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Comprehensive serotyping of *Mannheimia haemolytica* by a PCR system using the diversity of capsule biosynthesis genes

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The bovine respiratory disease complex (BRDC) is a global issue affecting dairy and beef farms and is of major concern due to the high morbidity and mortality rates in calves, as well as decreased production it causes, resulting in significant economic losses. *Mannheimia haemolytica* is one of the secondary pathogens associated with BRDC. *M. haemolytica* is classified into 12 serotypes based on capsular antigens. In addition to the prevalent serotypes A1, A2, and A6, strains belonging to other serotypes also cause respiratory diseases in cattle and other ruminants, necessitating a method for their rapid and easy identification. In this study, we organized the capsule biosynthesis genes based on genome information from all serotype strains and designed 11 PCR primer pairs targeting serotype-specific genes, which could individually identify serotypes A14/A16, which possess homologous genes, as well as all other serotypes. Additionally, we developed two multiplex PCR kits that include these serotype-specific and *M. haemolytica* species-specific primers. Specificity testing using reference strains confirmed that these kits can simultaneously and clearly identify both the species and their serotypes. The PCR-based system described here could be a valuable tool for subtyping *M. haemolytica* strains in epidemiological studies and surveillance efforts in cattle and other reservoir animals. This study also carefully compared and discussed the differences between the capsule synthesis genes of A8 and A14 from previously published and those obtained in this study.

Keywords *Mannheimia haemolytica*, Capsular antigen, Serotype, PCR

Mannheimia haemolytica, a member of the *Pasteurellaceae* family, is one of the secondary pathogens associated with the bovine respiratory disease complex (BRDC)[1]. *M. haemolytica* is a commensal bacterium that typically resides in the upper respiratory tract of cattle and can be found in both healthy and respiratory-diseased animals[1,2]. Stress factors such as transportation and co-infections with viruses and bacteria can promote the explosive proliferation of *M. haemolytica*, which is then inhaled into the lungs, leading to BRDC, including acute pleuropneumonia[3]. Its predominant virulence factor is leukotoxin belonging to the repeat-in-toxin family, which induces lysis of ruminant leukocytes[4]. BRDC results in significant economic losses for the dairy and beef farms due to the high morbidity and mortality rates in calves, as well as reduced weight gain and milk production in affected animals[5,6].

M. haemolytica is classified into twelve serotypes based on capsular antigens, designated as A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17. Five serotypes not found in this series were originally included in the *Pasteurella haemolytica* complex, but were subsequently excluded in a reclassification that showed that A11 was a type of *Mannheimia glucosida* and A3, A4, A10, and A15 were types of *Bibersteinia trehalosi*[7,8]. Serotype A1 is the most prevalent cause of BRDC worldwide, followed by serotypes A6 and A2[9]. In addition, although these serotypes are not often seen, cattle clinical strains of *M. haemolytica* belonging to serotypes A5, A7, and A16

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have been identified in Spain, while serotypes A7, A13, and A14 have been reported in Japan[10,11]. This pathogen is also responsible for pneumonia in other ruminant livestock besides cattle. In Ethiopia, *M. haemolytica* strains of serotypes A1, A2, A3, A5, A7, and A9 have been identified from sheep and goat with pneumonia[12,13]. In a Spanish study, *M. haemolytica* serotypes A1, A2, A7, A8, A9, and A13 were isolated from pneumonia lesions in sheep, while serotypes A2, A6, and A9 were found in goats[10].

To better understand the epidemiology of this pathogen and identify effective vaccine candidates, reliable testing methods covering a wide range of serotypes are required. The conventional method for determining serotypes is the agglutination reaction using specific antisera[14,15]. However, producing antisera is labor-intensive, and only a limited number of institutions can perform this method for all serotypes. As an alternative to agglutination methods, genetic serotyping approaches have been developed for various pathogenic bacteria[16–19]. For *M. haemolytica,* DNA-based methods have been introduced to identify the three clinically significant serotypes, A1, A2, and A6, and related genotypes[10,20,21]. However, no PCR method capable of identifying the other serotypes has been reported so far.

In this study, we compared the genome sequences of *M. haemolytica* serotype reference strains and confirmed that each serotype possesses unique capsule synthesis genes, which can serve as useful markers for DNA-based serotyping. Using this information, we designed PCR primers capable of identifying all serotypes, including the A14/A16 pair. Additionally, we proposed two multiplex PCR kits incorporating these serotype-specific primers along with an identification primer pair for *M. haemolytica* species, aimed at improving testing efficiency.

