Examination of the horizontal gene transfer dynamics of an integrative conjugative element encoding multi-drug resistance in Histophilus somni

Farghaly, Mai Mohammed

http://hdl.handle.net/1880/113703
master thesis

University of Calgary graduate students retain copyright ownership and moral rights for their thesis. You may use this material in any way that is permitted by the Copyright Act or through licensing that has been assigned to the document. For uses that are not allowable under copyright legislation or licensing, you are required to seek permission.

Downloaded from PRISM: https://prism.ucalgary.ca
Examination of the horizontal gene transfer dynamics of an integrative conjugative element encoding multi-drug resistance in *Histophilus somni* 

by 

Mai Farghaly 

A THESIS 
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES 
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE 
DEGREE OF MASTER OF SCIENCE 

GRADUATE PROGRAM IN VETERINARY MEDICAL SCIENCES 

CALGARY, ALBERTA 

JANUARY, 2021 

© Mai Farghaly 2021
Abstract

Integrative and conjugative elements (ICEs) are autonomous-transferred mobile genetic elements that play a significant role in disseminating antimicrobials between bacteria via horizontal gene transfer (HGT). One recently identified ICE in a clinical isolate of *H. somni* named ICEHs02 is 72,914 base pairs in length and harbours seven AMR genes which confer resistance to tetracycline (*tetR-tet(H)*), florfenicol (*floR*), sulfonamide (*sul2*), aminoglycoside (*APH(3’)-Ib*), (*APH(6)-Id*), and (*APH(3’)-Ia*), and copper (*mco*). This study investigated the host range of ICEHs02, assessed the effect of antimicrobial stressors on transfer frequency of ICEHs02, and examined effects of ICE acquisition on the host. *In vitro* conjugation assays were conducted to examine the frequency of transfer of ICEHs02 into other bacteria. PCR and sequence analysis confirmed the presence of ICEHs02 and its circular intermediate in recipient strains. The susceptibility of ICEHs02-carrying recipients was conducted using broth microdilution. The effect of tetracycline and ciprofloxacin on transfer frequency and excision rates was investigated using mating assays and qPCR. Growth curves and competition experiments were conducted to assess the fitness cost of ICEHs02 on the host. ICEHs02 was observed to transfer into *H. somni* and *Pasteurella multocida*. PCR assays confirmed the presence of the circular intermediate, ICE-associated core genes and accessory genes in recipient strains. Susceptibility testing confirmed the function of ICEHs02-associated AMR genes in recipient strains. Tetracycline and ciprofloxacin induction significantly increased the transfer rates of ICEHs02. The copy numbers of the circular intermediate of ICEHs02 per chromosome exhibited significant increases of ~37-fold after tetracycline exposure, and ~4-fold after ciprofloxacin treatment. The acquisition of ICEHs02 reduced the relative fitness of *H. somni TG* by 28% (*w = 0.72 ± 0.04*) and *P. multocida TG* relative fitness was decreased by 15% (*w = 0.85 ± 0.01*). In conclusion, this study provided information on the host range of the multi-drug
resistant ICEHs02, gave insight into the regulatory role of antibiotics on the HGT of ICEs and emphasized the importance of ICEs in the dissemination of AMR between bacteria.
Acknowledgements

I would like to express my deepest gratitude to all those who assisted me in completing this work. First of all, I am grateful to my supervisor, Dr. Karen Liljebjelke, who allowed me to be a part of her laboratory. I would like to express my most profound appreciation for her trust, guidance, encouragement and support throughout the past two years. She was not only a great supervisor; she was an excellent teacher and a fantastic friend. Dr. Karen had a significant influence on my research career, especially in my critical thinking, academic writing and organizing research-related ideas. I really appreciate all the effort and long hours that Dr. Karen has dedicated to me. I will always remember her words to me. I was lucky to get to know a person like Dr. Karen.

I would like to thank my committee members, Dr. Michael Francis Hynes and Dr. Frank van der Meer. I would like to express my deepest gratitude for the guidance you provided me as a part of my dissertation committee. Dr. Michael was a constant help and support during the past two years. I truly appreciate the time he dedicated to me and the technical advice he gave me. He allowed me to have meetings with him to answer my questions even in the hard times, when the COVID pandemic started. He was keen to answer all my questions via email. He offered to let me work in his laboratory and to use his equipment and kits. Dr. Michael's suggestions brought in threads of thought that made my research so much richer. My appreciation goes to Dr. Frank who guided me a lot at the start of my project. Dr. Frank was a fantastic instructor. He helped me in understating specific topics. Dr. Frank was always trying to direct me to express my ideas and thoughts clearly. I really appreciate his support, guidance, and constructive criticism during the course of my program. I am really proud that I had the opportunity to work with people like Dr. Michael and Dr. Frank.
I would like to thank Dr. Neil Rawlyk for sending us *H. somni* strains. Thanks to Dr. Tao Dong and Paul Gajda for granting access to their laboratories and allowing us to use their equipment. Thanks to Dr. Douglas Morck for providing us with the *H. somni* control strains. Thanks to Dr. Anthony B. Schryvers for providing us with the *M. haemolytica*, and *H. influenzae* strains. My appreciation goes to Dr. Sylvia Checkley for her technical advice in analyzing the data and leading the AMR research group with Dr. Karen. Thanks to Dr. David Hall for leading the one health research group with Dr. Sylvia Checkley. I really appreciate your efforts to guide us to explore new fields, express our opinions, and present research ideas.

I would like to extend my heartfelt gratitude and appreciation to my friends and lab mates Mohammad Mostafa Nazari and Fernando Guardado for their help and cooperation during the past two years. I would like to express my most profound appreciation to Alyssa Butters, who helped edit my thesis and supported me through the past year. Special thanks go to my friends from Iran who worked in our laboratory for a year, Dr. Mehdi Zarei and Dr. Somayeh Bahrami. Thank you for your help in lab techniques and your constant support. Thanks to ACWA lab members Steven Hersch, Kevin Manera, Fatima Kamal, and Maria Silvina Stietz for their help and technical advice with laboratory equipment and techniques. Thanks to my friends Lyndsay Rogers, Heather Van Esch, Kayla Strong, Abdallah Shahat, and Mohamed Saleh, for their encouragement and support.

Finally, I would like to thank my family and friends for their constant support through the past two years. Special thanks to my lovely sisters Nada Farghaly and Passant Farghly, and to my amazing brother Mohamed Aboubakr.
Dedication

This thesis work is dedicated to

My husband & My parents

To my husband, Amro Nour, my love and best friend, who has been a constant source of support and encouragement during my graduate school and my whole life.

To my father, Mohammed Farghaly, who has always loved me, supported me and rescued me in my most challenging days and to my mother, Ehsan El-Zaoawy, who taught me to be strong, patient, and never give up.
Disclaimer

This thesis was copy-edited for grammar and clarity by the graduate supervisor, Dr. Karen Liljebjelke. Technical and scientific recommendations were provided by Dr. Karen Liljebjelke, Dr. Michael Francis Hynes and Dr. Frank van der Meer.
Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.

*Thomas A. Edison*
Table of Contents

Abstract ................................................................................................................................................I
Acknowledgements .........................................................................................................................III
Dedication ..........................................................................................................................................V
Disclaimer .........................................................................................................................................VI
List of Tables ......................................................................................................................................XI
List of Figures ....................................................................................................................................XII
List of Symbols and abbreviation ......................................................................................................XIV

CHAPTER ONE: LITERATURE REVIEW .......................................................................................1
1.1 Introduction ................................................................................................................................1
1.2 Bovine respiratory disease .........................................................................................................1
1.3 Bovine histophilosis ....................................................................................................................2
   1.3.1 *Histophilus somni* disease complex (HSDC) ....................................................................2
   1.3.2 Clinical manifestation of *Histophilus somni* disease complex (HSDC) ..............................4
   1.3.3 Management and control of histophilosis in feedlot cattle ....................................................6
1.4 Antimicrobial usage in feedlot cattle ..........................................................................................7
   1.4.1 General overview on antimicrobial usage ............................................................................7
   1.4.2 Antimicrobials in feed or water .........................................................................................9
1.5 Antimicrobial resistance ...........................................................................................................13
   1.5.1 Overview on antimicrobial resistance ..............................................................................13
   1.5.2 Mechanisms of antimicrobial resistance ..........................................................................13
   1.5.3 The effect of using in-feed antimicrobials on antimicrobial resistance ..............................15
   1.5.4 Antimicrobial resistance in *H. somni* and other bovine respiratory pathogens ..............16
1.6 Horizontal gene transfer ..............................................................................................................18
1.7 Mobile genetic elements ............................................................................................................19
   1.7.1 Plasmids ..........................................................................................................................20
   1.7.2 Integrative and mobilizable elements ..................................................................................20
   1.7.3 Integrative and conjugal elements ......................................................................................21
1.8 Integrative and conjugal elements in the *Pasteurellaceae* family ...........................................25
   1.8.1 Integrative and conjugal elements in *M. haemolytica* and *P. multocida* .......................25
   1.8.2 Integrative and conjugal elements in *H. somni* ................................................................26
   1.8.3 Integrative and conjugal elements in *Haemophilus influenzae* .......................................27
1.9 Factors affecting the dissemination of ICEs and other MGEs ....................................................29
   1.9.1 Quorum sensing and the transfer of ICEs .........................................................................29
   1.9.2 The effect of SOS response on the transfer of ICEs............................................................30
   1.9.3 Role of antimicrobials in the dissemination of MGEs ..........................................................30
1.10 Fitness costs of integrative and conjugal elements ...................................................................32
1.11 Aims of the present master thesis ............................................................................................33
   1.11.1 Background information ..................................................................................................33
   1.11.2 Objectives .......................................................................................................................34
1.12 Research questions and hypotheses ..........................................................................................37
1.13 Significance of the research ......................................................................................................39

CHAPTER TWO: MATERIALS AND METHODS ........................................................................40

VIII
CHAPTER FIVE: CONCLUSION AND FUTURE DIRECTIONS

REFERENCES

APPENDICES
Appendix 1: Preparation of culture media
Appendix 2: Growth curve protocol for H. somni, P. multocida, and M. haemolytica
Appendix 3: Growth curves of P. multocida, M. haemolytica, H. somni
Appendix 4: Filter mating assay protocol
Appendix 5: E-strip test (Epsilometer test)
Appendix 6: Broth microdilution method using Sensititre® system
Appendix 7: Copper tolerance assay protocol
Appendix 8: Agarose gel electrophoresis
Appendix 9: Visualizing the DNA using ChemiDoc™ MP Imaging System
Appendix 10: Quantification of gDNA (genomic DNA) using ND-1000 UV/Vis Nanodrop spectrophotometer
Appendix 11: Purification of PCR-amplified products using GenepHlow™ Gel/PCR Kit
Appendix 12: Quantification of gDNA using Qubit® Fluorometer
Appendix 13: Growth curves and calculation of growth rate and doubling time
Appendix 14: DNA sequence alignments of PCR sequenced products for circular intermediate, chromosomal junction, and chromosome attachment sites to the sequence of ICEHsKLM014 (ICEHs02) and the whole genome sequence of H. somni KLM014
Appendix 15: Pair-wise sequence alignments of tRNA-Leu gene from H. somni KLM014 to other bacterial species to predict the host range of ICEHs02
Appendix 16: Copy-right agreement
List of Tables

