified 2,638 open reading frames (ORFs) on the chromosome and 31 on a previously unrecognised 36 kilobase (kb) plasmid. Of these ORFs, 314 (~12%) were annotated as having potential roles in pathogenesis and virulence (Bellgard et al., 2009), including six *rfb* genes on the plasmid that

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were predicted to be involved in rhamnose biosynthesis and incorporation into the spirochaete's lipooligosaccharide (LOS) in the cell wall.

The aim of the current study was to investigate whether the 36 kb plasmid of *B. hyodysenteriae* is likely to play a potential role in virulence.

2. Materials and methods

2.1. Animal ethics

The experimental infection was conducted with the approval of the Murdoch University Animal Ethics Committee (AEC number R2104/07).

2.2. Brachyspira hyodysenteriae strains

Strains of *B. hyodysenteriae* were obtained as frozen stock from the culture collection at the Reference Centre for Intestinal Spirochaetes at Murdoch University. They included virulent reference strains WA1 and B204, avirulent strain A1, and 264 Australian field isolates from diagnostic submissions over the period 1988-2007. The spirochaetes were confirmed to be *B. hyodysenteriae* based on their strong haemolysis on blood agar, production of indole and amplification in a species-specific PCR (La et al., 2003).

2.3. Bacterial culture

Spirochaete strains were propagated at 37^{o}C in Kunkle's pre-reduced anaerobic broth containing 2% (vol/vol) foetal bovine serum and a 1% (vol/vol) ethanolic cholesterol solution (Kunkle et al., 1996). Cells were harvested from cultures in mid-log phase by centrifuging at 10,000 x g. They were resuspended in phosphate buffered saline, placed in a counting chamber and enumerated under a phase contrast microscope.

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2.4. Visualization of plasmid bands

Brachyspira hyodysenteriae strains WA1, B204, A1 and Australian field isolate WA400 were grown in Kunkle's broth to a density of 10^8 cells/ml, and 10 ml of each was microfuged at 14,000 rpm for 5 min. The cells were resuspended in 200ul cold Tris-EDTA buffer (TE: 10mM Tris; 1mM EDTA; pH 8.0), to which was added 100μL lysostaphin (Sigma) and 300μL molten plug agarose consisting of 1.8% agarose in 0.5 x TBE (10.6 g Tris base; 5.5 g boric acid; 4 ml 0.5 M EDTA per litre: pH 8.0). This was pipetted into a pre-chilled plug mold, allowed to set and held at 4°C for 20 min. Plugs were dispensed into sterile 5ml tubes containing 3ml of TE and 12.5μL lysozyme (Sigma) and incubate at 37°C overnight. The TE was removed, replaced with fresh TE, held at 4^oC for 12 h, and the TE replaced. The plugs were stored at 4^oC until used for pulsed field gel electrophoresis (PFGE). A 1% agarose gel in TBE was prepared and pre-electrophoresed for 1.5h. Each plug was cut in half and one half was incubated in an eppendorf tube at 56°C for 8 min. The molecular mass standards Lambda ladder (Biorad) and Lambda DNA/*HindIII* marker (Promega) were similarly incubated. The plugs and standards were loaded into the gel and sealed with plug agarose. The gels were subjected to electrophoresis with a 1 – 6 s ramp for 11 h at 200 V. DNA in the gel was stained by immersion in 5μg/ml ethidium bromide for 30 min, destained for 45 min in 0.5 x TBE and photographed.

2.5. PCR analysis for plasmid genes

Details of the 31 genes located on the 36 kb plasmid of WA1 are available from the National Center for Biotechnology Information (NCBI; Refseq:

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125 NC_012226). Three sets of primers were designed to amplify portions of each of
126 nine of these genes (Table 1). Three of the genes (BHWA1_02688,
127 BHWA1_02687 and BHWA1_02686, respectively) are located around the origin
128 of replication of the plasmid, and PCRs for these were undertaken with DNA
129 from all strains and isolates. The sets of PCRs for the other six genes
130 (BHWA1_02675, BHWA1_02674, BHWA1_02673, BHWA1_02672,
131 BHWA1_02668 and BHWA1_02667 respectively) were used only with DNA
132 from strains WA1, B204, A1 and WA400.
133 The *B. hyodysenteriae* strains and isolates grown in Kunkle's broth were
134 harvested by centrifugation and the pellet resuspended in 1 ml sterile TE. The
135 resuspended cells were boiled for 2 min and the cellular debris was pelleted by
136 centrifugation. DNA in 2 μl of the supernatant was amplified by hot-start PCR in
137 a 20 μl total volume using HotStar*Taq* DNA polymerase (Qiagen), according to
138 the manufacturer's instructions. Briefly, amplification mixtures consisted of 1 x
139 PCR buffer (containing 1.5 mmol/l of $MgCl_2$), 1.25 U of HotStar*Taq* DNA
140 polymerase, 0.1 mmol/l of each dNTP (Promega) and 0.2 μmol/l of the
141 appropriate primer pair. Cycling conditions involved an initial 5 min HotStar*Taq*
142 DNA polymerase activation step at 95°C, followed by 30 cycles of denaturation at
143 95°C for 30 s, annealing at 52°C for 15 s, primer extension at 72°C for 1 min and
144 a holding step at 72°C for 5 min before holding at 10°C. The PCR products were
145 subjected to electrophoresis in 1% (w/v) agarose gels in 1 × TAE buffer (40
146 mmol/l Tris-acetate, 1 mmol/l EDTA), stained with ethidium bromide and viewed
147 over UV light.

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148 *2.6. Experimental infection*

149 *2.6.1. Strains used for infection*

150 Virulent *B. hyodysenteriae* strain WA1 containing the 36 kb plasmid and
151 WA400, an Australian field isolate that was identified as lacking the plasmid
152 during the screening process, were used in the experiment.

153 *2.6.2. Pigs and housing*

154 Thirty-six castrated male pigs (Large White x Landrace x Duroc) of
155 approximately 18 kg body weight were purchased from a commercial piggery that
156 had no history of SD being diagnosed, and from which *B. hyodysenteriae* had
157 never been isolated during routine screening. The pigs were weighed, ear-tagged,
158 and rectal faecal samples were taken and cultured for *Brachyspira* species on
159 selective Trypticase Soy Agar (BBL) containing 5% (vol/vol) defibrinated ovine
160 blood, 400 μg/ml of spectinomycin, and 25 μg/ml each of colistin and
161 vancomycin (Sigma-Aldrich) (Jenkinson and Wingar, 1981). The plates were
162 incubated for 5 to 7 d at 37°C in an anaerobic environment of 94% H_2 and 6%
163 CO_2 generated with anaerobic Gaspak plus sachets (BBL), and then were
164 examined for the presence of low, flat, spreading growth of spirochaetes. The pigs
165 were randomly assigned to two groups, A and B, comprising 24 and 12 animals
166 respectively. Each group was housed in a single pen in different rooms of an
167 isolation animal house. Strict biosecurity protocols, including the use of different
168 sets of protective clothing in the different rooms, were maintained to prevent
169 transmission of infection between the rooms. The pigs were fed *ad libitum* on a

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diet based on barley, triticale and canola meal in mash form that allows full expression of SD (Hansen et al., 2010).

2.6.3. Experimental challenge protocols

Seventeen days after arrival, the pigs of groups A and B were experimentally infected with *B. hyodysenteriae* WA400 and WA1 respectively. Briefly, a gastric tube was used to dose each pig, such that each animal received a slurry of 100 ml of a mid log-phase broth culture (~10^8 cells/ml) containing *B. hyodysenteriae* together with chopped agar from a blood agar plate with a dense spirochaete growth. This procedure was repeated daily on the following two days. On the next two days the food for the pigs was inoculated with more of the spirochaetes, such that each pig would receive ~ 2 blood agar plates with a dense spirochaete growth and 50 ml of broth culture. Following challenge, the pigs were observed daily for clinical signs consistent with SD, defined as the presence of diarrhoea that contained fresh blood and mucus. Pigs that developed these clinical signs were removed and subjected to post-mortem examination. Bacteriology swabs were taken from rectal faeces of all pigs every 3-4 days until 22 days after the last day of experimental challenge. The swabs were subjected to selective culture for *B. hyodysenteriae* as previously described. Faecal scores were as follows: 0 = no *B. hyodysenteriae*; 1 = spirochaetes on first streak of the plate; 2 = spirochaetes on the second streak; and so on until 5 = the whole surface of the plate covered in spirochaetes. The experiment was ended 27 days after the last day of experimental challenge. Single representative isolates from the faeces of all pigs with SD in

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both groups were checked for the presence of the plasmid by PCR amplification of the plasmid genes.