Results

Genome comparison

The genome sequences of all *M. haemolytica* serotype strains, except for A17, which we did not possess, were determined (Table 1). The concatenated sizes of the assembled sequences for each strain ranged from 2,475 to 2,632 kb (Supplementary Table S1). Phylogenetic analysis based on the core genes showed that the A1 and A6 strains were very closely related, while the A5, A7, A9, and A12 strains were also relatively closely related to them (Fig. 1). Strains belonging to A2, A8, A13, A14, and A16 were independent from each other, with the A2 and A13 strains, in particular, being evolutionarily distant from the other serotypes (Fig. 1).

Capsular biosynthesis gene clusters

The capsule biosynthesis gene clusters, which included four genes (*wzt*, *wzm*, *wzf*, and *wza*) that encode an ATP-binding cassette transport apparatus responsible for the secretion of capsule materials across the membranes, and two genes, *wbrA* and *wbrB*, involved in the phospholipid modification of capsular materials[22], were extracted from the assembled contigs of serotypes A1 to A16, and the A17 genome sequence obtained from the following database: https://ivsmlst.sund.ku.dk/ (Fig. 2A). The A2 gene cluster was extracted from two separate contigs. The six aforementioned genes were conserved (sharing ≥ 80% DNA sequence identity and ≥ 70% coverage) in the gene clusters of all serotype strains (Fig. 2A). Additionally, one gene encoding a glycosyltransferase located upstream of *wza* and two genes, *nmaAB*, encoding enzymes for the ManNAcA pathway located upstream of *wbrAB*, were conserved in serotypes A1, A5, A6, A8, and A9, while one gene encoding a hypothetical protein located upstream of *wza* was conserved in serotypes A7 and A12 (sharing ≥ 80% DNA sequence identity and ≥ 70% coverage) (Fig. 2A). Serotype A14 and A16 strains had capsule synthesis genes organized in exactly the same way, with their DNA sequences sharing 99% homology (Fig. 2B). As a result, each serotype or the A14/A16 pair had two to five unique genes with less than 50% homology to other capsule genes, flanked by conserved genes within the capsule synthesis gene cluster. Many of these unique genes encoded glycosyltransferases or hypothetical proteins.

Serotype-specific PCR primers

A gene unique to each serotype or the A14/A16 pair was selected as a marker gene, and PCR primers with lengths of 19 to 21 bases and a G + C content of 50–52.6% were designed for each gene (Fig. 2A, Table 2 and Supplementary text). The sizes of the PCR products amplified by these primers ranged from 132 to 990 bp. The specificity of these primers was confirmed using all serotype strains, except for A17 (one to three strains per serotype, including genome-analyzed strains) (Table 1), under the PCR condition described below. Additionally, the specificity of these primers was confirmed using non-*M. haemolytica* strains, including two *M. glucosida* (A11) strains and four *B. trehalosi* strains (A3, A4, A10, and A15), which were initially assigned serotypes under *M. haemolytica* but were later reclassified into different species and genus, respectively. The specificity was also tested using seven other *Mannheimia* species strains, including *Mannheimia varigena*, *Mannheimia granulomatis*, *Mannheimia pernigra*, *Mannheimia ruminalis, Mannheimia bovis*, *Mannheimia cairinae, Mannheimia caviae*, and *Mannheimia indoligenes* (Table 1), and no amplification was observed in any of them.

M. haemolytica species-specific PCR primers

We thought that adding *M. haemolytica*-specific primers to the multiplex PCR kits described below would improve the efficiency and reliability of the test. Based on a sequence comparison of the *recN* gene (an essential gene encoding a recombination/repair protein) from seven major *Mannheimia* species and *B. trehalosi* obtained from the DNA database, a primer pair specific to *M. haemolytica* was designed, producing an amplification product of 591 bp (Fig. 3, Table 2, and Supplementary Table S2). To evaluate the validity of this primer pair, all strains listed in Table 1 were used, and it was confirmed that only *M. haemolytica* strains could be specifically detected under the PCR condition described below.