Table 1.1: Types of antimicrobial use in food animal ................................................................. 11
Table 1.2: Antimicrobials used for prophylaxis, metaphylaxis, and treatment in feedlot Canada 12
Table 1.3: Comparison of recently identified ICEs in some members of the Pasteurellaceae family ............................................................................................................................. 27
Table 1.4: Genomic characteristics of the ICE identified in H. somni KLM014 ...................... 35
Table 2.1: Solvents and diluents used for the preparation of stock solutions ........................... 49
Table 2.2: PCR primers used in the detection of ICE associated core genes, accessory genes, circular intermediate, and species- specific primers ........................................................................... 60
Table 3.1: Conjugative transfer frequency of ICEHs02, and genotypic characteristics of donors (H. somni KLM 014, P. multocida DSM 16031ICEHs02), recipients (RifR H. somni KLM 08, RifR H. somni ATCC700025, RifR P. multocida DSM16031, nalR P. multocida DSM16031, nalR H. somni ATCC700025) and transconjugant strains using PCR primers to detect four core genes (traI, ssDNA, tnp, and int) and two accessory genes( tetH, mco) ............................................................................................................................... 71
Table 3.2: Phenotypic characteristics of ICEHs02- carrying donor (H. somni KLM014), recipient strains (RifR H. somni KLM08, RifR H. somni ATCC700025, and RifR P. multocida DSM16031) and transconjugant strains .................................................................................................................. 72
Table 3.3: Conjugal transfer frequency and fold change in the transfer frequency upon tetracycline and ciprofloxacin induction of the donor H. somni KLM014, H. somni ATCC700025, H. somni KLM08, P. multocida DSM16031 were used as recipient strains ......................................................................................... 79
Table 3.4: Quantification of ICEHs02 circular form and chromosomal junction with tetracycline and ciprofloxacin induction and without induction using real-time PCR ........................................ 81
Table A.1: AMR profile of H. somni KLM014, H. somni KLM08, H. somni ATCC700025, and P.multocida DSM16031 using broth microdilution assay (sensititre ®) ........................................ 148
List of Figures

Figure 1.1: Life cycle of integrative and conjugative elements (Johnson and Grossman 2015)...23

Figure 1.2: A schematic map of ICEHsKLM014 (ICEHs02) showing the resistance gene regions 1 and 2......................................................................................................................36

Figure 2.1: Tetracycline antimicrobial susceptibility of H. somni KLM08 using M.I.C.E. method ............................................................................................................................................45

Figure 2.2: A schematic diagram illustrating the location of PCR primers used to detect the excision and integration of the ICEHs02 sequence ........................................................................................................58

Figure 2.3: A graph illustrating the mathematical points on the best fitted line used for calculating the growth rate of bacteria ........................................................................................................................................66

Figure 3.1: Agarose gel electrophoresis visualizing products of PCR assays to confirm the transfer of ICEHs02 from the donor strain H. somni KLM014 to the recipient strain H. somni ATCC700025 ........................................................................................................................................73

Figure 3.2 A: Diagram illustrating the excision (formation of circular intermediate) and integration of ICEHs02 in H. somni KLM014 chromosome ........................................................................................................75

Figure 3.2 B: Agarose gel electrophoresis visualizing PCR products of the circular intermediate, chromosomal junction, and chromosomal attachment sites ........................................................................................................75

Figure 3.3: Agarose gel electrophoresis visualizing the circular intermediate form of ICEHs02 in the donor strain H. somni KLM014 and the transconjugant strains H. somni KLM08, H. somni ATCC700025, and P. multocida DSM16031 using the primer pair (PCIF, PCIR) .................76

Figure 3.4: Bar graph illustrating a comparison of the copy numbers of ICEHs02 circular intermediate upon tetracycline and ciprofloxacin induction (treatment groups), and without induction (control group) ........................................................................................................................................82

Figure 3.5 A: Comparison of the growth curves of H. somni ATCC700025 transconjugant (TG) and H. somni ATCC700025 isogenic ancestor ........................................................................................................84

Figure 3.5 B & C: Exponential growth curves used for the calculation of the growth rate of H. somni ATCC700025 TG and H. somni ATCC700025 isogenic ancestor ........................................................................................................84

Figure 3.6 A: Comparison of the growth curves of P. multocida DSM16031 transconjugant (TG) and P. multocida DSM 16031 isogenic ancestor ........................................................................................................85

Figure 3.6 B & C: Exponential growth curves used for the calculation of the growth rate of P. multocida DSM16031 TG and P. multocida DSM16031 isogenic ancestor ........................................................................................................85
Figure 37: Bar graph illustrating a comparison of the relative fitness cost of the newly acquired \textit{ICEHs02} on the recipient strains \textit{H. somni} ATCC700025 and \textit{P. multocida} DSM16031...........87

Figure 3.8: long-term serial passage experiment to detect the change of \textit{ICEHs02} carriage in \textit{H. somni} ATCC700025 transconjugants (TGs) grown on BHITT with and without tetracycline selection........................................................................................................90

Figure A.1: Growth curves of \textit{P. multocida} DSM16031 and \textit{M. haemolytica} h186 showing the mid-log phase.........................................................................................................................................................141

Figure A.2: Growth curve of \textit{H. somni} KLM014 showing the mid-log phase used in the conjugation experiment .........................................................................................................................................................................................142

Figure A.3: DNA sequence alignment of the circular intermediate sequenced PCR amplicons to the sequence of \textit{ICEHs02} using SnapGene ® version 5.1.5 .................................................................................................157

Figure A.4: DNA sequence alignment of the chromosomal junction sequenced PCR amplicon to the whole genome sequence of \textit{H. somni} KLM014 using snapGene ® version 5.1.5 .................158

Figure A.5: DNA sequence alignment of the left chromosomal attachment site sequenced PCR amplicon to the whole genome sequence of \textit{H.somni} KLM014 using snapGene ®version 5.1.5 ...........................................................159

Figure A.6: DNA sequence alignment of the right chromosomal attachment site sequenced PCR amplicon to the whole genome sequence of \textit{H. somni} KLM014 using snapGene ®version 5.1.5 ........................................................................................................................................................................................................160
<table>
<thead>
<tr>
<th>Symbols</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMR</td>
<td>Antimicrobial resistance</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AGPs</td>
<td>Antimicrobial growth promoters</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BRD</td>
<td>Bovine respiratory disease</td>
</tr>
<tr>
<td>BVDV</td>
<td>Bovine viral diarrhea virus</td>
</tr>
<tr>
<td>BRSV</td>
<td>Bovine respiratory syncytial virus</td>
</tr>
<tr>
<td>BoHV-1</td>
<td>Bovine alphaherpesvirus 1</td>
</tr>
<tr>
<td>BPIV-3</td>
<td>Bovine parainfluenza virus 3</td>
</tr>
<tr>
<td>CTX</td>
<td>Cefotaxime</td>
</tr>
<tr>
<td>CIP</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CuSO4</td>
<td>Copper sulfate</td>
</tr>
<tr>
<td>CBA</td>
<td>Columbia Blood Agar</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dtr</td>
<td>DNA-transfer replication</td>
</tr>
<tr>
<td>DRs</td>
<td>Direct repeats</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ETOH</td>
<td>Ethyl alcohol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EB</td>
<td>Elution buffer</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>HSDC</td>
<td><em>Histophilus somni</em> disease complex</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
</tr>
<tr>
<td>HTM</td>
<td>Haemophilus Test Medium</td>
</tr>
<tr>
<td>ICEs</td>
<td>Integrative and conjugative elements</td>
</tr>
<tr>
<td>IMEs</td>
<td>Integrative and mobilizable elements</td>
</tr>
<tr>
<td>IS</td>
<td>Insertion sequence</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MGEs</td>
<td>Mobile genetic elements</td>
</tr>
<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>Mpf</td>
<td>Mating pair/pore formation</td>
</tr>
<tr>
<td>NAL</td>
<td>Nalidixic acid</td>
</tr>
<tr>
<td>Ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-template control</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>OriT</td>
<td>Origin of transfer</td>
</tr>
<tr>
<td>PT</td>
<td>Plasmid transfer</td>
</tr>
<tr>
<td>PCI-F</td>
<td>Primer of chromosomal junction-forward</td>
</tr>
<tr>
<td>PCI-R</td>
<td>Primer of chromosomal junction-reverse</td>
</tr>
<tr>
<td>PCJ-F</td>
<td>Primer of circular intermediate-forward</td>
</tr>
<tr>
<td>PCJ-R</td>
<td>Primer of circular intermediate-reverse</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Pmol</td>
<td>Picomoles</td>
</tr>
<tr>
<td>QS</td>
<td>Quorum sensing</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>SI</td>
<td>Sub-inhibitory</td>
</tr>
<tr>
<td>sBHI</td>
<td>Supplemented brain heart infusion</td>
</tr>
<tr>
<td>TME</td>
<td>Thrombotic meningoencephalitis</td>
</tr>
<tr>
<td>T4SS</td>
<td>Type 4 secretion system</td>
</tr>
<tr>
<td>T4CP</td>
<td>Type 4 coupling protein</td>
</tr>
<tr>
<td>Tn</td>
<td>Transposon</td>
</tr>
<tr>
<td>TET</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TG</td>
<td>Transconjugant</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
</tr>
<tr>
<td>WB</td>
<td>Wash buffer</td>
</tr>
</tbody>
</table>
Chapter One: Literature Review

1.1 Introduction

Antimicrobial resistance (AMR) is a global concern threatening animal and human health [1]. Antimicrobials are a crucial weapon against infections in both humans and animals. However, they are losing their effect more quickly than we are producing new treatments [2]. In May 2016, Jim O’Neill published a report predicting that 700,000 lives are endangered every year due to antimicrobial-resistant infections. If there were no solution by 2050, the numbers would increase, reaching 10 million deaths a year [3]. Antibiotic resistance in animal and human pathogens can complicate the prevention and treatment of infectious diseases, and could adversely impact human health, animal health and welfare, food safety, the environment and the economy [4]. There are many factors that might contribute to the emergence of AMR [5]. Understanding more about the development and the transmission of resistance genes will help in tackling problems associated with AMR and will inform antimicrobial stewardship and mitigation strategies that will be critical to maximize therapeutic options.

