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1 # Method for culturing *Candidatus* Ornithobacterium hominis

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23 **Abstract**

24 *Candidatus* Ornithobacterium hominis has been detected in nasopharyngeal microbiota
25 sequence data from around the world. This report provides the first description of culture
26 conditions for isolating this bacterium. The availability of an easily reproducible culture
27 method is expected to facilitate deeper understanding of the clinical significance of this
28 species.

29

30 **Manuscript text**

31 *Candidatus* Ornithobacterium hominis (OH) is a bacterium that has been detected in
32 nasopharyngeal microbiota sequence data from around the world but has never been cultured
33 (Salter et al., 2019; Salter et al., 2017). This bacterium is of growing interest as polymerase
34 chain reaction (PCR)-based studies found that OH was prevalent and persistent in the
35 nasopharynx of a paediatric population at high-risk of respiratory infection (Salter et al.,
36 2019; Salter et al., 2017). Additionally, the closest known relative of OH is *Ornithobacterium*
37 *rhinotracheale*; a respiratory pathogen of birds (Zahra et al., 2013). These observations
38 prompt research to understand the pathogenic potential and clinical significance of OH.
39 Although genomes can be derived from metagenomic data, OH isolates are needed to deepen
40 understanding of the bacterium's role in human respiratory infections. The aim of this study
41 was to determine culture conditions for recovery of OH isolates.

42

43 The study was approved by the Human Research Ethics Committee of the Northern Territory
44 Department of Health and Menzies School of Health Research (Approval number: 0785).
45 Culture was performed using biobanked nasopharyngeal swabs that were collected from four
46 Australian children (age 1-2 years) immediately prior to bronchoscopy for investigation for
47 chronic suppurative lung disease (Marsh et al., 2016). The swabs had been stored in skim

48 milk-tryptone-glucose-glycerol broth (STGGB) at -80°C for up to 10 years and had two
49 freeze-thaw cycles prior to OH culture. These swabs were selected as all were OH-positive
50 by 16S rRNA gene sequencing at 5-55% relative abundance (Marsh et al., 2016).
51
52 Ten microlitres of the STGGB swab media was inoculated onto Tryptic Soy Agar with 5%
53 Sheep Blood (TSA), Horse Blood Columbia agar (HBA), Chocolate agar and Brain Heart
54 Infusion agar (BHI). The plates were incubated aerobically, microaerophillically (Campygen,
55 Oxoid) and anaerobically (Anaerogen, Oxoid) at 35°C for up to five days. Aerobic culture
56 was also performed in the presence of a wet sponge to provide increased humidity (Mayahi et
57 al., 2016). Oxidase testing was done using oxidase test strips (Oxoid). Tributyrin hydrolysis
58 was determined using Catarrhalis discs (Remel). Production of β-lactamase was determined
59 using nitrocefin (Oxoid).
60
61 Primary cultures were reviewed for colonies resembling *O. rhinotracheale* (van Empel and
62 Hafez, 1999). Colonies of oxidase-positive, Gram-negative pleomorphic bacilli were
63 screened using PCR targeting OH-specific regions of the 16S rRNA and *toxA* genes, as
64 described previously (Salter et al., 2019). PCR-positive isolates were confirmed using
65 genome sequencing. Genomes were assembled *de novo* using the Microbial Genome
66 Assembly Pipeline (MGAP) v1.0 (https://github.com/dsarov/MGAP---Microbial-Genome-
67 Assembler-Pipeline) (Chapple et al., 2016). OH identification was confirmed where isolates
68 had >96% average nucleotide identity (Kim et al., 2014; Richter and Rossello-Mora, 2009)
69 when compared to draft OH genomes OH-22767 (GenBank accession
70 NZ_UNSC00000000.1) and OH-22803 (GenBank accession UNSD00000000.1). Both draft
71 genomes were derived from metagenomic analysis of nasopharyngeal swabs from Thai
72 children (Salter et al., 2019) . Isolate genomes were mapped against the draft OH genomes

73 using the Synergised Pipeline for Analysis of Next Generation Sequencing Data in Linux
74 (SPANDx) v3.2.1 (Sarovich and Price, 2014) , which wraps Burrows-Wheeler Aligner (Li
75 and Durbin, 2009), Sequence Alignment/Map (SAM) tools (Li et al., 2009), Picard Tools and
76 Genome Analysis Tool Kit (McKenna et al., 2010). Genomes were aligned using draft OH
77 genome OH-22803 as the reference with an *O. rhinotracheale* genome (ORT-UMN 88,
78 GenBank accession CP006828.1) included as an outgroup. Maximum parsimony
79 phylogenomic trees were generated using Phylogenetic Analysis Using Parsimony (PAUP)
80 v4.0a153 (Swofford, 1998) and visualised using FigTree
81 (http://tree.bio.ed.ac.uk/software/figtree/). Bootstrapping was performed in PAUP with 1000
82 replicates. Lipopolysaccharide comparisons were generated using Easyfig (Sullivan et al.,
83 2011). The OH isolate genomes are available from the Sequence Read Archive (SRA;
84 BioProject number: PRJNA510696).
85
86 OH was successfully cultured from all four swabs. Primary isolation was challenging due to
87 substantial overgrowth by other taxa (Figure 1). Of the conditions tested, optimal primary
88 culture was achieved using TSA incubated in a microaerophilic atmosphere at 35°C for up to
89 five days. OH also grew on HBA, Chocolate agar and BHI; however, isolates were not
90 consistently recovered from these media. Aerobic growth was possible but required
91 additional humidity (e.g. incubating plates in a box containing a wet sponge).
92
93 Under microaerophilic conditions, OH colonies were pleomorphic, glistening, grey and
94 concave. Colonies ranged in size from 1-3 mm after 48-120 hours incubation. All isolates
95 were pleomorphic Gram-negative bacilli. Consistent with the phenotype predicted by the
96 draft genomes (Salter et al., 2019), OH isolates were oxidase-positive, catalase-negative and
97 all produced β-lactamase. All isolates also hydrolysed tributyrin. Some pure isolates