Strain ID	Species	Serotype**	Results of PCR***	
			Mh species	Mh serotype
I29*	*Mannheimia haemolytica*	A1	+	A1
ATCC 29696	*Mannheimia haemolytica*	A1	+	A1
IBR3-169-8	*Mannheimia haemolytica*	A1	+	A1
J28*	*Mannheimia haemolytica*	A2	+	A2
IBR3-169-6	*Mannheimia haemolytica*	A2	+	A2
G13*	*Mannheimia haemolytica*	A5	+	A5
ATCC 29695	*Mannheimia haemolytica*	A5	+	A5
A30*/ATCC 33370	*Mannheimia haemolytica*	A6	+	A6
ATCC 29697	*Mannheimia haemolytica*	A6	+	A6
IBR3-169-3	*Mannheimia haemolytica*	A6	+	A6
H1*	*Mannheimia haemolytica*	A7	+	A7
ATCC 29698	*Mannheimia haemolytica*	A7	+	A7
H21*/ATCC 29699	*Mannheimia haemolytica*	A8	+	A8
B1*/ATCC 33373	*Mannheimia haemolytica*	A9	+	A9
ATCC 29,700	*Mannheimia haemolytica*	A9	+	A9
S209*/ATCC 33376	*Mannheimia haemolytica*	A12	+	A12
ATCC 29702	*Mannheimia haemolytica*	A12	+	A12
A13*/NCTC 11302	*Mannheimia haemolytica*	A13	+	A13
A14*/NCTC 11303	*Mannheimia haemolytica*	A14	+	A14
A16*	*Mannheimia haemolytica*	A16	+	A16
KC282	*Mannheimia glucosida*	(A11)	-	AN
ATCC 29701	*Mannheimia glucosida*	(A11)	-	AN
BD977	*Mannheimia varigena*	-	-	AN
B256	*Mannheimia varigena*	-	-	AN
BD949	*Mannheimia granulomatis*	-	-	AN
B350	*Mannheimia granulomatis*	-	-	AN
BD1705	*Mannheimia pernigra*	-	-	AN
C343	*Mannheimia pernigra*	-	-	AN
B234	*Mannheimia ruminalis*	-	-	AN
CCUG 38470	*Mannheimia ruminalis*	-	-	AN
KCTC 25018	*Mannheimia bovis*	-	-	AN
CCUG 76754	*Mannheimia cairinae*	-	-	AN
CCUG 59995	*Mannheimia caviae*	-	-	AN
CCUG 77347	*Mannheimia indoligenes*	-	-	AN
ATCC 29703	*Bibersteinia trehalosi*	(A3)	-	AN
ATCC 29704	*Bibersteinia trehalosi*	(A4)	-	AN
JF2	*Bibersteinia trehalosi*	(A10)	-	AN
T15	*Bibersteinia trehalosi*	(A15)	-	AN

Table 1. Strains used in this study. *Strains used for genome analysis. ** Serotypes in parentheses indicate those that were excluded, because they belonged to species other than *M. haemolytica*. *** Results of multiplex PCR kits developed in this study. Mh: *M. haemolytica*, AN: all-negative.

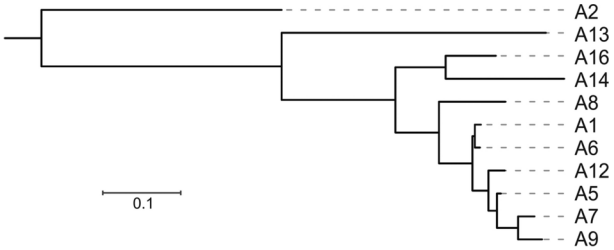

Fig. 1. Phylogenetic tree based on core-genes of *M. haemolytica* serotype reference strains.

