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Comparison of Beta-lactamase Genes in Environmental Rahnella Isolates

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Abstract

Antibiotic resistance is a growing problem in the field of healthcare. Antibiotics are becoming less effective as species of bacteria adapt and share resistance mechanisms. If transmission of mechanisms can be better understood at the molecular level, inhibitors could be developed to lessen the likelihood of antibiotic resistance. In this study, *Rahnella* spp were isolated from environmental sources on MacConkey plates containing 100 μg ml^{-1} ampicillin, and confirmed by 16S rRNA gene sequencing. Whole genomic DNA was extracted from isolates and initial amplifications were performed by polymerase chain reaction (PCR) using primers specific for 16S amplification. New primers were designed based on the sequence of a β-lactamase gene identified in a *Rahnella* genome. These primers provided strong amplification. The products of these amplifications were sequenced, with the predicted protein products showing high sequence similarities to a previously identified *Rahnella* β-lactamase gene. Individual sequences were compared and found to cluster into two distinct groups, with each being distinct from the known *Rahnella* β-lactamase. Additional sequence data was used to determine the full sequences of this class A beta-lactamase gene predicted to be responsible for beta-lactam resistance. Primers were produced to amplify the full gene and a High Fidelity PCR Kit by Qiagen was used to amplify the gene and furthermore, sequence the full gene. Ongoing research is being conducted to understand more about the mechanism by which the class A beta-lactamase gene confers resistance and additionally how this resistance is transferred between bacteria.

Introduction

Antibiotic resistance is a growing problem in the treatment of bacterial infection. Once easily treatable infections are becoming increasingly more dangerous and antibiotics are becoming less effective. In the 19th century, before antibiotics, it is believed that more than one-third of the population died from some sort of infectious, now treatable, disease.[1] With the discovery of penicillin these numbers fell drastically, but as antibiotic resistance increases among pathogenic organisms, death by bacterial infection is becoming more common and harder to prevent.[1] One important factor to consider when thinking about antibiotic resistance is the role antibiotics play in agriculture. Almost 70% of the antibiotics used for farming are of human importance and are used medically to treat human infections.[2] Antibiotic resistance in humans is now thought to be a byproduct of this "nontherapeutic use" of antibiotics in agriculture.[2] Resistant bacterial strains are being easily transmitted from animals to humans through direct contact, ingestion of food carrying resistant pathogens, and exposure to animal manure.[2] Antibiotics were once thought to be a sort of "miracle" drug but are now of little use in certain treatments of bacterial infections. For example, diagnosis of multidrug-resistant Tuberculosis (MDR-TB), caused by *Mycobacterium tuberculosis,* accounts for about 3% of all new TB diagnoses.[4] MDR-TB is resistant to two front-line treatments for the disease: rifampicin and isoniazid.[4] Because of multidrug-resistance, treatment of MDR-TB is longer, more intense and leads to worse outcomes than traditional TB treatment.[4]

Additionally, bacteria strains commonly found in soil confer resistance to front-line antibiotics. *Rahnella* species confer resistance to many beta-lactam antibiotics and

have the ability to spread this resistance to other more pathogenic bacteria. *Rahnella* strains are naturally occurring soil organisms that confer resistance to beta-lactam antibiotics through use of beta-lactamases.[3] Beta-lactamases are gene products that are secreted from gram-negative cells upon introduction of antibiotics. Beta-lactams contain a highly reactive four-membered beta-lactam ring that is responsible for the efficacy of this class of antibiotic.[5] Beta-lactamases have the ability to hydrolyze this four-membered beta-lactam ring and therefore impede the success of the antibiotic.[5] Many bacteria species confer such resistance through translation of inherent genes imbedded in their genome; some species confer resistance through easily transmittable genes residing on a plasmid.[3,5] *Rahnella* is thought to use a class A beta-lactamase gene imbedded on a large plasmid, about 500,000 bps in length. Understanding the genetic basis of bacterial resistance to antibiotics plays an important role in preventing the irreversible spread of resistance. *Rahnella* is an organism that can be used as a laboratory model to identify and understand resistance mechanisms in more pathogenic strains of bacteria. Amplification and sequencing of the full class A beta-lactamase gene responsible for resistance against beta-lactam antibiotics is the first step in gaining pertinent knowledge regarding how to effectively continue the fight against antibiotic resistance.