1.2 Bovine respiratory disease

Bovine respiratory disease (BRD) is one of the most economically significant animal health problems in the beef industry in North America [6], [7]. The annual incidence of BRD in North America ranges from 5 to 44%, with an estimated yearly loss of about 1 billion dollars from death, reduced feed efficiency, prevention and treatment costs [8]–[11]. Bovine respiratory disease complex is considered the primary cause of death and illness in feedlot cattle [12]. BRD is responsible for 75% of feedlot morbidity, and 50 to 70% of all mortality in the feedlot [10], [13].
BRD has critical long-term consequences on production by adversely affecting growth [14], reproductive function, and survival rates [15].

BRD is a multifactorial disease involving the complex interaction of several factors; host factors, environmental factors, and infectious agents [16], [17]. Host stressors such as weaning, transporting, comingling, crowding, and temperature fluctuations are considered some of the environmental factors that influence the development and severity of the disease [7], [16], [18], [19]. The manifestation of disease typically occurs within one week to 10 days after shipment of calves to feedlots, which is why the descriptive name "shipping fever" is commonly used [20].

Multiple pathogens are associated with BRD, including viral agents such as bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine alphaherpesvirus 1 (BoHV-1), and bovine parainfluenza virus 3 (BPIV-3) [6], [7], [21]–[23]. A primary viral infection may increase susceptibility of the respiratory tract to secondary bacterial infection [11]. The key bacterial pathogens linked with BRD include *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Trueperella pyogenes*, and *Mycoplasma bovis* [6], [7], [21]–[23].

1.3 Bovine histophilosis

1.3.1 *Histophilus somni* disease complex (HSDC)

"*Histophilus somni* disease complex" or histophilosis is a multisystemic syndrome in cattle caused by *H. somni* [24], [25]. *Histophilosis* has been a significant problem of cattle in North America [26]. *H. somni* is a Gram-negative, pleomorphic coccobacillus, non-spore forming, non-capsulated bacterium of the *Pasteurellaceae* family [24], [27], [28], previously known as *Haemophilus somnus* [24]. *H. somni* usually inhabits the upper respiratory and reproductive systems of dairy and beef cattle [24], [27], [28]. The reproductive tract is considered the main
reservoir of H. somni. It was reported that both female and male cattle have H. somni in their reproductive system and urinary tract [29], [30]. The urine or discharges can contaminate the environment, and other cattle can be infected by inhaling aerosols that contain the bacteria [30]. Coughing and nasal discharges are also considered a potential source of infection [29].

Bovine histophilosis usually happens in late fall and early winter, but it can flare-up at all times of the year [31]. The incubation period ranges from two to 21 days, depending on the stress and immune status of the infected calf [32]. H. somni can infect multiple organ systems, causing pneumonia, necrotizing myocarditis, pericarditis, pleuritis, thrombotic polyarthritis, conjunctivitis, meningoencephalitis (TME), and otitis media [24], [33], [34]. Identification of H. somni is challenging as it can be found in the animal as a commensal organism. The tendency to occur in combination with other pathogens and fastidious growth characteristics further complicate diagnosis/identification of disease caused by H. somni [31].

H. somni is a fastidious, capnophilic organism, and it only grows on enriched media supplemented with blood such as Trypticase Soy Agar (TSA) with 5% defibrinated sheep blood or Columbia Blood Agar (CBA) with 5% defibrinated sheep blood [24], [35]. H. somni shows enhanced growth in the presence of high concentrations of CO₂. Unlike other Haemophilus species, H. somni does not require factor X (Hemin) and factor V (Nicotinamide Adenine Dinucleotide) for growth [36]. In the laboratory, H. somni is slow-growing on agar plates supplemented with 5% defibrinated sheep blood, with an incubation period of 24 to 48 hours at 37 °C in the presence of 5-10% CO₂. H. somni forms small, circular, yellowish, mucoid, convex, and shiny colonies in CBA supplemented with 5% sheep blood. H. somni can be either beta-hemolytic, alpha-hemolytic, or non-hemolytic on blood agar [24], [37]. It is challenging to identify H. somni using common biochemical tests due to its poor growth characteristics [29].
1.3.2 Clinical manifestation of *Histophilus somni* disease complex (HSDC)

1.3.2.1 Respiratory disease

*H. somni* can be found as a commensal in the upper respiratory tract of healthy cattle. Alteration of the normal flora of the upper respiratory tract may lead to infection in the upper airway, which can extend to the lower airway [38]. It was reported that *H. somni* infects the upper respiratory tract causing laryngitis and tracheitis, and it can also cause severe fibrinous pleuritis [30].

*H. somni* tends to be an opportunistic pathogen that causes bovine respiratory pneumonia either independently or in combination with *M. haemolytica, P. multocida*, and with viral pathogens such as bovine respiratory syncytial virus (BRSV)[33], [34], [39]. Many studies showed that *H. somni* infected calves developed a more severe clinical disease and pneumonic lesions when previously infected with BRSV [40]–[42].

The signs of *H. somni* bronchopneumonia in calves and adult cattle cannot be differentiated from pneumonia caused by *P. multocida or M. haemolytica* [38]. Respiratory disease signs typical of BRD are: tachypnea, cough, nasal discharge, and depression [31]. *H. somni* can also cause septicemia by passing into circulation by crossing the alveolar barrier [41].

1.3.2.2 Central nervous system disease

Thrombotic meningoencephalitis-myelitis (TME) is a fatal neurological disease of cattle [43], [44]. TME affects feedlot cattle more frequently than dairy cattle [43], [45] and most commonly affects older calves and yearlings [31], [45], [46]. The mortality rates from *H. somni* encephalitis are very high [43], [45], [47].

Clinical signs are typical of meningoencephalitis as neurologic damage is usually severe. The manifestations include depression, blindness, ataxia, weakness, and fever. In addition,
symptomatic cattle may demonstrate closed or semi-closed eyelids and hence the disease is frequently called “sleeper syndrome” [43], [45], [47]. The infected animals typically die within 12 to 48 hours [38], [47]. Treatment is effective only during the early stages of the disease [31], [45].

1.3.2.3 Myocarditis

Myocarditis is one of the lethal syndromes associated with *H. somni* infection in cattle [32], [48]. One study indicated that *H. somni* was the leading cause of myocardial disease in feedlot cattle [49], and recently, myocarditis has seemed to be of higher frequency than thrombotic meningoencephalitis in western Canada [30], [50]–[52]. Cardiac *histophilosis* usually occurs between 50 to 60 days after arrival to feedlots [49]. Infected animals are usually yearlings rather than weaned calves, and they often die suddenly [30]. Sudden death associated with myocarditis was first defined as a sign related to septicemia with *H. somni* in the late 1980s [30], [53]. Infected cattle usually appear depressed, febrile, and lethargic. The presence of gross lesions in papillary muscles of the left ventricular myocardium in feedlot cattle generally assist the differential diagnosis of *H. somni* myocarditis [48].

1.3.2.4 Other manifestation of histophilosis

*H. somni* can invade the circulatory system and then proliferate in different organs and areas of the body, causing clinical signs that depend on the affected region [30]. In female cattle, *H. somni* can cause vaginitis, endometritis, infertility, and abortion [54], [55]. Otitis associated with *H. somni* has been reported in feedlot cattle [30], [56], [57]. Infected animals were febrile and showed extensive draining of clear yellow fluid from their ears [30], [57]. The association between *H. somni* and conjunctivitis was reported in 1982. Several calves in a pen of 40 suffered from serous conjunctival discharge that yielded pure cultures of *H. somni* when sampled [57]. In addition to these conditions, polyarthritis in pen-mates has been reported two days to several weeks
after a TME outbreak. Infected animals showed lameness and stiffness. Edema was noted in infected joints, and joint capsules were distended with excessive and turbid synovial fluid [58].

1.3.3 Management and control of histophilosis in feedlot cattle

Management of *H. somni* in feedlot cattle is challenging as it can invade multiple host tissues [30] and can avoid clearance by host defences [59]. *H. somni* can colonize host tissues forming biofilms and is able to survive intracellularly by modulating the phagocytic function of immune cells [59], [60]. There are two strategies to manage expected outbreaks of *histophilosis* in feedlot cattle; vaccination, and mass medication with antimicrobials [31].

1.3.3.1 Vaccination

Vaccination for prevention of *H. somni* infection has been employed for decades. There are eight vaccines available for use in feedlot cattle in Canada [61]. Vaccine products are usually composed of live attenuated (bacterins) or inactivated bacterial toxins (toxoids) [61]. Most commercial products for *H. somni* are composed of either killed cells or outer membrane proteins [62]. The data evaluating effectiveness of vaccines against *histophilosis* are controversial. Some studies suggest that immunization with vaccines could reduce morbidity and mortality in feedlot cattle [63]–[66]. On the other hand, a study conducted in 2012 using three separate field trials to analyze the effectiveness of commercial vaccines against *H. somni* indicated a limited impact of vaccination against *histophilosis* in feedlot cattle [67]. The reason behind these conflicting data is unclear. Some studies suggest that the recently developed vaccines would only protect from the planktonic form of *H. somni* and not the biofilm form of the organism [68], [69]. Biofilms are a complicating factor in *H. somni* infection as they enhance the tolerance of the bacteria to treatment with antimicrobials and against host defence mechanisms [69]. Some reports indicated adverse effects of *H. somni* vaccines on calves [70], [71]. These problems might be related to the endotoxin
activity of the lipooligosaccharides that can cause anaphylactic shock [70]. Some studies have discussed new approaches to improving vaccines, but all these efforts are still in development [71]–[73].

1.3.3.2 Metaphylaxis

Metaphylaxis is the term used for mass medication of a group of high-risk animals to prevent the manifestation of disease and to enhance overall health and production [74]. Many factors are considered to evaluate groups considered high-risk, including long transport distance, mixed origin cattle, mixed group pens, and weight [75]. These factors might increase the susceptibility of the group to infections. In addition, multiple studies have shown that assessing clinical signs is not always a reliable indicator of the disease [76]–[78]. For these reasons metaphylaxis is considered essential for reducing the number of infected animals and limiting disease spread [74].

Since the 1980s many studies have discussed the positive effect of metaphylactic use of antimicrobials on health and performance of newly received cattle [79]–[81]. Several antibiotics have shown good activity against bacterial BRD pathogens, including H. somni [79], [82], [83]. Long-acting oxytetracycline (tetracycline), tulathromycin and tilmicosin (macrolides) are the most common antimicrobials used in Canadian feedlots [83], [84]. Some studies reported that cattle receiving chlortetracycline or tilmicosin had lower morbidity and mortality rates than animals not receiving medications [80]–[82].