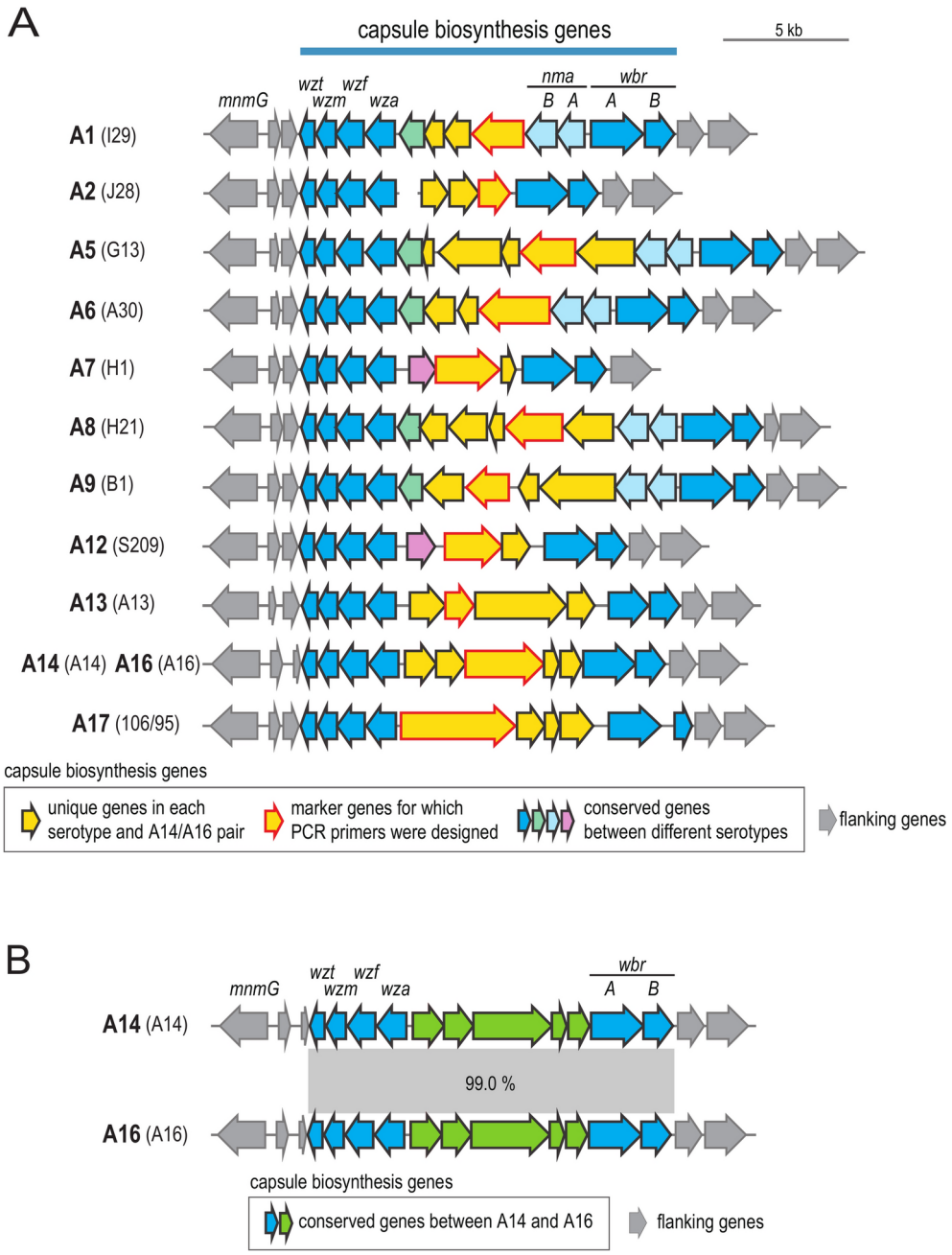

Fig. 2. Comparison of capsule biosynthesis gene clusters of different serotypes of *M. haemolytica*. (**A**) Composition of the synthetic gene clusters and marker genes for each serotype. (**B**) Comparison of synthetic gene clusters between A14 and A16.

Multiplex PCR kits

Two types of multiplex PCR kits containing the 11 serotype-specific and *M. haemolytica*-specific primer pairs mentioned above were constructed. The A kit contained primer pairs for A1, A2, A6, A8, A9, and A12, while the B kit contained primer pairs for A5, A7, A13, A17, and A14/16. The PCR products generated by these primer pairs were of different sizes, forming step-like patterns on agarose gels (Fig. 4). Both kits also included the *Mannheimia*-specific primer pair (Fig. 4 and Supplementary Table S3). Evaluation of all strains listed in Table 1 confirmed that the two kits could simultaneously and specifically detect *Mannheimia* species and their serotypes. Evaluation using serial dilutions of a pure culture of a *M. haemolytica* A2 strain confirmed that the A kit could barely detect both the species and serotype of the strain in saline at a concentration of 2.2×10^4 CFU/ml (Supplementary Figure S2).