2.6.5. Post-mortem examination

The pigs were stunned using a captive bolt pistol and then exsanguinated by severing the carotid artery. The carcases were opened and the intestinal tract was removed. The large intestine was opened along its length and gross pathological changes and their distribution were recorded. Lesions were subjectively recorded as mild patchy inflammation, moderate haemorrhagic colitis or severe mucohaemorhagic colitis. Intestinal contents were collected from the caecum and from four points evenly dispersed along the colon, and were used for spirochaete culture. Scoring of spirochaete growth was as for faecal samples. Portions of the colonic wall at the same sites were placed in 10% buffered formalin for histological examination. The fixed sections were blocked, embedded in paraffin and cut at 4μm. They were stained with haematoxylin and eosin and examined by an American board-certified veterinary pathologist who was blinded to the origin of the sections. Each section was examined for the presence of mucosal erosion or ulceration, and the number of lymphoid follicles within the submucosa was counted. Inflammatory changes within the mucosa were characterised by the type of inflammatory cell present, and were grouped according to the severity of the inflammatory cell infiltrate. Well preserved sections of intestine with the mucosal glands and enterocytes cut in longitudinal section were examined under 400x magnification. The number of intra-epithelial lymphocytes (granulated and non-granulated) and granulocytes (eosinophils and neutrophils) and the severity of

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inflammation within the mucosa were recorded. An estimate of mucosal thickness was made. The presence of surface or intraglandular bacteria, *Balantidium coli*, and any other infectious agents or abnormal histological changes were recorded.

2.6.6. Analysis

Raw results were tabulated and Fisher's exact test was used to make comparisons between the two experimentally infected groups in terms of the incidence of disease, culture and post-mortem results.

3. Results

3.1. PCR analysis

Of the 264 Australian field isolates tested, only one (WA400; 0.4%) failed to show amplification with any of the plasmid-specific PCR reactions directed at the three genes around the origin of replication. Of the remaining 263 isolates, 231 were PCR positive for all three genes, 17 were positive only for BHWA1_02688 and BHWA1_02686, six were positive only for BHWA1_02687 and BHWA1_02686, and nine were positive only for BHWA1_02686.

Strains WA1 and B204 gave amplifications in the PCRs for all nine plasmid genes tested, whilst A1 and WA400 showed no amplification with any of the nine PCRs.

3.2. PFGE results

DNA bands of ~36 kb were observed in strains WA1 and B204, but not in A1 or field isolate WA400. Judging from the intensity of the bands, the plasmid appeared to be in low copy number (Fig. 1). All four strains also showed the presence of a ~ 7.5 kb extra-chromosomal band that likely represented the

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prophage-like gene transfer agent VSH-1 (Humphrey et al., 1997).

3.3. Experimental infection

Spirochaetes were not found in the faeces of the pigs prior to infection. Following experimental challenge, 13 of the 24 pigs (54%) of group A shed *B. hyodysenteriae* in their faeces and developed mucohaemorrhagic diarrhoea consistent with SD. The other pigs did not develop diarrhoea. In contrast, 11 of the 12 pigs (92%) of group B shed *B. hyodysenteriae* in their faeces during the experimental period and developed signs of SD. The difference in disease incidence between the two groups was statistically significant ($P = 0.031$). For the pigs infected with WA400, faecal samples from 38 of the 117 sampling days were positive compared to 40 of 54 for the pigs challenged with WA1. This difference was highly significant ($P < 0.0001$). All pigs that developed SD had faecal scores of 5 on at least two sampling occasions before they were clinical affected, and similar high levels of colonisation were present in affected pigs from both groups. The pigs that did not develop SD remained culture negative. All but one of the pigs that had lesions consistent with SD at post-mortem had culture scores of 4 or 5 at all five intestinal sites; the exception was one pig from group A that was culture negative in the caecum. This pig also did not have any lesions in the caecum. PCR analysis of the representative isolates from the pigs with SD confirmed that the strains had not been accidentally transmitted between the rooms.

The extent and distribution of gross lesions in the large intestine differed between the two groups. The 11 pigs from group B that developed SD had severe

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lesions recorded at all five sites. On the other hand, of the 13 pigs from group A that developed SD, only two had lesions in the caecum, and these were only moderate. Two pigs had severe lesions at the two most proximal colonic sites, while at all the other sites in all the other pigs from group A only had mild or moderate gross lesions.