1.4 Antimicrobial usage in feedlot cattle

1.4.1 General overview on antimicrobial usage

Antimicrobials play a significant role in food animal production. Cattle are vulnerable to many infectious diseases, which may spread quickly in the feedlot environment [85]. Herd diseases have devastating outcomes on animal health, which consequently affect national and foreign trade,
and that creates urgent economic demand for antimicrobial usage to limit bovine infectious diseases [85]. Using antimicrobials is useful for fighting infectious diseases mainly when no effective vaccine is available [86].

Antimicrobials are used for treatment, prophylaxis, metaphylaxis, and until recently for growth promotion [21], [87], [88](Table 1.1). Prophylaxis is treatment used for calves at high-risk upon their arrival to feedlots to prevent diseases. Metaphylaxis refers to the treatment of the entire pen group when only a small number of animals display clinical signs to treat infected animals and protect uninfected animals [88], [89]. Growth promotion is the use of antimicrobial growth promoters (AGPs) to improve the growth rate and production performance [88], [90]; however, the use of AGPs was banned in Canada in 2018 [91]. The use of AGPs was first banned in the EU in 2006 and then in 2012 in the USA [88]. Banning the use of AGPs is controversial as some antibiotics were approved for both treatment and growth promotion [92]. Some drugs approved only for growth promotions were believed to prevent diseases, and some antimicrobial mainly used for prophylaxis may indirectly enhance the growth [88], [92]. Additionally, it seems that the EU ban for the AGPs has resulted in unintended consequences, including increased use of some antimicrobials more important in human medicine [93].

Tetracyclines and macrolides are usually used for disease prevention in feedlot cattle [94]. Florfenicol is also used upon arrival, although prophylactic use of this antibiotic is less prevalent; it is usually used metaphylactically for control of BRD once detected in cattle pens [33]. The antibiotics most commonly used for the clinical treatment of respiratory disease feedlot cattle are macrolides, including tilmicosin, and tulathromycin, tetracyclines, such as oxytetracycline, and chlortetracycline, the cephalosporin ceftiofur, and the sulfonamide sulfamethazine [74], [92], [95]. Antimicrobials can be delivered to the cattle via injection, for example oxytetracycline, penicillin,
tylosin [92] or delivered in feed or water (mass medication) such as ionophores, tylosin, and chlortetracycline [33], [59], [95]. Antimicrobials used in feedlot cattle in Canada are illustrated in Table 1.2.

1.4.2 Antimicrobials in feed or water

Treatments may be administered to individual animals; however, it is usually easier to treat the whole group by placing the medication in the feed or drinking water [92]. In general, feedlot beef cattle are routinely fed rations medicated with an ionophore [96] to prevent coccidiosis, and improve feed conversion and weight gain [92]. Ionophores also have antibacterial properties and can be used to prevent ketosis in cattle [92].

Tylosin and oxytetracycline are mainly used to control liver abscesses [96]–[98]. Many factors might contribute to the incidence of liver abscesses. High grain content diet is one of the significant factors that influence formation of liver abscesses. Rapidly fermented grains such as wheat, barley, and high-moisture corn can alter the ruminal pH; this usually leads to acidosis, rumenitis, and consequently liver abscesses. Tylosin feeding was shown to decrease abscess frequency by 40 to 70% [97].

In western Canada, many calves are mass medicated with oxytetracycline or tilmicosin upon arrival at feedlots, and this prophylactic treatment has been shown to significantly decrease morbidity and mortality in feedlot cattle [79], [99], [100]. Chlortetracycline is usually given to cattle during the first month in the feedlot for respiratory disease prevention and control [33], [59], [95], [101].

Despite the importance of antimicrobials in metaphylaxis, prophylaxis, and growth promotion in some countries, there is a concern that antimicrobials in animal feed might contribute to the antimicrobial resistance crisis. It is believed that using antibiotics in feed enhances the
development and selection of antimicrobial-resistant strains [102]. Antimicrobial resistance will be discussed in detail in the following section.
Table 1.1: Types of antimicrobial use in food animal

<table>
<thead>
<tr>
<th>Types of Antimicrobial Use</th>
<th>Purpose of use</th>
<th>Route of Administration</th>
<th>Administration to Individuals or Groups</th>
<th>Diseased Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Therapeutic</td>
<td>Therapy</td>
<td>Injection, feed, water</td>
<td>Individual or group</td>
<td>Diseased individuals or some of the individuals in groups</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metaphylaxis</td>
<td>Disease</td>
<td>Injection, feed, water</td>
<td>Group</td>
<td>Some</td>
</tr>
<tr>
<td></td>
<td>Prophylaxis/Therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Disease prevention</td>
<td>Feed</td>
<td>Group</td>
<td>Not evident although some infections may be subclinical</td>
</tr>
<tr>
<td>Prophylaxis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth promotion</td>
<td>Growth promotion</td>
<td>Feed</td>
<td>Group</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Feed efficiency</td>
<td>Feed</td>
<td>Group</td>
<td>None</td>
</tr>
</tbody>
</table>

Adapted from Reports and Publications – Veterinary Drugs, Government of Canada. Uses of Antimicrobials in Food Animals in Canada: Impact on Resistance and Human Health [Health Canada, 2002][92].
Table 1.2: Antimicrobials used for prophylaxis, metaphylaxis, and treatment in feedlot Canada

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>Purpose of use</th>
<th>Antimicrobial class</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tylosin</td>
<td>Liver Abscess Prevention</td>
<td>Macrolides</td>
<td>Injection/ In feed</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>BRD prevention/ treatment</td>
<td>Macrolides</td>
<td>Injection/ In feed</td>
</tr>
<tr>
<td>Tulathromycin</td>
<td>BRD prevention/ treatment</td>
<td>Macrolides</td>
<td>Injection</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>BRD prevention/ treatment</td>
<td>Phenics</td>
<td>Injection</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>BRD prevention/ treatment</td>
<td>Tetracyclines</td>
<td>Injection/ In feed</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>Liver abscess prevention/</td>
<td>Tetracyclines</td>
<td>In feed</td>
</tr>
<tr>
<td></td>
<td>Histophilosis prevention/treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danofloxacin</td>
<td>BRD treatment</td>
<td>Quinolones</td>
<td>Injection</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>BRD treatment</td>
<td>Quinolones</td>
<td>Injection</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxine</td>
<td>BRD treatment</td>
<td>Sulfonamides</td>
<td>Injection</td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>BRD treatment</td>
<td>Sulfonamides</td>
<td>Injection</td>
</tr>
<tr>
<td>Penicillin</td>
<td>BRD treatment</td>
<td>β-lactams</td>
<td>Injection</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>BRD treatment</td>
<td>β-lactams</td>
<td>Injection</td>
</tr>
</tbody>
</table>

Adapted from Compendium of Veterinary Products, Canada, Online version (last updated 2020) [61].
1.5 Antimicrobial resistance

1.5.1 Overview on antimicrobial resistance

Antimicrobial resistance (AMR) is a global concern threatening animal and human health. According to an estimation by the World Health Organization (WHO), throughout the past decade the human mortalities caused by some antibiotic-resistant strains surpassed the combined number of losses caused by influenza, Human Immunodeficiency Virus (HIV) and traffic accidents [103]. The agricultural sector is considered the most extensive user of antimicrobials worldwide [21]. According to “The State of the World’s Antibiotics 2015” at least 63,200 tons of antibiotics were used in animal husbandry worldwide in 2010, and that amount exceeded all human consumption [104].

AMR in bacteria can be disseminated either by vertical or horizontal gene transfer. Vertical gene transfer is the inheritance of genes from parents to offspring. The genes are passed on by the replication of the bacteria. In contrast, horizontal gene transfer is the transfer of genes between different strains of bacteria [105], [106].

1.5.2 Mechanisms of antimicrobial resistance

Bacteria have a variety of sophisticated mechanisms to overcome the adverse effects of antimicrobials. There are four major modes of action: 1) limiting drug uptake (decreased permeability), 2) modification of drug target sites, 3) antimicrobial inactivation, and 4) drug efflux or reduced intracellular concentration [107]–[109].

1.5.2.1 Limiting drug uptake

Gram-positive and gram-negative bacteria may differ in their ability to limit the uptake of antimicrobials [107]. The outer membrane, which is composed of lipopolysaccharide (LPS) in
Gram-negative bacteria, acts as a natural barrier to protecting bacteria from a particular group of antimicrobials [107], [108]. In Gram-negative bacteria, the antimicrobials can access the cell through porins which are channels that allow the diffusion of hydrophilic molecules. Antimicrobial resistance can occur via changes in porin number and mutations which limit the uptake of drugs [107]. In addition, some bacteria are able to form a biofilm that protects the bacteria and decreases the ability of antimicrobials to reach the bacteria [107].

1.5.2.2 Modification of drug target sites

Antimicrobials attack a specific target in the bacteria; these targets include the cell wall, ribosomes, DNA, RNA polymerases and cellular peptides [107]. Bacteria can modify these sites so that the antimicrobial will not bind to them and in result the bacteria will be resistant to the antimicrobial [107], [108]. Resistance to drugs that target the cell wall usually happens through mutation in the structure of penicillin-binding proteins, while resistance to antimicrobials that target the ribosomal subunits occur through changes to ribosomal methylation or by mutations in genes encoding ribosomal proteins [107]. Resistance to antimicrobials that target DNA might be caused by modification of the bacterial DNA gyrase or topoisomerase, decreasing the antimicrobial’s ability to bind to the nucleic acid [108].

1.5.2.3 Drug inactivation

Bacteria can enzymatically inactivate antimicrobials either by degradation of the drug or by adding a chemical group to the drug rendering it inactive [107], [108]. Drug inactivation by transfer of a chemical group usually happens through three main mechanisms; phosphorylation, adenylation, and acetylation. The most common example of enzymatic destruction of
antimicrobials is the β-lactamases, drug hydrolyzing enzymes that destroy the amide bond of the β-lactam ring [107], [108].

1.5.2.4 Drug efflux

Drug efflux is a key mechanism of resistance that allows the bacteria to regulate their internal environment and eliminate toxic substances via an energy utilizing pump [110]. The efflux pumps may be specific for one substrate or may transport a wide variety of compounds; this latter type is called a multidrug efflux pump (MDR) [110].

1.5.3 The effect of using in-feed antimicrobials on antimicrobial resistance

As mentioned earlier, antibiotics are used for treatment, growth promotion, improving feed conversion efficiency, and preventing diseases in animal production. The application of antimicrobials in animal feed raises concerns that these practices might contribute to AMR in bacterial pathogens and diminish the efficacy of antimicrobials applied to manage infectious diseases in cattle [21], [23] and human [95]. Some antimicrobials are used for veterinary medicine, and others are used for human medicine; however, most classes are used in both areas [93]. Using some antimicrobials in animal feed may result in cross-resistance with critical antimicrobials for humans [115]. Antimicrobial administration creates intense selective pressure in microbial communities allowing selection of resistance genes and resistant bacteria, which can transfer to the broader environment via horizontal gene transfer (HGT) [90]. Mobile genetic elements (MGEs) conferring drug resistance may transfer easily from one environment to another [116], [117]. Transferring resistance genes from bacteria of animal origin to human origin bacteria has been proved in an animal model [118]. It is logical to use a One-Health approach when discussing
the complex problem of AMR, as there are no boundaries between the microbiota of the environment, humans, and animals.