Serotype	Target gene	Primer name	Sequence (5'—3')	Product size
A1	glycosyltransferase	Mh_A1_F	TCCCCTGTTGCAAATACACC	990 bp
		Mh_A1_R	TACTTCACGTATGCCCAGCA	
A2	hypothetical protein	Mh_A2_F	GGAACAGGGGCTTTGTATCT	332 bp
		Mh_A2_R	GTTGCATGCCCAGAATAAGG	
A5	glycosyltransferase	Mh_A5_F	ATGTGCGAGAGAGGTTGCTT	408 bp
		Mh_A5_R	CGTCACATTGCACCTCAGAT	
A6	glycosyltransferase	Mh_A6_F	GGCATGCGGTACACCTGTAAT	250 bp
		Mh_A6_R	CAATGGGGCTTCAGGCTTA	
A7	glycosyltransferase	Mh_A7_F	ATGGTGGGCTCTCAAGTTCA	307 bp
		Mh_A7_R	TATACTGCTGTTGCCGCAGA	
A8	glycosyltransferase	Mh_A8_F	GGTGTATCTCATGCGGTTGGA	429 bp
		Mh_A8_R	GCTTTGCGTTGAAGAGGCT	
A9	hypothetical protein	Mh_A9_F	GCCTTCCAATTTTGGAGCTG	760 bp
		Mh_A9_R	GCCTCCACCAAGTATACGAA	
A12	glycosyltransferase	Mh_A12_F	ATACTTCCGGTTCTGCTGGA	169 bp
		Mh_A12_R	CCCAGAAATGGGCTATCACT	
A13	CDP-glycerol glycerophosphotransferase	Mh_A13_F	CTCATGATGATGGGGGATAC	221 bp
		Mh_A13_R	TGCCCTGTATTAGGGTGAGA	
A14/A16	glycosyltransferase	Mh_A14/A16_F	GTGCTTATTGGTCTGCAGGT	132 bp
		Mh_A14/A16_R	CACCATAAGCTCTTGCTGGA	
A17	glycosyltransferase	Mh_A17_F	AAGGGCAGGTTAAAGCACCT	791 bp
		Mh_A17_R	TCTTGCTACCTGCCTTTGCT	
-	*recN* (Mh-specific sequence)	Mh-spe_recN_F	CCTTTACTATGCAGCCACAT	591 bp
		Mh-spe_recN_R	GGCTATCAACCTCAGCCAAG	

Table 2. PCR primers designed in this study.

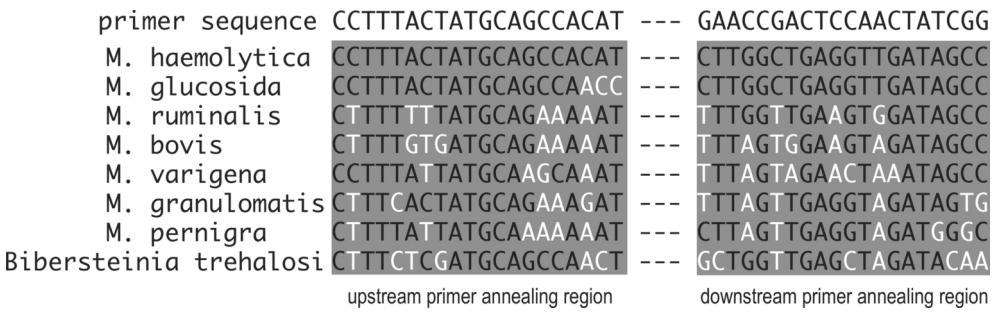

Fig. 3. *M. haemolytica*-specific primers designed on *recN*.

Discussion

Although serotyping is a standard method for subtyping certain pathogenic bacteria, DNA-based typing approaches utilizing the specificity of antigen-encoding genes have become increasingly widespread as alternatives to these methods[16–19]. In this study, to efficiently screen *M. haemolytica* serotypes, two multiplex PCR kits were developed based on the results of a genomic comparison of serotype reference strains. This system may enhance the efficiency of *M. haemolytica* investigations and provide valuable information for detecting the emergence of new epizootic disease and selecting appropriate vaccine candidates, thereby aiding infection control efforts. In addition, the sequence set of marker genes extracted in this study may be useful for in silico serotyping by homology search using the whole genome sequence of *M. haemolytica*. Further evaluation of the validity of these methods using strains with defined serotypes will be required, including evaluation of the PCR method using actual A17 strains. The A kit was confirmed to be capable of detecting the species and serotype of a strain present at 2.2×10^4 CFU/ml in saline. Although these kits are intended for use in identifying isolated strains, they may also be useful for direct diagnosis of specimens such as nasal swab suspensions from diseased cattle.