When the histological sections were examined lesions consistent with SD were noted in all the sections derived from pigs with gross lesions. Erosions were recorded in most sections, but there was no ulceration. All affected sections showed broadly similar numbers of intra-epithelial lymphocytes, as well as lymphocytes, plasma cells, macrophages and granulocytes in the lamina propria. There were minor differences between sections from individual pigs and between pigs, but no consistent pathological differences were recorded that could be attributable to the strain used for infection.

4. Discussion

In this study it was demonstrated that a plasmid of ~ 36 kb that was first described in strain WA1 was also present in strain B204, and the two shared at least nine ORFs. On the other hand strain A1 lacked the plasmid, demonstrating that it is a true plasmid and is not essential for the survival of *B. hyodysenteriae*. Strain A1 previously has been shown to colonise pigs but not cause disease (Hudson et al., 1974; Achacha et al 1996), whereas strains WA1 and B204 have been shown to be virulent, colonising experimentally challenged pigs and inducing typical lesions of SD (Jensen and Stanton, 1993; Siba et al., 1996).

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These observations suggest that genes on the plasmid may contribute to virulence in *B. hyodysenteriae.*

To determine whether *B. hyodysenteriae* isolates that lack the plasmid are widespread in the field, a large collection of Australian isolates was examined using a set of PCRs. Many of these isolates had been used in population studies using multilocus enzyme electrophoresis and/or multilocus sequence typing in our laboratory (Trott et al., 1997; La et al., 2009), and they came from a wide range of genetic backgrounds within the species. Thirty-two (12%) of the isolates amplified in only one or two of the PCRs, and this suggests that there is heterogeneity in the plasmid sequence in these isolates around the primer sites. Again there was no consistent grouping of these isolates according to their genetic background, suggesting that these differences are likely to have arisen from localised mutations. It would be useful to sequence plasmids from different *B. hyodysenteriae* strains to determine the extent of heterogeneity over the whole plasmid. A single isolate that lacked the plasmid was identified, and this was recovered from a faecal sample sent to our laboratory as a diagnostic sample in the mid-1990s. The herd of origin had a clinical history consistent with SD, but unfortunately no other details were recorded. This finding does demonstrate that strains lacking the plasmid may be present in pigs in a small minority of herds with clinical signs of SD. It is not known whether such strains may be present in other herds where SD is not a clinical problem - as most such herds are not routinely examined for *B. hyodysenteriae*.

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To help test the hypothesis that *B. hyodysenteriae* isolates lacking ~ 36 kb plasmid may have reduced virulence, the Australian isolate WA400 that lacked the plasmid was used to experimentally infect pigs. Pigs in both groups developed SD, but significantly fewer animals challenged with WA400 were colonised and developed disease compared to those that were challenged with strain WA1. It was not possible to determine whether the clinical severity of the disease in the two groups differed, as, for welfare reasons, the pigs were removed for post-mortem examination as soon as blood and mucus appeared in the faeces. The gross intestinal lesions in the pigs that became colonised with WA400 appeared milder than occurred with WA1, and only two pigs infected with WA400 had lesions in the caecum. These results suggested that the strains had different capacities to colonise the intestinal tract - although paradoxically the culture scores at most intestinal sites were similar and were high for both strains. In future work it would be useful to examine this in more detail by quantifying the spirochaetal load in the caecum and colon of infected pigs using a more sensitive technique such as quantitative PCR (Song and Hampson, 2009).

Despite gross differences in lesion severity, it was surprising that sections from pigs in both groups showed similar evidence of increased mucosal thickness, inflammatory cell infiltration and erosions. This suggested that the underlying process involved in lesion development was not altered by the absence of the plasmid, and therefore that expression of other genes located on the chromosome is important for lesions to occur. The chromosomal backgrounds of strains WA1 and WA400 have not been compared. Ideally to help dissect out the importance of

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the plasmid-encoded genes, variants of the same strain that differed only in whether or not they possessed the plasmid should have been compared - but these were not available.