98 produced two colony morphologies (Figure 1D). This phenotype is suggestive of small-cell
99 variants (Zahra et al., 2013) as both colony types were positive by OH 16S rRNA and *toxA*
100 PCR.
101
102 The OH isolate genomes had average nucleotide identity of 97.86-98.23% with draft
103 genomes OH-22803 and OH-22767, indicating that they are members of the same species.
104 Phylogenomic analysis demonstrating the high similarity between the Australian isolates and
105 draft OH genomes from Thailand is shown in Figure 2. All isolate genomes contained distinct
106 lipopolysaccharide (LPS) biosynthesis clusters which differed to those of the draft genomes
107 (Figure 3). β-lactamase production was associated with mobile genetic elements that were
108 different in each isolate and occurred at different loci. All isolates also had genes encoding
109 efflux pumps associated with multi-drug resistance.
110
111 In summary, following identification of OH *in silico*, we now report culture conditions for its
112 propagation. Of the conditions tested, optimal growth was achieved using TSA with
113 incubation for up to five days in a microaerophilic atmosphere; conditions which are not part
114 of standard culture used to recover respiratory pathogens from nasopharyngeal swabs (Satzke
115 et al., 2013). Primary isolation was challenging due to extensive overgrowth by other flora.
116 We recommend OH-specific PCRs (Salter et al., 2019) are used to confirm isolate identity.
117 The OH colonial morphology was similar to that reported previously for *O. rhinotracheale*
118 (van Empel and Hafez, 1999), including growth of multiple colony morphologies suggestive
119 of small-cell variants (Zahra et al., 2013). The significance of this observation is unknown;
120 however, small-cell variants of other bacteria (e.g. *Staphylococcus aureus*) have been
121 associated with poorer clinical outcomes in patients with respiratory disease (Wolter et al.,
122 2013). Association of β-lactamase genes with multiple mobile genetic elements indicates that

123 OH β-lactam resistance has been acquired through several independent events. Heterogeneity
124 among the LPS cluster is suggestive of multiple capsular types, consistent with observations
125 from earlier DNA-based studies (Salter et al., 2019; Salter et al., 2017). The availability of an
126 easily reproducible culture method is expected to facilitate deeper understanding of the
127 clinical significance of OH.

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184

185 **Figure 1: *Ca.* Ornithobacterium hominis colony morphology**

186 A) Primary isolation of *Ca.* O. hominis isolate 903C1 on TSA after 120 hours aerobic
187 incubation in the presence of a wet sponge. Arrow indicates a *Ca.* O. hominis colony.
188 B) Purified *Ca.* O. hominis isolate 903C1 after 120 hours microaerophillic incubation on
189 TSA. Pure culture of this strain produced a uniform colony size.
190 C) Primary isolation of *Ca.* O. hominis isolate 902C1 on TSA after 120 hours
191 microaerophillic incubation. Arrow indicates a *Ca.* O. hominis colony.
192 D) Purified *Ca*. O. hominis isolate 902C1 after 120 hours microaerophillic incubation on
193 TSA. Pure culture of this isolate producted two colony morphotypes.
194

195 **Figure 2: Phylogenomic analysis of the *Ca.* Ornithobacterium hominis isolates**

196 A midpoint-rooted maximum parsimony tree was constructed based on 764 biallelic single
197 nucleotide polymorphisms (SNPs) orthologous to the four Australian *Ca.* O. hominis isolates
198 (900C2, 902C1, 903C1 and 916C1); two previously reported draft *Ca.* O. hominis genomes
199 from Thailand (OH-22767 and OH-22803); and an *O. rhinotracheale* outgroup (ORT-UMN
200 88). Bar indicates a distance of 50 SNPs.

201

202 **Figure 3: Comparison of *Ca.* Ornithobacterium hominis lipopolysaccharide biosynthesis**

203 **loci.**

204 A tblastx alignment of the lipopolysaccharide biosynthesis clusters in the four Australian OH

205 isolates compared to draft genomes OH-22767 and OH-22803 derived from Thailand(2).

206

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