The reference strains of *M. haemolytica* serotypes A1 to A12 and A13 to A16 were obtained from the National Animal Disease Center, USA, and the Moredun Research Institute, UK, respectively, more than 30 years ago. For confirmation, we compared our *M. haemolytica* genomes with those of serotype strains published in a previous study by Christensen et al.[23]. Sequences were obtained from the following database: https://ivsmlst.sund.ku.dk/.

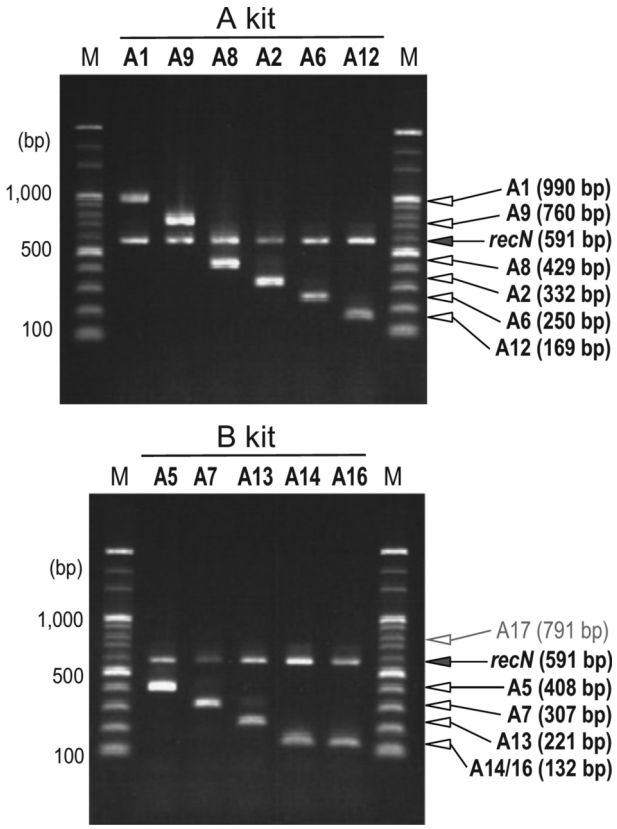

Fig. 4. PCR products of serotype reference strains by two multiplex PCR kits.

It was confirmed that all strain pairs within the same serotype, except for those belonging to A8, A13, and A14, possessed nearly homologous genomes (Supplementary Figure S1). The two A13 strains were phylogenetically distant but branched off from a common ancestor. For some reason, the A8 and A14 strains were expected to be mixed up. Therefore, we re-evaluated the phylogenetic relationship by adding the genome sequence of the A8 strain NCTC 10636 (= ATCC 29699) (accession number CP097336), which was registered in a public DNA database by a group in the United States. As a result, it was found to be homologous to the genome of our A8 strain (Supplementary Fig. 1). No genome information for the other A8 and A14 *M. haemolytica* strains was found in public DNA databases. Although evaluation by a third party may be necessary in the future, based on the above evaluation, we determined that the A8 and A14 strains and their genomic information used in this study were appropriate as references, albeit indirectly.

Serotype A14 and A16 strains shared homologous capsule synthesis genes (Fig. 2A). Of the five genes specific to this pair compared with other serotypes, the first, second, and fifth genes (encoding α- 2,8-polysialyl transferase, a glycosyltransferase, and a hypothetical protein, respectively) each had one amino acid difference between the two strains, while the third gene (encoding a glycosyltransferase) had 22 amino acid differences (data not shown). *Escherichia coli* O107 and O117 share an O-antigen synthesis region consisting of 11 genes with 98.6% nucleotide sequence identity, and it has been shown that the difference in their O-antigen glycan structure (or antigenicity) was due to only three amino acid substitutions in one gene (*wclY*) encoding a glycosyltransferase[24]. Although the structures of the capsular glycans of A14 and A16 are unknown, slight amino acid differences in the shared capsule synthesis genes may result in differences in the glycan structures they synthesize.