The WA1 plasmid contains genes encoding enzymes forming part of the rhamnose biosynthesis pathway (*rfb* genes) that are predicted to function in incorporation of rhamnose in the O-antigen backbone of the cell wall LOS. Other glycosyltransferases were encoded by the plasmid, and these may be involved in incorporating other sugars into the LOS, or glycosylating proteins. It seems likely that differences in LOS structure or other cell wall components could influence the ability of the spirochaete to survive in the intestinal milieu and/or to interact with the colonic epithelium. As LOS from *B. hyodysenteriae* is known to have toxic effects, and may contribute to inflammation and lesion development (Nuessen et al. 1983; Greer and Wannemuehler, 1989; Nibbelink et al. 1997), it is also possible that changes in the LOS composition may influence exposure of the toxic lipid-A components.

5. Conclusions

The findings of this study support the likelihood that the 36 kb plasmid of *B. hyodysenteriae* is involved in facilitating colonisation of the large intestine and hence allowing the development of disease. Strains lacking plasmids are predicted to have reduced virulence. Besides the plasmid-associated differences that are likely to occur in the LOS/glycosylation patterns of *B. hyodysenteriae* strains, other chromosomally encoded functions are undoubtedly involved in lesion production.

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Figure legend

Fig 1. Agarose gel showing the presence of a DNA band corresponding to the 36 kb plasmid in DNA extracted from *B. hyodysenteriae* strain WA1, and separated by pulsed field gel electrophoresis. Genomic DNA and DNA from the gene transfer agent VSH-1 are also shown. A similar plasmid band was observed in DNA from strain B204, but not in DNA extracted from A1 or in WA400. Lambda ladder is shown in lane 1 (λ1) and lambda DNA/*HindIII* marker in lane 2 (λ2).

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Table 1. Oligonucleotide primers used in the PCRs for detection of the *B. hyodysenteriae* 36 kb plasmid.

Primer name*	Sequence (5'-3')	Product size (bp)
BHWA1_02688-F	AGCAAAACGTCTTGGTTTTGAA	933
BHWA1_02688-R	TCATTAATGTCTTTTACTTCTTTATCATCA	
BHWA1_02688-F	AGCTGCGGAGCTAGCGGAGA	717
BHWA1_02688-R	TGCTTTATCATTGTCTGAAAAGCTG	
BHWA1_02688-F	TTTTGTAGATTTACTTAATAATGAAGAAGC	417
BHWA1_02688-R	AGGCAGTCTATAACACCTTCGCA	
BHWA1_02687-F	AGCAGCCGTATTGCTATATTTGA	540
BHWA1_02687-R	ACTCTCTTCGCTGTAAGATTCTTCT	
BHWA1_02687-F	GCTTCAAGCAAATTAAATATTAAAGAACC	373
BHWA1_02687-R	TGCTGAATCTGTATTCATTTCTTAAACTGG	
BHWA1_02687-F	CAGCAGTAATTTACGTGAGGGA	212
BHWA1_02687-R	AGCTTTCCTGATTATTAATGCTTTCT	
BHWA1_02686-F	AGGAGGAGTAGGAAAAACTACACT	676
BHWA1_02686-R	TTCTTCTATTGTAGTGCTTTTAGGA	
BHWA1_02686-F	AGCTGATTACGAATTAGCAGACTGT	494
BHWA1_02686-R	TGTCAGCTATTTTTCTGTCCTGTCCT	
BHWA1_02686-F	GCTGAAACAAAACTTTTCCAAGAGCC	186
BHWA1_02686-R	ACCATCAAAAGAAAAGTATTCAGGTGT	
BHWA1_02675a-F	ATTGGATAGAACATAGAGGGAG	301 bp
BHWA1_02675a-R	ACTGTATCATTTGCTATTTCATTAG	
BHWA1_02675b-F	TATAAAAACTATAAGAATATCTCTACAAGG	367 bp
BHWA1_02675b-R	AACATATAAGGTATAAAATGGTTGAG	
BHWA1_02675c-F	CCTCAACCATTTTATACCTTATATG	184 bp
BHWA1_02675c-R	TAACTATATTTTCTCGTTTTCCTTG	
BHWA1_02674a-F	ATTTAGAAGATGTAATACCTTTAGAGG	249 bp
BHWA1_02674a-R	TCATTTTCGCTATATTTTTATTTAC	
BHWA1_02674b-F	TTATACAAAATAGGAGAGCCTTTAG	363 bp
BHWA1_02674b-R	ATCGCAATAATCTGAAAATG	
BHWA1_02674c-F	GTATGTACTTATCTTTTTTATTCTATTGTC	194 bp