Materials and Methods

Soil samples were collected from Northeast Ohio coordinates 40°46’30’’N 81°20’09’’ W. Soil samples were diluted with distilled water and spread plated onto MacConkey with ampicillin agar. Plates were incubated overnight at 30 degrees Celsius. Lactose fermenting (red) bacteria colonies, a total of 9 morphologically different

colonies, were isolated from the plates and streaked for isolation on additional MacConkey with ampicillin plates. All 9 isolates (named S1-S9) were grown in individual overnight cultures using large glass test tubes with 3 mL liquid LB broth in a 30 degree Celsius shaker. Polymerase chain reaction (PCR) was done using 1 µl of overnight culture growth and 16S amplification protocol. Flat cap PCR tubes (0.2 mL) by USA Scientific were used to conduct PCR. Each flat cap PCR tube contained a total of 50 µl, made up of 5 µl 10X Taq buffer, 1 µl dNTPs, 1 µl 16S forward primer 27F, 1 µl 16S reverse primer 1492R, 40 µl ddH20, 1 µl Taq. Polymerase, and 1 µl overnight broth culture from each of the 9 samples. PCR was run using 16S amplification protocol in a BIO-RAC T100 thermal cycler. 16S amplification protocol consisted of 34 cycles of 30 seconds at 94°C followed by 30 seconds at 60°C, followed by 1 min 15 seconds at 72°C. This was followed by 5 min at 72°C and an infinite hold at 12°C**.** Gel electrophoresis was completed by adding 2 µl of loading buffer dye to 10 µl of PCR products and running on a 1% agar gel. Agar gel was made using .8g of agar and 80 mL of Tris/Borate/EDTA buffer. Agar mixture was microwaved for 1.5 minutes until solid agar was dissolved in buffer. 4 µl of ethidium bromide was added to the mixture and the gel was poured and let solidify for 15 minutes. Wells were loaded with 2-log ladder size standard in lane 1, negative control in lane 2, and S1-S9 samples sequentially in the following lanes (3-11). Gel electrophoresis was conducted at 120 volts for 1 hour. Sample S1-S9 PCR products (12 µl of each) were sent with 27F and 1492 primers (to the University of Chicago for CRC DNA sequencing.

Samples S1-S9 were amplified using primers specific for a class A beta-lactamase gene in *Rahnella aquatilis*. PCR was conducted using custom *Rahnella* primers named

RAHN-Up and RAHN-Down. The sequence of RAHN-Up was 5′-CTGGAAAAAGAAAGCGGCG-3′.[6] The sequence of RAHN-Down was 5′ -TCAATAACCCTGCGTCACA-3'.[6] Flat top PCR tubes were filled to 50 µl by adding 5 µl 10X Taq buffer, 40 µl ddH20, 1 µl RAHN-Up primer (50 picomol concentration), 1 µl RAHN-down primer (50 picomol concentration), 1 µl dNTPS, 1 µl Taq polymerase, and 1 µl of S1-S9 overnight culture. PCR was run in a BIO-RAD T100 thermal cycler on the RAHN protocol with cycles as follows: 34 cycles of 30 seconds at 94°C followed by 30 seconds at 60°C, followed by 1 min 15 seconds at 72°C. This was followed by 5 min at 72°C and an infinite hold at 12°C**.** Gel electrophoresis was conducted using a 1% agar gel prepared by adding 80 ml of TBE buffer to 0.8g of agar. Size standard 2-log ladder was used along with a negative control. 4 µl of ethidium bromide was added to the gel and gel electrophoresis was run at 120V for 1 hour. PCR products (12 µl of each) were sent along with 90 µl RAHN-Up primer at a concentration of 5 picomol to the University of Chicago for CRC DNA sequencing.