Methods

Strains used in this study

To obtain genome sequences and evaluate the validity of the PCR methods developed in this study, 20 M. *haemolytica* strains belonging to serotypes A1 to A16 were used (Table 1). The reference strains of *M. haemolytica* serotypes A1 to A12 and A13 to A16 used in this genome analysis were provided to the National Institute of Animal Health, Japan, by the National Animal Disease Center, USA, in 1979, and by the Moredun Research Institute, UK, in 1992, respectively. In addition, nine *Mannheimia* species strains other than *M. haemolytica*, including *M. varigena*, *M. granulomatis*, *M. pernigra*, and *M. ruminalis*, *M. bovis*, *M. cairinae*, *M. caviae*, *M. indoligenes* and four *B. trehalosi* strains were used (Table 1). DNA for genome sequencing and PCR was purified from cultures grown on BD Difco™ Brain Heart Infusion Agar (BD Difco, NJ, USA) supplemented with 5% defibrinated sheep blood and 0.5% yeast extract (BD Difco) or in AccuDia™ Brain Heart Infusion Broth (Shimadzu Diagnostics, Tokyo, Japan) using the Wizard® Genomic DNA Purification Kit (Promega, WI, USA).

Genome analysis

Genome information of serotype A1 to A17 strains used in the previous study was obtained from the following database, https://ivsmlst.sund.ku.dk/[23]. We performed the whole genome sequencing of eleven *M. haemolytica* strains belonging to serotypes A1 to A16 by the using Illumina platform. Low quality and adapter sequences in the reads were trimmed using Platanus_trim v1.1.0 (http://platanus.bio.titech.ac.jp/pltanus_trim) with the default parameters for subsequent analyses. For de novo assembly the sequence reads were downsampled to a total read length of 500 Mbp, corresponding to 200 ×coverage of the genome, using the seqkit sample command[25], and then assemblies were performed using Platanus_B v1.3.2 with the default parameters[26]. The draft genomes were assessed using the CheckM (v1.1.3)[27] taxonomy workflow with the specific marker set for the genus *Mannheimia*, confirming >97% completeness and <3% contamination. Gene annotation for each genome was performed using Prokka v1.14.5[28]. Pangenome analysis was carried out using Roary v. 3.13.0[29]with the parameters -i 95, -e, -n. Phylogenetic analysis based on the single nucleotide variants in the core genes was performed using RaxML-NG v. 1.0.1[30]with the parameters -all, -bs-trees 100, -model GTR + G4. The tree was rooted at the midpoint and visualized using iTol[31]. Capsule synthesis gene clusters and their surrounding regions were identified and extracted from the assembled contigs. *recN* sequences were obtained from the DNA database (Supplementary Table S2). Comparative analyses of them were performed using in silico Molecular Cloning software GE v. 7.29 (in silico biology, Kanagawa, Japan).

Simplex and multiplex PCR

Simplex PCR was performed using the following protocol: each 15-µl reaction mixture contained 7.5 µl of DreamTaq Green PCR master mix (2 ×) (Thermo Scientific, MA, USA), primers (final concentration of 0.2 µM each), and 1 µl of genomic DNA (2–10 ng/µl). Multiplex PCR was performed using a similar protocol: each 15-µl reaction mixture contained 7.5 µl of DreamTaq Green PCR master mix (2 ×), primers (final concentration of 0.1 or 0.2 µM each), and 1 µl of genomic DNA (2–10 ng/µl). The pre-preparation method for the primer mixes that can be used for the two types of multiplex PCR is shown in Supplementary Table S3. The thermocycling conditions were the same for both simplex and multiplex PCR: 25 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s. PCR products (2 µl) were electrophoresed in 2% agarose gels in 0.5 ×Tris-borate-EDTA buffer (25 mM Tris-borate, 0.5 mM EDTA) and photographed under UV light after staining the gel with ethidium bromide (1 µg/ml). The bacterial suspension used to evaluate the sensitivity of the PCR kit was prepared as follows: the *M. haemolytica* B123 strain, which belongs to A2, was inoculated into Brain Heart Infusion broth and cultured overnight at 37 °C, and the bacterial suspension was serially diluted with physiological saline. These bacterial dilutions were added directly to the same PCR reaction mixture as described above. A 100 µl aliquot of each dilution was inoculated onto two Brain Heart Infusion agar plates and cultured, and the bacterial concentration was estimated based on the number of grown colonies.

Data availability

All sequence data determined in this study are available in the DDBJ/EMBL/GenBank BioProject under accession number PRJDB19202.

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Author contributions

A.I. and D.T. administrated the project; Y.U., K.H. and R.U. collected the strains; A.I., Y.U., K.H. and G.Y. performed the experiments; A.I., M.O. and Y.O. analyzed the data; A.I. wrote the original draft, the other authors reviewed and edited the paper. All authors read and approved the final manuscript.

Declaration

Competing interests

The authors declare no competing interests.

Additional information

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