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BHWA1_02674c-R	CATATTGGATTTTTATCTCTATGTC	
BHWA1_02673a-F	AAATACTTGTCAATAATCTTAGTGG	1819 bp
BHWA1_02673a-R	TTTCATCATAAGCAAAAATAATATC	
BHWA1_02673b-F	GTAAGTGGAAAAAGAATGAAACATAC	1032 bp
BHWA1_02673b-F	AGATTGTCTTGACGAATAAAAG	
BHWA1_02673c-F	AATAAATATGACATTAAAGGAATAAAAATC	805 bp
BHWA1_02673c-R	CTATTGTTAGTAGCAAAATAATAAAAATAC	
BHWA1_02672a-F	AAATGTAGAAGATATTGTATTGCC	417 bp
BHWA1_02672a-R	ACCTCTCCTATATGTTTTTTATACTTAG	
BHWA1_02672b-F	ATTACTACAAAATGTACTCTAAAATGTAAG	546 bp
BHWA1_02672b-R	CCATACTATATGACAAAAATAAAATCTAG	
BHWA1_02672c-F	TATCTAAGTATAAAAAACATATAGGAGAGG	498 bp
BHWA1_02672c-R	CAGCACAAAACTCACATAGTG	
BHWA1_02668a-F	GTTCATACCATTTAGAAAAAGAAGAG	701 bp
BHWA1_02668a-R	GTTCATACCATTTAGAAAAAGAAGAG	
BHWA1_02668b-F	AGAACAAAACAACATAAAGCATC	206 bp
BHWA1_02668b-R	CATCAGTAAAACAAATATAATCCC	
BHWA1_02668c-F	CCTGAGCATTATGGACTTTC	240 bp
BHWA1_02668c-R	TGTACTGTCTGATTTTTTATGGTC	
BHWA1_02667a-F	ACTGGAGTTGCTGGATTTATAGGATC	560 bp
BHWA1_02667a-R	AAGTCAGGTCTCTGTCTCTTTCC	
BHWA1_02667b-F	CAAATAAAGATCATACTGTTATAGGAATAG	597 bp
BHWA1_02667b-R	ATGTATAGTCACGCATAGTGG	
BHWA1_02667c-F	TGTAATACATTTAGCAGGATATGG	384 bp
BHWA1_02667c-R	GGTATAGGATTATTTTCAAGTATCAG	

*Primers named according to the plasmid gene they are designed to amplify (NCBI; Refseq: NC_012226).

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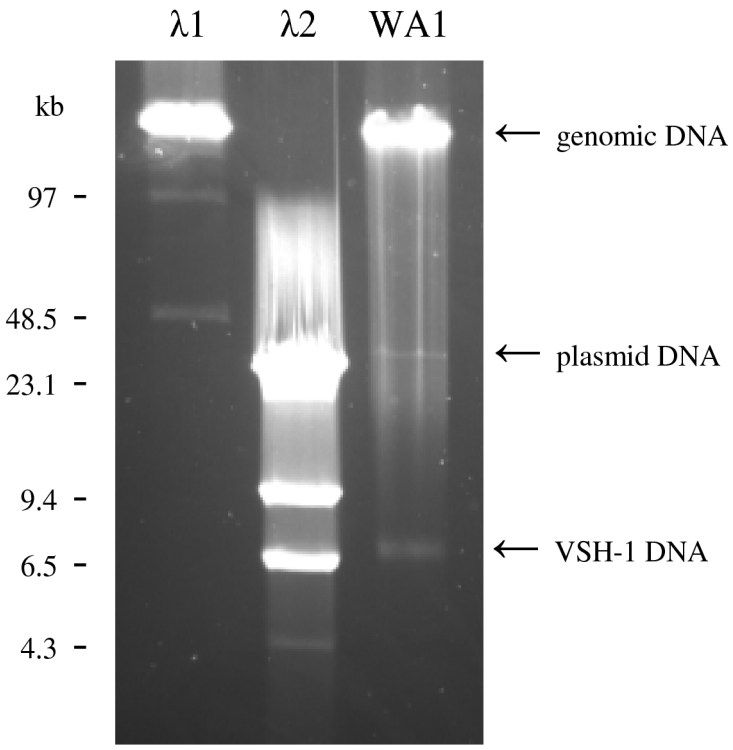

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