Custom primers were made to amplify the full beta-lactamase class A gene. The forward primer, named ORA0711, had the following sequence: 5'-CGCGAATTCACCATGATGAAAAATACCCTG CG-3'. ORA0712, the reverse primer, had a sequence of 5'-GCGTTCGAATCAATAACCC TGCGTCACAA-3'. Before amplification of the full gene, DNA purification of S1-S9 was done using a Wizard® Genomic DNA Kit by Promega. Wizard® genomic DNA purification instructions found on page 14 and 15 of the following protocol was used: https://www.promega.com/-/media/files/resources/protocols/technical-manuals/0/wizard-genomic-dna-purification-kit-protocol.pdf. PCR amplification of S1-S3 samples with primers ORA0711 and

ORA0711 was done using a Phusion High Fidelity PCR kit. Each flat top PCR tube was filled with 34.5µl ddH20, 10µl Phusion 5X high fidelity buffer, 1µl dNTPs, 1 µl ORA0711 primer, 1µl ORA0712 primer, 0.5 µl Phusion polymerase, 1µl DMSO, and 1µl of DNA from S1-S3 in 9 tubes total. One control tube was made using the above amounts without the addition of 1µl DNA. PCR was run in a BIO-RAD T100 thermal cycler on the RAHNHF protocol. RAHNHF protocol consisted of the following: 34 cycles of 30 seconds at 96°C followed by 30 seconds at 60°C, followed by 2 min 30 seconds at 72°C. This was followed by 5 min at 72°C and an infinite hold at 5°C**.** Gel electrophoresis was conducted using a 1% agar gel, prepared as described previously. Phusion Lambda ladder was used as a size standard and the gel was run at 130V for 30 minutes. Using QIAquick PCR Purification Kit by Qiagen PCR products from S1-S9 amplified with ORA0711 and ORA0712 primers were purified. S1-S9 purified PCR products were sent to the University of Chicago for CRC DNA sequencing.

Results

Upon initial spread plating of soil samples collected, 9 phenotypically distinct lactose-fermenting bacteria colonies were observed (named S1-S9). Distinctions were made based on size and color. Amplification and gel electrophoresis of the 16S gene of S1-S9 resulted in positive results: bands in every lane of the 1% agar gel with exception of the negative control lane. Bands signify amplification of 1500 bps in length.

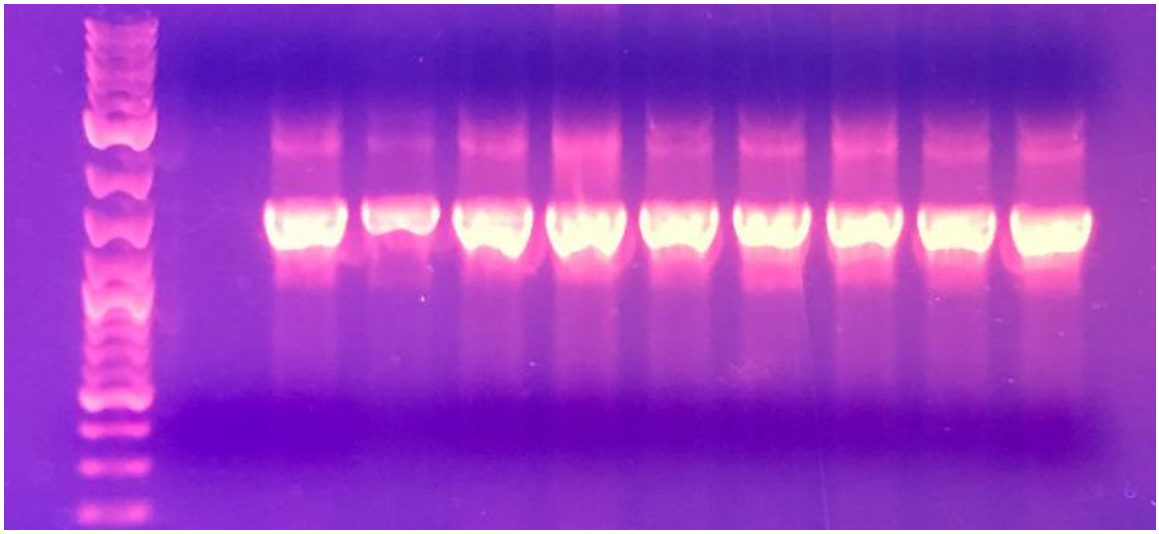

Fig. 1. Gel Electrophoresis results for amplification of 16S gene of S1-S9, numbered sequentially from left to right starting in lane 3. Amplification shown by fluorescent bands suggests the 16S gene to be about 1500 bps in length, as indicated by the 2-log ladder in lane 1 (furthest left).

University of Chicago CRC DNA sequencing of the 16S gene resulted in 8 *Rahnella aquatilis* organisms and 1 *pseudomonas sp.* (sample S4). All samples, with the exception of S4, were 100% identical.