The route of administration of antimicrobials may also contribute to AMR prevalence. There is an association between in-feed antimicrobial use, increasing resistance to antibiotics [119] and the effect of antibiotics on developing AMR in gut microbiota [120]. C. Varga el al. (2008) found that the use of in-feed antimicrobials was linked with the increase in resistance of *E. coli* to many antimicrobials [119]. In 2013, a study done by Lu Zhang et al. indicated that oral exposure to antimicrobials plays a significant role in increasing resistance and development of gut microbiota [120]. Another study demonstrated that antibiotics in animal feed might induce phage-mediated gene transfer and consequently dissemination of antibiotic resistance [113]. The latter studies were done on swine and mouse models indicating the need for similar studies in cattle.

1.5.4 Antimicrobial resistance in *H. somni* and other bovine respiratory pathogens

The emergence of antibiotic-resistant strains is most often associated with the use of antibiotics. Klima et al. (2014) found that multi-drug resistance occurred more frequently in *M. haemolytica* isolates obtained from cattle with BRD than from healthy animals [114]. There is evidence of decreasing susceptibility of *M. haemolytica, P. multocida, and H. somni* to some antimicrobials due to frequent use in feedlot settings to treat BRD [115]. A recent study conducted on healthy and BRD diagnosed cattle in Alberta detected high levels of resistance (more than 70%) to tulathromycin and oxytetracycline in *M. haemolytica* and *P. multocida* isolates and high levels of resistance to oxytetracycline (67%) and penicillin (52%) in *H. somni* isolates [116]. A retrospective study to determine the prevalence of multidrug-resistance in *M. haemolytica* isolates from BRD cases evaluated records from 2009 to 2011 from the Kansas State Diagnostic Laboratory. They observed that in 2009, approximately 5% of isolates were resistant to 5 or more
antimicrobials, while more than 35% of isolates were resistant to more antimicrobials in 2011[117]. A recent study compared historical isolates and contemporary isolates of H. somni from feedlot cattle in Alberta during the years 1980-1990 and 2012-2016, respectively. The study observed that resistance to antimicrobials has significantly increased over time [25]. A cross-sectional study was conducted in 2017 in southern Alberta to estimate the prevalence of antimicrobial-resistant BRD pathogens using data from seven private veterinary practices. The study noted that 100% of the M. haemolytica, M. bovis, P. multocida, and T. pyogenes isolates and 66.7% of the H. somni isolates were resistant to at least one antimicrobial class. The report indicated that 90.2% of isolates were resistant to macrolides, and 47.2% of isolates were resistant to multiple drug classes. In addition, the study compared the multi-drug resistance (MDR) profiles to recent reviews and suggested that AMR is increasing [118].

It is essential to note that some AMR phenotypes are associated with MGEs such as integrative and conjugative elements (ICEs) and plasmids. Some of these MGEs carry antimicrobial resistance genes (ARGs) that confer resistance to most of the drugs used to manage BRD [25], [115], [119]–[121]. Plasmid-mediated resistance to florfenicol was first identified in M. haemolytica in 2011. The study identified a 7.7 Kb plasmid that carries the floR gene and is easily transformed into E. coli recipient strains conferring resistance to florfenicol [122]. Related plasmids identified earlier in P. multocida conferred floR resistance gene and showed similar results to the study done on M. haemolytica [123], [124]. Several ICEs were recently identified in BRD pathogens and it was noticed that the AMR genes were organized as tandem arrays on the ICEs [120], [121]. More details about the recently identifiedICEs in BRD pathogens are discussed in section 1.8.
In addition to MGEs, AMR was also confirmed to occur due to mutagenesis. A research study done on *P. multocida* and *M. haemolytica* explained that high-level macrolide resistance could result from 23s rRNA mutations [125]. Another study recently performed on *P. multocida* detected a mutation in the *parC* gene that conferred high resistance to quinolones [126].

Although metaphylaxis and prophylaxis treatment approaches have advantages in controlling BRD dissemination and increasing survival rate, such approaches have the disadvantage of subjecting a large group of animals to unnecessary antimicrobials which can contaminate the environment with antimicrobials and resistant bacteria [89]. Several studies suggested that the presence of antimicrobial and metal residuals contaminating the environment and in treated animals and humans can produce a selective pressure for horizontal gene transfer (HGT) and that contributes to the dissemination of AMR [88], [127], [128].

1.6 Horizontal gene transfer

New genetic material can be acquired by bacteria either *de novo* via genetic mutations or by uptake of extra-genomic DNA. Horizontal gene transfer (HGT) or lateral gene transfer is the transfer of genetic materials between bacteria. Horizontal gene transfer enables bacteria to obtain multiple unique traits to cope with environmental challenges commanded by human actions such as misuse of antimicrobials and industrial pollution [129]–[131].

There are three known mechanisms of HGT; transformation, conjugation and transduction [34], [129], [132]–[135]. Transformation is the natural bacterial uptake of DNA fragments from the exogenous environment. Transduction involves the transfer of small fragments of bacterial genomes via a bacteriophage. Conjugation is a complex process that requires close contact between bacteria leading to the unidirectional transfer of genetic material from one cell to another.
through a conjugation pore [130], [132], [134], [136]. Natural transformation is regulated by the recipient cell, while conjugation and transduction are regulated by the donor cell.

Conjugation can result in the transmission of substantial portions of genomes and even whole chromosomes in a single event [133]. Horizontal gene transfer by conjugation requires complex multiprotein cellular machinery. The conjugation machinery is synthesized by donor strains and connects the donor and recipient [21]. During conjugation the donor produces a pilus which pulls the donor and recipient together, and then a pore is formed between the two bacteria allowing the transfer of genetic material from the donor to the recipient [137].

Each conjugative transfer requires transfer genes (tra genes), which are essential for DNA transfer and replication (Dtr), and mating pair formation genes (Mpf genes), which are responsible for the formation of the Type IV Secretion System (T4SS) complex [138]. Around 10–20 proteins establish the architecture of the T4SS devoted to ssDNA and protein transfer [139]. The type IV secretion system consists of four main components; a) ATPase, the fueling device of T4SS machinery and DNA transfer, b) Translocation proteins of the inner membrane, T4 Coupling proteins (T4CP), c) Core proteins traversing the cell envelope, d) Pilus proteins or adhesins that initiate contact between cells [139]–[141].

1.7 Mobile genetic elements

Mobile genetic elements (MGEs) are considered messengers for the transmission of many traits among bacteria [142]. Many kinds of MGEs contribute to the adaptation and evolution of bacteria. The mobilome contains numerous types of genetic systems that can relocate from one place to another within a genome or between bacterial genomes. Mobile genetic elements can be classified into two main groups; the first group can transfer from one bacterium to another; i.e., plasmids, and integrative and conjugative elements (ICEs), and the second group cannot initiate
transfer by themselves; i.e., transposons (Tn), insertion sequences (IS), and integrative and mobilizable elements (IMEs). These elements usually transfer by integrating or using the machinery of the first group [143].

1.7.1 Plasmids

Plasmids are self-replicating extrachromosomal double-stranded DNA molecules that can transfer between bacterial population- [138], [142], [144]–[146]. Some plasmids might represent up to 10-20% of the total bacterial genome. There are two types of plasmids; self-transmissible conjugative plasmids which are able to transfer between bacteria, and non-conjugative plasmids which lack this ability [145]. Self-transmissible conjugative plasmids encode all genes needed for their horizontal gene transfer including origin of transfer site (oriT), Dtr and Mpf [138], [139], [146]. Plasmids vary significantly in size, copy number, and in the genes they carry [138]. Large plasmids usually have small copy numbers, while small plasmids may have a large copy number [145]. Plasmids usually exist autonomously in their host cell, however, some plasmids can integrate immediately into the chromosome [144]. Plasmids may possess genes conferring resistance to antibiotics, metals, utilization of carbon compounds, virulence, and symbiosis determinants [138].

1.7.2 Integrative and mobilizable elements

Integrative and mobilizable elements (IMEs) are a highly wide-spread mobile genetic elements (MGEs) that encode their excision and integration machinery, but are incapable of transferring by themselves. IMEs hijack the conjugation machinery of independent conjugative elements for their transfer [142], [147]. Most IMEs integrate into specific sites on the chromosome including tRNAs and AT-rich sites [142]. Multiple IMEs encode tyrosine recombinase, while some of them encode serine recombinase or DDE transposase [142], [147], [148]. IMEs carry
accessory genes conferring numerous benefits that can improve the overall capacity of their bacterial host to survive, such as antibiotic resistance [142].

The detection of IMEs is challenging for two reasons. Firstly, various IMEs do not encode markers that would be valuable for selecting transconjugants. Additionally, IME transfer requires a supporter element such as ICE that may be in some cases incompatible with the IME [142].

1.7.3 Integrative and conjugative elements

Integrative and conjugative elements (ICEs) are a distinct group of mobile genetic elements (MGEs) that can be found in both Gram-positive and Gram-negative bacteria [149]. These mobile genetic elements are integrated into the host chromosome; however, they have the ability to excise from the host genome, develop a circular intermediate, initiate transfer of themselves by conjugation to a new host and re-integrate into the new host chromosome [21], [25], [121]. ICEs integrate into a particular site, usually a tRNA, in the chromosomal DNA of the host [121]. ICEs are composed of conserved core genes (structural genes) and variable accessory genes (cargo genes). The core genes are essential for the dissemination and maintenance of ICEs. The accessory genes include genes for antibiotic resistance, metal tolerance, virulence, biofilm formation, and metabolic activities [21], [25], [121], [134]. The transfer mechanism of ICEs is assumed to be comparable to that of conjugative plasmids, with the exception of the first and last steps of chromosomal excision and integration [142], [150].

The mechanism of the transfer of ICEs occurs in four main steps. First, the relaxase (mob) nicks the ICE DNA sequence at the origin of transfer (oriT). Next the relaxase (mob) binds covalently to one of the DNA strands forming a nucleoprotein complex (relaxasome). The nucleoprotein complex (Dtr) then interacts with a type 4 secretion system (T4SS) through the T4SS coupling protein (T4CP) and a ssDNA version of the ICE is transported to the recipient cell to be
integrated into the host chromosome. Lastly, the single-stranded DNA is replicated by DNA polymerase enzyme in the primary and new hosts leading to double-stranded DNA molecules in each cell [133], [139], [140], [150]. All ICEs encode an integrase that facilitates their integration into the host chromosome [131]. Integrases are able to determine the insertion site of ICEs [131]. The integration of ICEs usually relies on tyrosine recombinases. However, some ICEs utilize serine or DDE recombinases [131], [133], [134], [142], [150], [151].
Figure 1.1: Life cycle of integrative and conjugative elements (Johnson and Grossman 2015) [134].