Cross-referencing BLAST database entries with the sequencing data received from the University of Chicago determined the full gene sequence, approximately 900 bps long. Amplification of the full class A beta-lactamase gene using ORA0711 and ORA0712 primers resulted in positive bands for S1-S3 and S5-S9 upon gel electrophoresis.

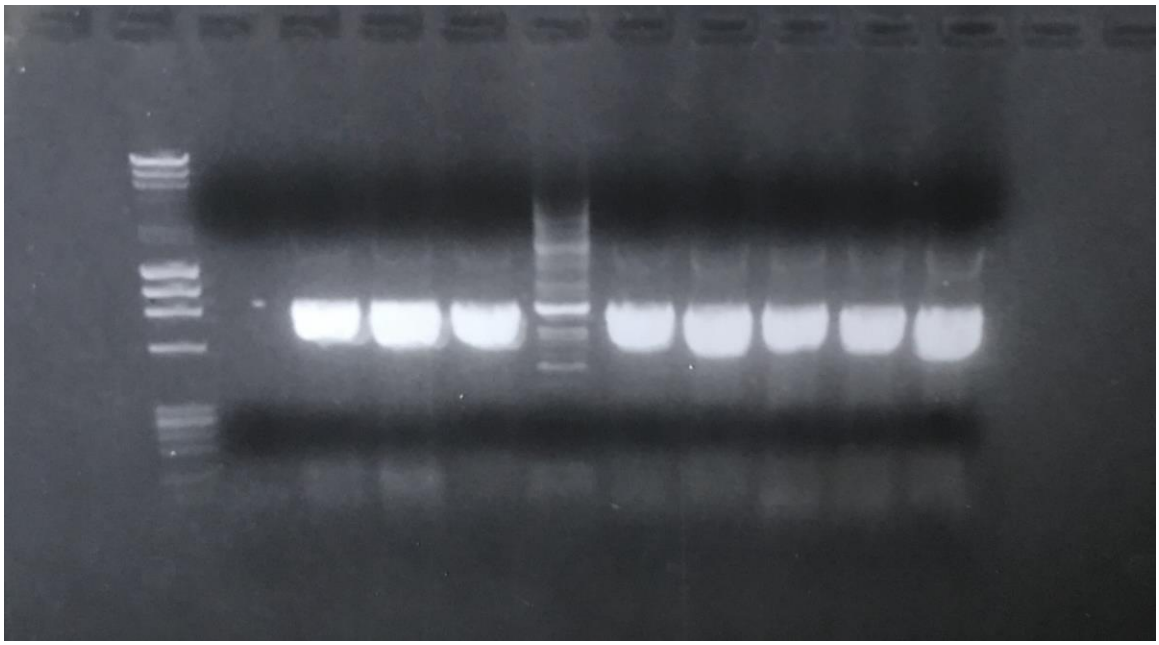

Fig. 2. Gel Electrophoresis results of High fidelity PCR kit amplification of S1-S9 with ORA0711 and ORA0712 primers. Bands show positive result for S1-S3 and S5-S9. No band seen in lane 2 signifying a negative result for negative control. S4 shows degradation of the amplified gene by experimenter error.

CRC DNA sequencing of High Fidelity Kit PCR products by the University of Chicago resulted in clean sequences that were 100% identical, with the exception of S3 (appendix 1).

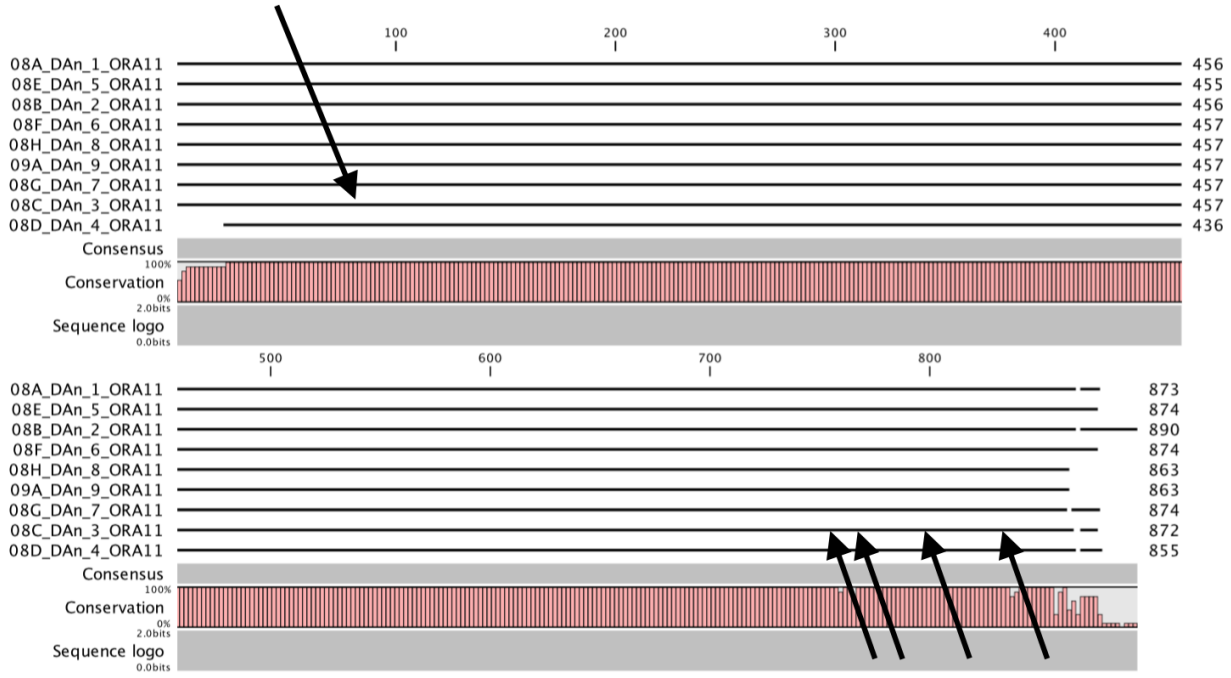

Fig. 3. Gross alignment of S1-S9 class A beta-lactamase genes. Arrows show base pair differences in S3 from the consensus sequence of S1-S2, and S4-S9. S1, S2, and S4-S9 were all identical in sequence.

Gene sequencing results of 16S amplification compared to gene sequences results of class A beta-lactamase amplification reveal discrepancies in similarity. Sample S4 is a *pseudomonas sp*. sharing an identical class A beta-lactamase gene with S1, S2 and S4-S9, all of which are *Rahnella sp*.. Additionally, S3 carries a class A beta-lactamase gene with multiple base pair differences from the consensus sequence of S1, S2 and S4-S9. However, S3 has a 16S gene sequence identical to the other *Rahnella sp.* samples (S1, S2 and S5-S9).

Discussion

In the present study, amplification of the 16S gene and subsequent gel electrophoresis gave way to sequencing of the species-specific gene that proved the bacteria to be all *Rahnella aquatilis* with one *Pseudomonas spp.*. Further comparison revealed the full sequence of the gene responsible for beta-lactam resistance. Knowledge of the full sequence was used to produce new primers to amplify the entire gene sequence. This amplification could lead to insertion into a plasmid and cloning in order to prove that this gene is in fact responsible for the antibiotic resistance. Successful amplification of the full gene proved possible, and showed distinctions between one strain in particular and the rest of the samples. These differences in genetic make up could help explain how transmission of genetic material such as beta-lactamase genes occurs.

There is little data regarding the antibiotic resistance patterns of *R. aquatilis* due to its scarcity in clinical cases and antimicrobial resistance studies.[7] However, the present study confirmed the use of a class A beta-lactamase gene within a 500,000 base pair plasmid as a means of antibiotic resistance for *Rahnella aquatilis and Pseudomonas spp.* Through amplification and sequencing it was found that *Pseudomonas spp.* carried a genetically identical beta-lactamase gene as *R. aquatilis*. Additionally, it was found that one sample of *R. aquatilis* housed a class A beta-lactamase gene that was genetically different in numerous places than the majority of *R. aquatilis* strains. Discrepancies in sequence data suggest uncomplicated gene transmission between species. Though Ruimy et al. suggests the class A beta-lactamase amplified in the present study to be

chromosomal, this study found the gene sequence on a large plasmid and not within the *R. aquatilis* chromosome.