The grey figure represents the donor and the green represent the recipient. a) The ICE (represented by blue line and blue circle) is integrated into the host chromosome, b) ICE excision and formation of mating pore, c) Relaxase (represented by yellow half circle) nicks the ICE DNA sequence at the origin of transfer (oriT) and binds covalently to one of the DNA strands forming a nucleoprotein complex, d) The nucleoprotein complex then interacts with a type 4 secretion system (T4SS) through the T4SS coupling protein (T4CP) and is transported to the recipient cell, e) The single-stranded DNA is replicated by DNA polymerase enzyme in the primary and new hosts leading to double-stranded DNA molecules in each cell, f) The double stranded ICE is then integrated in to the host chromosome.
Under normal circumstances, integrative conjugative elements are maintained integrated into the host chromosome, and conjugation genes are not expressed until they are stimulated by signal molecules [134], [139]. The inducing signals are variable for different ICEs, but there are common triggers associated with activating various ICEs. These include the SOS response, cell-cell signalling, induction during stationary phase, phenotype dependant induction, and others [134]. ICE excision might also occur randomly without induction in some cases [134]. Information about factors that induces the excision of ICE is scarce, but it has been recognized that stress conditions may contribute to increased frequencies of HGT of ICEs [129]. The effect of different stressors that induce the transfer of ICEs will be discussed in detail in section 1.9.

An integrative and conjugative element (ICE) is integrated into a specific site on the host chromosome and is flanked by identical direct repeats on the right (attR) and left (attL), which recombine with the aid of the integrase forming a covalently closed circular molecule. The attL and attR sequences merge to yield the attP site on the ICE and attB on the host chromosome [131], [134], [152], [153]. ICEs have a conserved modular structure constituted of three functional units providing genes essential for ICE function, and that includes integration, regulation, and conjugation [152]. In a study done by Haskett et al. (2016), they reported the finding of tripartite ICEs which are present as three separate chromosomal regions when integrated into their hosts and have the ability to recombine as a circular ICE for conjugative transfer [153].

Some ICEs can upregulate the transfer of other mobile genetic elements such as integrative and mobilizable elements (IMEs) and Cis-mobilizable elements (CIMEs)[131]. IMEs encode a functional integrase but require complete T4SS machinery for their transfer [131], [142], [147]. CIMEs lack both an integrase and T4SS but maintain functional flanking attL and attR sites which
help in a process called accretion in which the ICEs combine with the CIMEs creating a larger and more complex ICE [131], [154].

1.8 Integrative and conjugative elements in the *Pasteurellaceae* family

The *Pasteurellaceae* are a broad and distinct group of Gram-negative bacteria, that can cause many diseases in humans and animals [155]. As discussed earlier in section 1.2 some members of the *Pasteurellaceae* family play a significant role in BRD in cattle. Integrative conjugative elements carrying multiple antimicrobial resistance genes have been identified in many members of the *Pasteurellaceae* family using whole-genome sequencing (WGS) [25], [120], [121], [156]. WGS is a powerful tool for analyzing the entire genome to better understand the molecular evolution of strains and aids in the detection of mobile genetic elements involved in multi-drug resistance in bacteria [155].

1.8.1 Integrative and conjugative elements in *M. haemolytica* and *P. multocida*

ICEs identified in *M. haemolytica* and *P. multocida* share related features but the size might vary between the two species and in different strains. The size of ICE identified in *P. multocida* (*ICEPmu1*) and *M. haemolytica* (*ICEMh1*) is 82,214 bp and 92,345 bp, respectively. *ICEPmu1* and *ICEMh1* share similar characteristics; both of them integrate into a tRNA-Leu gene sequence. *ICEPmu1* is flanked by 13 bp direct repeats (DRs) while the DRs flanking the *ICEMh1* are two bp shorter than the DRs of *ICEPmu1* and consist of only 11 bp. Both *ICEMh1* and *ICEPmu1* carry multiple resistance genes that confer phenotypic resistance to various antibiotics [120], [121]. *ICEPmu1* harboured 12 antimicrobial resistance genes found in two regions on the ICE [157]. However, *ICEMh1* harboured five antimicrobial resistance genes which were found in two accessory gene regions. *ICEMh1* was shown to transfer to *P. multocida* conferring resistance to
neomycin, streptomycin, and tetracycline, while ICEPmu1 transferred to *P. multocida*, *M. haemolytica*, and *E. coli* conferring resistance corresponding to all antimicrobial resistance genes identified in the donor [120], [121].

### 1.8.2 Integrative and conjugative elements in *H. somni*

A putative integrative and conjugative element encoding the tetracycline resistance gene (*tetH*) has previously been described in *H. somni* 2336 (*ICEHso2336*). The size of that ICE is 66,463 bp. It is integrated into the tRNA-Leu site and is flanked by 11 bp terminal DRs. *ICEHso2336* possesses comparable characteristics with *ICEMh1* and *ICEPmu1*. The nucleotide identity between *ICEMh1*, *ICEPmu1*, and *ICEHso2336* is about 99% [156]. The host range of *ICEHso2336* in *H. somni* is as yet unexplored [25].

An integrative conjugative element (*ICEHs1*) of 64,932 bp in size has been identified in *H. somni AVI* [25]. *ICEHs1* has 83 genes including tetracycline resistance genes (*tetH/tetR*), a multidrug efflux pump gene (*ebrB*), copper (*mco*), zinc (*czcD*), and arsenic (*acr3*) resistance genes. Similar to *ICEPmu1* and *ICEMh1*, *ICEHs1* is integrated into the tRNA-Leu sequence and is flanked by 11 bp direct repeats (DRs) like *ICEMh1* and *ICEHso2336* [25]. The sequence of *ICEHs1* is highly similar to *ICEPmu1*, *ICEMh1* and *ICEHso2336* sequences. There is about 99% identity of the core genes between *ICEHs1* and the three ICEs [25]. *ICEHs1* has only one variable region containing tetracycline resistance and metal tolerance, while *ICEMh1* and *ICEPmu1* have two variable regions containing the multidrug resistance genes [25], [120], [121]. *ICEHs1* was confirmed to transfer to *H. somni* and *P. multocida*, and to confer the tetracycline resistance phenotype [25].
1.8.3 Integrative and conjugative elements in *Haemophilus influenzae*

*Haemophilus influenzae* is a member of the *Pasteurellaceae* family that can cause diseases to humans [158]. A study done in 2004 indicated that *ICE*Hin1056 in *H. influenzae* type b strain 1056 and the putative *ICE* in *H. somni* 2336 share the same evolutionary history and they have a single common ancestor [159]. The size of *ICE*Hin1056 is about 59,393 bp, and it harbours 64 open reading frames (ORFs) and carries resistance to a variety of antimicrobials including ampicillin, tetracycline, and chloramphenicol [160]. Like *ICE*Hs1, *ICE*Hin1056 was found integrated into tRNA-Leu gene sequence [161] and encoded related proteins that regulate the T4SS machinery [162]. *ICE*Hin1056 was shown to transfer to *H. influenzae* species [160], but its transfer to other species has not been investigated.
Table 1.3: Comparison of recently identified ICEs in some members of the **Pasteurellaceae** family

<table>
<thead>
<tr>
<th>Member of the <strong>Pasteurellaceae</strong> Family</th>
<th>Name of ICE</th>
<th>Size in bp(^1)</th>
<th>No.(^2) of genes</th>
<th>Integration site</th>
<th>Direct repeats</th>
<th>Resistance genes</th>
<th>Host range</th>
<th>Ref.(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. haemolytica</em> 42548</td>
<td>ICEMh1</td>
<td>92,345</td>
<td>107</td>
<td>tRNA-Leu</td>
<td>11 bp</td>
<td>5 genes(^a)</td>
<td><em>P. multocida</em></td>
<td>[120]</td>
</tr>
<tr>
<td><em>P. multocida</em> 36950</td>
<td>ICEPmu1</td>
<td>82,214</td>
<td>88</td>
<td>tRNA-Leu</td>
<td>13 bp</td>
<td>12 genes(^b)</td>
<td><em>P. multocida</em></td>
<td></td>
</tr>
<tr>
<td><em>H. somni</em> 2336</td>
<td>ICEHso2336</td>
<td>66,463</td>
<td>-</td>
<td>tRNA-Leu</td>
<td>11 bp</td>
<td>3 genes(^c)</td>
<td>Not-explored</td>
<td>[156]</td>
</tr>
<tr>
<td><em>H. somni</em> Av31</td>
<td>ICEHs1</td>
<td>64,932</td>
<td>83</td>
<td>tRNA-Leu</td>
<td>11 bp</td>
<td>4 genes(^d)</td>
<td><em>P. multocida</em></td>
<td>[25]</td>
</tr>
<tr>
<td><em>H. influenzae</em> 1056</td>
<td>ICEHin1056</td>
<td>59,393</td>
<td>64</td>
<td>tRNA-Leu</td>
<td>-</td>
<td>3 genes(^e)</td>
<td><em>H. influenzae</em></td>
<td>[160]</td>
</tr>
</tbody>
</table>

**Table 1.3:** Comparison of recently identified ICEs in some members of the *Pasteurellaceae* family

1. basepair; 2. Number; 3. References

- a. streptomycin (*strA and strB*), kanamycin/neomycin (*aphA1*), tetracycline (*tetR-tet(H)*) and sulphonamides (*sul2*)
- b. streptomycin/spectinomycin (*addA25*), streptomycin (*strA and strB*), gentamicin (*aadB*), kanamycin/neomycin (*aphA1*), tetracycline (*tetR-tet(H)*), chloramphenicol/florfenicol (*floR*), sulphonamides (*sul2*), tilmicosin/clindamycin (*erm*) or tilmicosin/tulathromycin [*msr(E)-mph(E)*], Beta-lactamase (*bla\(^{OXA-2}\)*)
- c. tetracycline (*tetR-tet(H)*), copper (*mco*), zinc (*czcD*)
- d. tetracycline (*tetH*), multidrug efflux pump gene (*ebrB*), and metal tolerance genes; copper (*mco*), zinc (*czcD*), and arsenic (*acr3*).
- e. ampicillin, tetracycline, and chloramphenicol
1.9 Factors affecting the dissemination of ICEs and other MGEs

Many factors induce the transfer of ICEs. Some of these factors might directly affect the propagation of the ICE including SOS responses to DNA damage, the presence of potential recipients, and the selective advantage that some ICEs might provide their hosts [134], [139]. These factors are not universal, and each ICE might behave differently. However, many factors are known to induce a wide variety of ICEs, and some ICEs could be induced by a variety of factors [134]. The following section will briefly address quorum sensing, SOS response, and antimicrobials and their role as regulators for dissemination of ICEs.

1.9.1 Quorum sensing and the transfer of ICEs

Quorum sensing (QS) is the ability of the bacteria to monitor cell population density by secretion of hormone-like molecules called autoinducers. When these molecules reach a threshold outside the bacterial cell, it indicates a high cell density in the surrounding environment. Quorum sensing plays an essential role in the regulation of many physiological activities like virulence, extracellular enzyme production, antimicrobial synthesis, conjugation, and others in both Gram-positive and Gram-negative bacteria [163]–[165].

Recent studies have identified the importance of QS in the regulation and dissemination of ICEs [166], [167]. Ramsay et al. (2015) found that QS activates the excision of ICEMLSym^{R7A} in *Mesorhizobium* loti [167]. The process involves the production of acyl-homoserine lactones (AHLs), which activate the *traR* gene that in turn leads to the expression of other genes needed for conjugation [167], [168]. Another study performed in 2017 confirmed that QS can control the assembly and excision of a tripartite integrative and conjugative element (*ICE3*) in the same bacterial species [166].
1.9.2 The effect of SOS response on the transfer of ICEs

The SOS response is an inducible DNA repair system that enables bacteria to survive DNA damage caused by numerous agents like UV, antibiotics, ionizing radiation, high temperature, and others [129], [169], [170]. Unrepaired DNA damage is considered a severe challenge to the bacterial cell and it might lead to harmful mutations and even cell destruction [170]. SOS response is regulated by two main proteins; a repressor called LexA and an inducer called RecA. Under normal conditions the repressor LexA binds to the operator of the SOS operon to prevent expression of the genes. Upon induction RecA provokes the cleavage of LexA allowing the expression of the SOS operon genes to repair DNA damage [134], [139], [169].

Induction of ICEs by the SOS response means that the host is encountering fatal challenges; therefore the ICE must depend on horizontal transfer rather than vertical transmission to survive and disseminate [134]. The SOS response when induced by ciprofloxacin, stimulated the excision of ICEBs1 and consequently increased the frequency of transfer of the ICE. The study showed that the enhanced gene expression and excision were RecA-dependent [171].

1.9.3 Role of antimicrobials in the dissemination of MGEs

Antimicrobials are known to play a significant role in the treatment and the control of many infectious diseases. However, many studies suggest that low concentrations of antimicrobials may play a regulatory function in the environment [172]–[175]. A review from 2006 indicated that antimicrobials follow the hormesis phenomenon. According to this phenomenon antimicrobials have the ability to confer different characteristics when used at sub-inhibitory (SI) compared to high concentrations [172]. The review discussed the effect of SI concentrations of antimicrobials on gene transcription and consequently impact many activities including the conjugation rate, biofilm formation, mutation frequency, bacterial adhesions and others [172].
There is evidence that sub-inhibitory concentrations of antibiotics may significantly enhance the horizontal transfer frequency of many types of MGEs [174], [176]–[179]. The role of antimicrobials in the dissemination of MGEs including ICEs can be classified into three major concepts, 1) Regulation in a SOS-dependent manner [171], [180], 2) Regulation in an SOS-independent manner [174], [176]–[178], and 3) Selection pressure resulted from the selective advantage that some MGEs provide to their hosts [127], [181], [182].

It was noticed two decades ago that SI concentration of beta-lactam antimicrobials significantly increased the rate of plasmid transfer in *Staphylococcus aureus* between 100 to 1000-fold [176]. It is worth noting that other tested antimicrobials in this study; erythromycin, gentamicin, vancomycin, teicoplanin, and aztreonam, had no such effect on the transfer frequency of the plasmid [176]. Two years later, Valentine et al. (1988) indicated that pre-growth of the donor cells of *Bacteroides* species in a sub-inhibitory concentration of tetracycline enhanced the transfer frequency of tetracycline-resistant conjugal element (TCr) [177]. Another study indicated that the presence of sub-inhibitory concentrations of tetracycline in conjugation media enhanced the transfer of conjugative transposons in *Bacteroides* [178]. A similar effect of tetracycline was observed in *Bacillus subtilis* and *Bacillus thuringiensis* [174]. It was also noticed that pre-incubating the donor with SI concentrations of tetracycline or incorporation of tetracycline in the mating media significantly increased the frequency of transfer of the *ICE Tn916* [174]. Pre-incubation of the donor of *E. coli* in a sub-lethal concentration of cefotaxime (CTX) enhanced the transfer rate of *blaCTX–M–1* plasmid in an SOS-independent mode [183].

The role of antimicrobials in the dissemination of ICEs in the *Pasteurellaceae* family is still unknown. Further experiments are needed to uncover the effect of different antimicrobials and the underlying mechanism behind ICEs-mediated conjugation. It is imperative to address this gap
in knowledge since the increased conjugation frequency of MDR ICEs will adversely affect the therapeutic options in animals and humans.

1.10 Fitness costs of integrative and conjugative elements

Despite the beneficial traits that could be conferred by MGEs they may also have a fitness cost for their hosts [184]. The demonstration of the fitness cost could be either a decreased growth rate or failure of the strains-carrying MGEs to compete in the absence of selection pressure [184], [185]. The biological burden of newly acquired mobile genetic elements usually includes the cost of the backbone of the element and the resistance genes they carry [185], [186]. The cost might be due to the disruption of host genes during the integration of the MGE into the chromosome, the replication of the element, or the expression of MGEs-encoded genes [184]. Many studies observed the cost associated with plasmids in the absence of a selective environment [185], [186] and the ability of the bacteria to acquire compensatory mutations to reduce the host-fitness cost of the resistance trait [186], [187]. However, little is known about the fitness cost of the ICEs on their hosts and the compensatory evolution that enable the bacterial cells to overcome the biological cost caused by the acquired elements [188].

In a recent study on the \textit{ICE Tn5801} in \textit{Enterococcus faecalis} that carries Tet (M) resistant gene it was noticed that in the absence of selective pressure the ICE imposed a fitness cost on its host. In contrast, in the presence of even low concentration of tetracycline in the environment, the fitness cost was reduced and disappeared entirely with a higher concentration of tetracycline [189]. Likewise, it was observed from a co-culture competition experiment that \textit{ICEMh1}\textsubscript{PM22} - carrying transconjugants were less fit than their isogenic ancestors in the absence of selection pressure [190]. On the other hand, the \textit{ICE\textsubscript{CIC}} in \textit{Pseudomonas aeruginosa PAO1} did not impose a fitness cost to its host [191]. A similar result was observed in a copper resistance-encoding ICE in
*Pseudomonas syringae*. The study showed that there was no obvious fitness burden associated with the ICE in the absence of copper sulphate [192]. The information regarding the fitness cost imposed by ICEs is still scarce so further research is needed to fill this knowledge gap.

### 1.11 Aims of the present master thesis

#### 1.11.1 Background information

A recent study by our laboratory identified a new ICE in *H. somni* KLM014 isolated from feedlot cattle with BRD which may encode multidrug resistance [193]. The sequence for this ICE (*ICEHsKLM-014*, also known as *ICEHs02*) has been submitted to the GenBank database [https://www.ncbi.nlm.nih.gov/nuccore/MN401320.1](https://www.ncbi.nlm.nih.gov/nuccore/MN401320.1) with accession number (MN401320). The sequence length of *ICEHs02* is 72,914 bp and it encodes 79 genes. *ICEHs02* is integrated into the tRNA-Leu and is flanked by 21 base-pair direct repeats base pairs [193]. The genomic characteristics of *ICEHs02* are shown in Table 1.4.

The identified *ICEHs02* core genes include genes responsible for integration, excision, transfer, regulation, and stability of the ICE. The identified core genes include; single-stranded DNA binding proteins (*ssDNA*), integrases from the tyrosine recombinase family (*XerC, XerD*), Type 4 conjugative transfer coupling protein (*T4CP*), transposase (*ISL3 family*), relaxase (*TraI*), conjugal transfer protein (*traG*), and conjugal transfer ATPase (*T4SS ATPase*) [193].

The accessory genes that have been identified may provide a competitive advantage for persistence of the host bacteria. The annotated cargo genes in the variable region of *ICEHs02* contains one metal tolerance gene and six multidrug resistance genes. The resistance genes were identified within two cargo gene regions. Cargo gene region 1 includes tetracycline efflux MFS transporter (*tetR-tet(H)*), and the multicopper oxidase gene (*mco*), while cargo gene region 2
includes predicted genes for a florfenicol efflux MFS transporter \((floR)\), a sulfonamide-resistant dihydropteroate synthase \((sul2/folP)\), and aminoglycoside-O-phosphotransferases \((APH(6)-Id)\), \((APH(3')-Ia)\), and \((APH(3'')-Ib)\) (Figure 1.2).

### 1.11.2 Objectives

The overall objective of this study is to understand the role of \(ICEHs02\) in the maintenance and dissemination of antimicrobial resistance among various bacteria in the feedlot ecosystem. In order to achieve that objective, four aims have been established:

1. Determine the host range of the \(ICEHs02\) to understand the role of ICE in disseminating resistance phenotypes into a broader environment.
2. Characterize the phenotype transferred with the ICE to confirm that the new hosts' competitive advantage is functional.
3. Examine the effect of different antimicrobial stressors (for example, exposure to sub-lethal concentrations of tetracycline or ciprofloxacin) on the frequency of transfer of the ICE in order to understand the regulatory role of antibiotics in this ecosystem that might affect the HGT frequency.
4. Examine the fitness cost of ICE acquisition on its new host to better understand the emergence and management of antibiotic resistance.
Table 1.4: Genomic characteristics of the ICE identified in *H. somni KLM014*

<table>
<thead>
<tr>
<th>Name of the ICE</th>
<th>ICEHsKLM014-ICEHs02</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Bank accession number</td>
<td>MN401320.1</td>
</tr>
<tr>
<td>Length (bp)</td>
<td>72918</td>
</tr>
<tr>
<td>GC content (%GC)</td>
<td>41.82</td>
</tr>
<tr>
<td>Number of coding genes</td>
<td>79</td>
</tr>
<tr>
<td>Direct repeats</td>
<td>CGTGTCCGGTTCTCGAGTCCGACC (21 bp)</td>
</tr>
<tr>
<td>Insertion site</td>
<td>tRNA-Leu</td>
</tr>
<tr>
<td>The location of integrated tRNA gene</td>
<td>687445...687530</td>
</tr>
<tr>
<td>The location of ICE on the chromosome</td>
<td>687500...760413</td>
</tr>
</tbody>
</table>
Figure 1.2: A schematic map of *ICEHsKLM014 (ICEHs02)* showing the resistance gene regions 1 and 2

The resistance gene regions 1 (left) and 2 (right) are shown in boxes. Genes are represented by arrows, with the arrowhead indicating the direction of the transcription. Antimicrobial resistance genes are represented by red arrows, blue arrows represent metal resistance genes, orange arrows represent the core genes and grey arrows represent the hypothetical proteins. Resistance region 1 includes tetracycline efflux MFS transporter (*tetR-tet(H)*), and multicopper oxidase gene (*mco*), resistance region 2 that includes florfenicol efflux MFS transporter (*floR*), sulfonamide-resistant dihydropteroate synthase (*sul2*), and aminoglycoside-O-phosphotransferases (*APH(3'″)-Ib*, *APH(6)-Id*, and *APH(3')-Ia*). The map was created using SnapGene ® version 5.1.5.
1.12 Research questions and hypotheses

1. The first research question is to investigate the host range of ICEHs02.

- The first specific aim was to investigate whether ICEHs02 would transfer from the donor strain *H. somni* KLM014 to other bacteria in the BRD complex (*P. multocida, M. haemolytica*). In addition, investigate if the ICEHs02 would transfer to other environmental bacteria such as *E. coli* and the human respiratory pathogen *H. influenzae*. Lastly, we aimed to investigate the frequency of the transfer of ICEHs02.