Further studies should be conducted to prove that the class A beta-lactamase gene amplified in this study is responsible for resistance against beta-lactam antibiotics. Additionally, cloning should be completed to better understand the evolution and transmission of genetic material, specifically beta-lactamase genes, in *Rahnella sp.* The data from this study is helpful in understanding the genetic similarities and differences between bacteria species housing the same plasmid with resistance genes. Limitations when conducting the present study relate to the low pathogenicity of *R. aquatilis.* Antibiotic resistance mechanisms in *Rahnella sp.* specifically are not of clinical significance.[7] however, the class A beta-lactamase gene focused on for this study is within a plasmid capable of transmission to more pathogenic species of bacteria. Studying the mechanisms in less pathogenic organisms, such as *Rahnella sp.,* as laboratory models can be helpful in gaining knowledge about more pathogenic species and can aid in the fight against antibiotic resistance.

References

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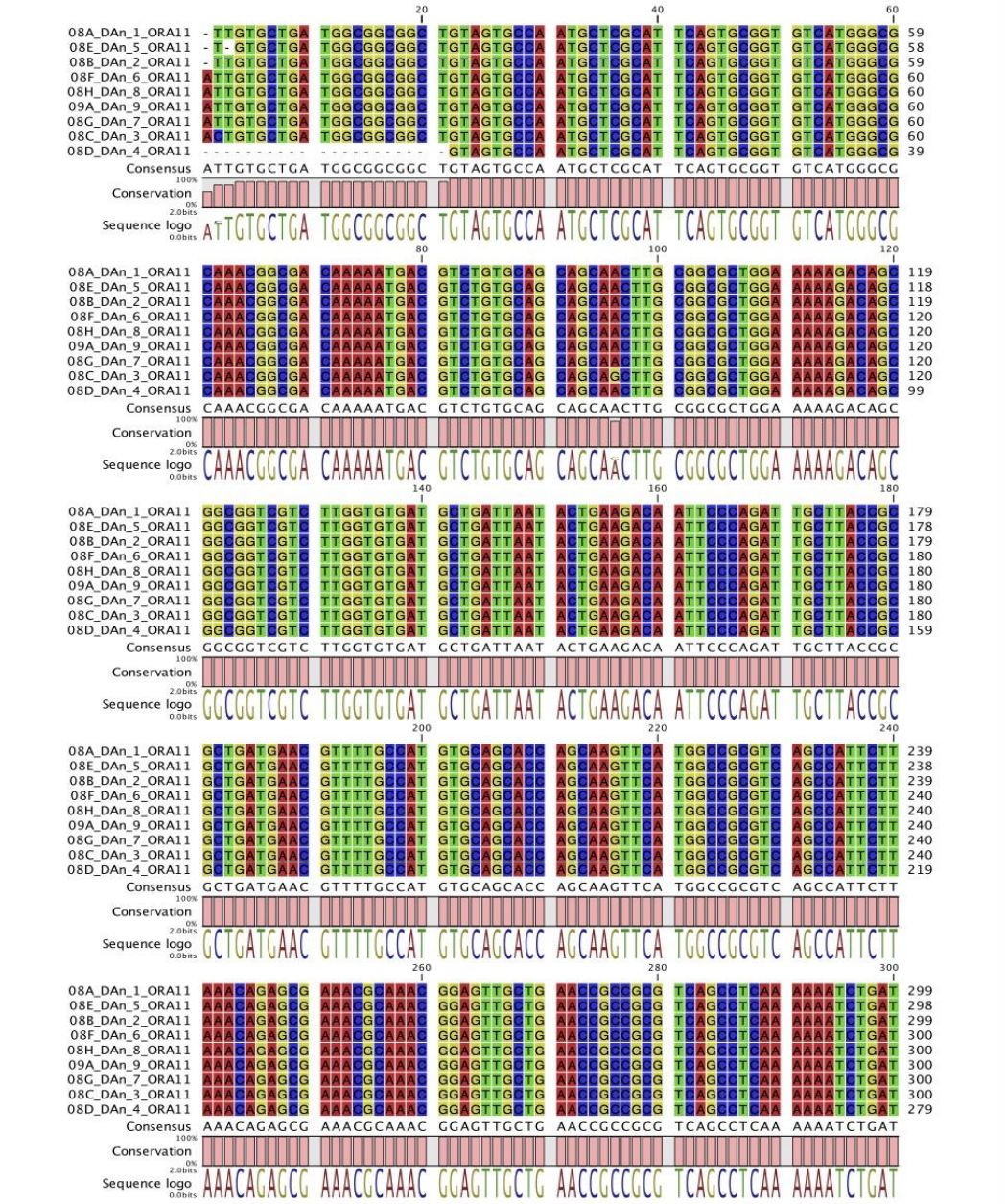
Appendix 1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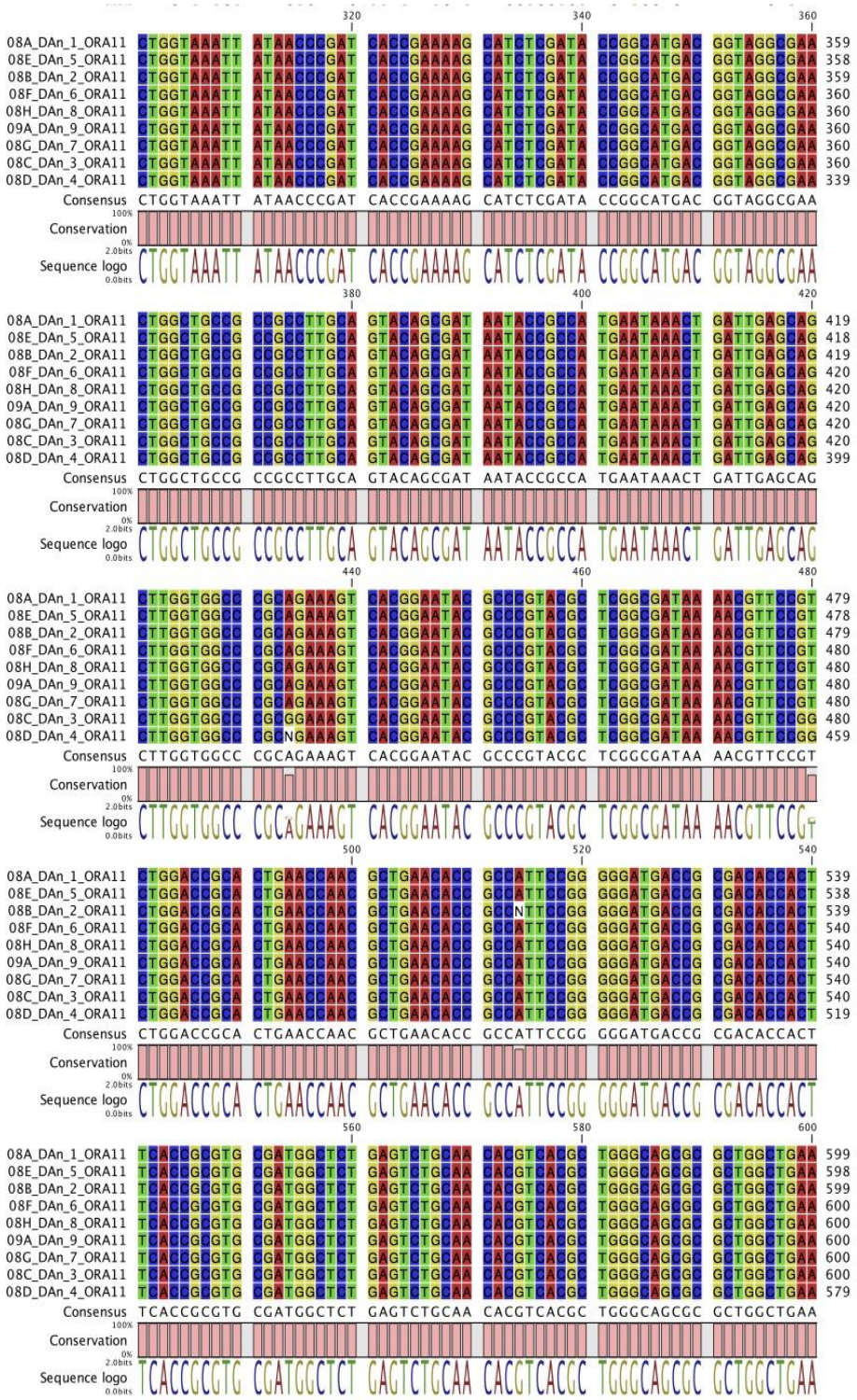
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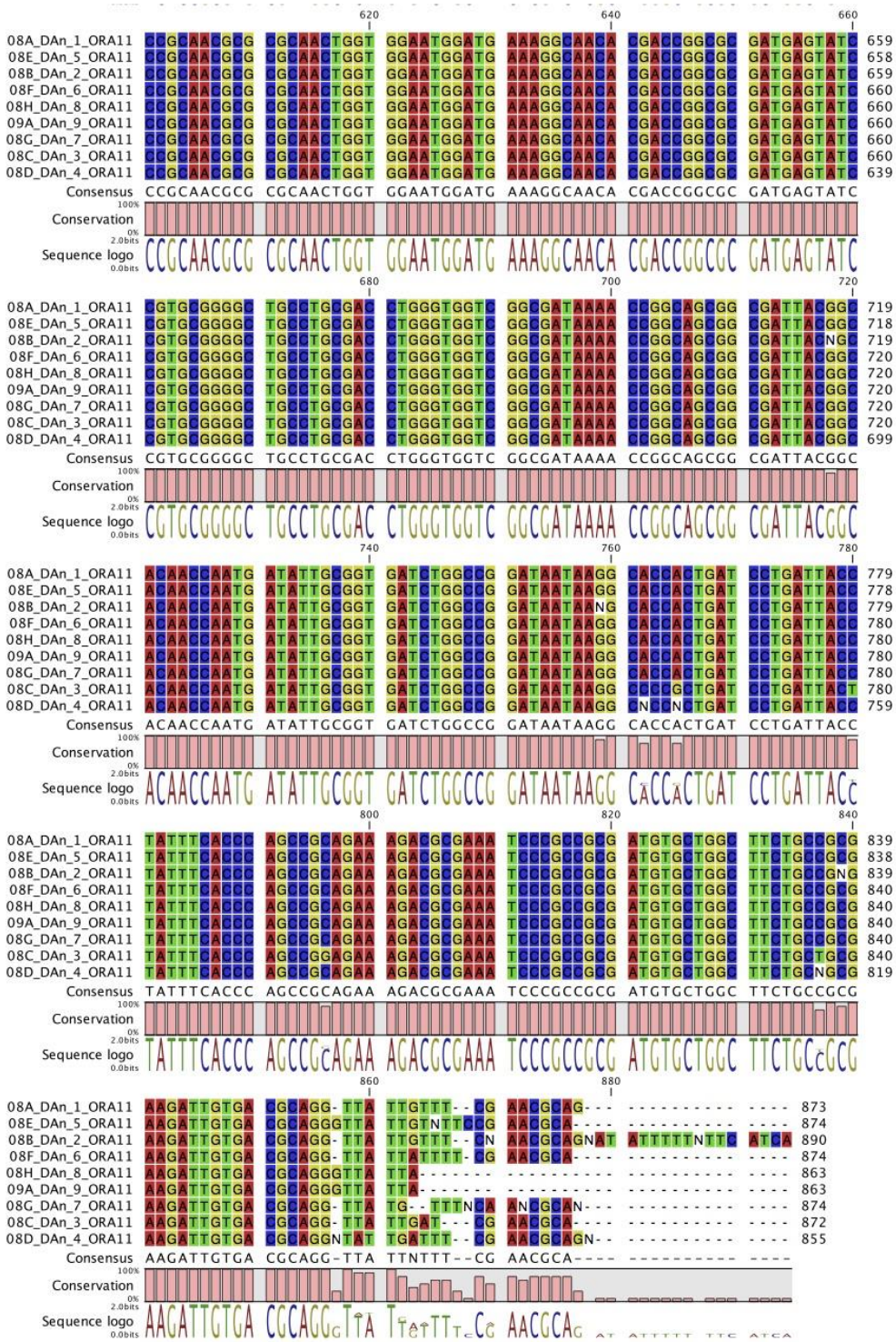
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08B_DAn_2_ORA11 890
08F_DAn_6_ORA11 874
08H_DAn_8_ORA11 863
09A_DAn_9_ORA11 863
08G_DAn_7_ORA11 874
08C_DAn_3_ORA11 872
08D_DAn_4_ORA11 855
Consensus AAGATTGTGA CGCAGG-TTA TTNTTT--CG AACGCA---- ----------
Conservation
Sequence logo