- We hypothesize that that ICEHs02 would move more frequently from *H. somni* to *H. somni*, and the closely related bacteria *P. multocida, M. haemolytica*. Besides, we hypothesized that ICEHs02 would move with a lower transfer frequency to the distantly related bacteria *E. coli* and *H. influenzae*.

- In order to answer these questions, several experiments were conducted:
  1) Filter mating assays were conducted between the donor *H. somni* KLM014 and multiple bacterial species to investigate the host range of the ICEHs02 and determine the conjugal transfer frequency.
  2) Tetracycline E-strip test was performed to screen the transconjugants that have the ICE.
  3) PCR assays were conducted to confirm the transfer of the ICE by detecting the presence of core genes and accessory genes in the transconjugants.
  4) *In silico* prediction of the host range was conducted to further investigate the ICE's host range, since it is challenging to conjugate many bacterial strains in the laboratory.
2. The second research question was to investigate whether the ICEHs02 would confer a resistance phenotype to the recipient strains

- **ICEHs02** carries multiple drug resistance genes and one metal tolerance gene. We wanted to investigate if the ICE will confer all the antimicrobial resistance phenotypes to the recipient strains.
- We hypothesized that the ICE would confer all the resistance phenotypes to all the recipient strains.
- To achieve that objective, we conducted microdilution susceptibility assays to determine the MIC of several antimicrobials in the donor, recipient and transconjugants.

3. The third research question was to investigate whether the presence of stressors such as sub-inhibitory concentrations of tetracycline or ciprofloxacin would affect the transfer frequency of **ICEHs02**.

- We hypothesized that the presence of sublethal concentrations of antimicrobials would increase the transfer frequency of the ICE.
- To achieve that objective, we conducted conjugation assays with some modifications. The donor strain was incubated with a sub-inhibitory concentration of ciprofloxacin or tetracycline prior to conjugation.

4. The fourth question was to investigate whether the **ICEHs02** would impose a fitness cost to its host and subsequently whether the ICE will be lost from the population in the absence of selection pressure.

- We hypothesized that the ICE would impose a fitness cost on the recipient strains, and consequently, due to the fitness cost, the ICE will be lost from the population in the absence of selection pressure.
To investigate that objective, the following experiments were conducted:

1) growth curve assays were conducted to compare the growth rate of the ICE-carrying strains with their isogenic parents.
2) co-culture competition experiments were conducted between the ICE-carrying strains and their isogenic ancestors.
3) Serial passage experiments were conducted to investigate if the ICE will be lost from the host strains in the absence of selection pressure.

1.13 Significance of the research

AMR is a global concern threatening animal and human health. MGEs play a significant role in the dissemination of antimicrobials between bacteria. ICEs can convey many traits such as AMR and enhanced virulence via HGT which may increase the survival of the recipient bacteria. The host range of ICEHs02 in H. somni has not been examined yet. Investigating the host range of ICEHs02 will help in understanding the role of ICE in the reservoir of AMR in this environment, and the dissemination of antimicrobial resistance by the horizontal gene transfer. The transfer of ICE carrying multiple drug resistance genes (ICEHs02) into other bacteria in the BRD complex might decrease the therapeutic options for bovine respiratory disease. The role of sub-inhibitory concentrations of antimicrobials in the dissemination of ICEs in the Pasteurellaceae family has not been investigated yet. Addressing this knowledge gap will help in understanding the regulatory role played by antimicrobials in the maintenance and dissemination of ICEs among bacteria in the feedlot ecosystem. In addition, there is scarce information about the fitness cost imposed by the newly acquired ICEs on their new host. The fitness cost of ICEs in H.somni strains have not been explained yet. This information will help understanding the fate of the ICE once it enters a recipient cell and how the selective environments might affect the persistence of ICEs.
Chapter Two : Materials and Methods

2.1 Bacterial strains and growth conditions

*H. somni* strains (KLM014, KLM08) used for this study were among a collection obtained from the Vaccine and Infectious Disease Organization-International Vaccine Center (VIDO-InterVac), University of Saskatchewan, SK, Canada. *H. somni* isolates were collected from necropsy tissue samples of feedlot cattle in Alberta that died displaying clinical signs of histophilosis or BRD between 2012-2016. *M. haemolytica* h186, *Haemophilus influenzae* h211, h040 were kindly provided by Dr. Anthony Schryvers. *E. coli* K12 MG15655 was kindly provided by Dr. Tao Dong.

*H. somni* strains were grown in Tryptic Soy Agar (TSA) (Becton, Dickinson and company, MD, USA) or Columbia Blood Agar base (CBA) (Hardy Diagnostics, Santa Maria, CA) supplemented with 5% sheep blood (Rockland, PA, USA) and were incubated at 37°C in the presence of 5-10% CO₂ for 24 hours. *P. multocida* and *M. haemolytica* were grown on the same media and incubated at 37°C for 24 hours. Liquid cultures of *H. somni* strains were done using Brain Heart Infusion broth supplemented with 0.1% trizma base (Sigma-Aldrich, St Louis, USA) and 0.01% thiamine monophosphate (BHITT) (Sigma-Aldrich, St Louis, USA) while both *P. multocida* and *M. haemolytica* were grown in BHI (Becton, Dickinson and company, MD, USA) with no supplementation. Lysogeny/Luria broth (LB) and LB agar (Becton, Dickinson and company, MD, USA) were used for *E. coli*. *Haemophilus influenzae* strains were grown on chocolate agar plates (Hardy Diagnostics, Santa Maria, CA) and supplemented Brain Heart Infusion (sBHI) containing NAD (3.32 μg/mL) and hemin (10 μg/mL) and were incubated at 37°C in a CO₂ incubator. Veterinary Fastidious Medium (VFM) (Oxoid, Ontario, Canada) or Mueller-Hinton Fastidious Medium with Yeast Extract (MHF-Y) (Oxoid, Ontario, Canada) were used as
recommended by CLSI for *H. somni* strains [194]. When the selection was required, the following antibiotic concentrations were used: tetracycline 8 µg/mL, rifampicin 50 µg/mL, and nalidixic acid 30 µg/mL. When induction was done, the following antibiotic concentrations were used: tetracycline 8 µg/mL, or ciprofloxacin 2 µg/mL.

### 2.2 Filter mating assay

Many studies suggested that filter mating is the best technique for conjugative transfer [146], [195], [196]. An early study was done in Japan (1988) indicated that donor and recipient contact each other in the filter surface more efficiently than in liquid and solid mating assays [196]. After a few years, another study was done by Joanna Lampkowska et al. (2008) to optimize the conjugation protocol which compared the conjugation frequencies in solid, liquid, and filter mating and indicated that the filter mating is the most reproducible technique for conjugation [195].

A tetracycline-resistant plasmid-free *H. somni* KLM014 was used as a donor. A rifampicin-resistant, and tetracycline-susceptible *H. somni* ATCC700025, *H. somni* KLM08, *P. multocida DSM 16031*, *M. haemolytica 186*, *E. coli K12MG 15655*, *H. influenzae h040, h211* were used as recipients. Selection of spontaneous rifampicin-resistant mutants of recipient strains was performed by selecting and purifying colonies on CBA or BHI having increasing concentrations (1, 5, 10, 25, 50, 100, 150, 200 µg/mL) of rifampicin (Sigma-Aldrich, St Louis, USA). Selection of spontaneous rifampicin mutants for *H. influenzae* was done using sBHI and chocolate agar plates while LB medium was used for *E. coli*.

The tetracycline-resistant, rifampicin-resistant *P. multocida DSM16031* transconjugant (*P. multocida DSM16031 ICEHd02*) was used a donor in the second round of conjugations. Nalidixic acid-resistant, rifampicin and tetracycline susceptible *P. multocida DSM16031* and *H. somni ATCC700025* were used as recipients. The selection of nalidixic acid-resistant mutants of recipient
strains was made by inoculating *H. somni* ATCC700025 and *P. multocida* DSM16031 in BHITT and BHI respectively at 37°C for 24 hours and spreading aliquots of 200 μL on CBA plates having 30 μg/mL nalidixic acid. The plates were incubated at 37°C for 48 hours. The resistant colonies were selected and purified on CBA plates with the same concentration of the antimicrobial.

Bacterial growth curves were performed prior to conjugation experiments to detect the mid-exponential phase of the bacteria. Growth curves of *H. somni* KLM014, *P. multocida* DSM16031, *M. haemolytica* 186 were done using single colonies from overnight cultures on TSA or CBA blood agar plates. The single colonies were inoculated into 10 mL of BHITT or BHI and incubated at 37 °C with 5% CO₂ for 24 hours with shaking at 225 RPM; then, 5 mL of the overnight grown culture was inoculated in 250 mL of BHITT or BHI. The initial optical density (OD₆₀₀) at zero hours was read using a spectrophotometer (Spectramax M2, Molecular Devices, USA). The Broth cultures were incubated at 37 °C with 5% CO₂ for 24 hours with shaking at 225 RPM. The OD₆₀₀ of the culture suspensions were read at an interval of 1 hour (*P. multocida*, and *M. haemolytica*) and 2 hours for (*H. somni*) till the reading becomes static. The detailed protocol for growth curves is discussed in appendix 2.

Conjugation experiments were conducted as previously explained [121], [151], with some adjustments. Briefly, donor and recipient bacteria were grown distinctly in BHITT broth (*H. somni*), BHI broth (*M. haemolytica, P. multocida*), sBHI broth (*H. influenzae*) or LB broth (*E. coli*) at 37°C with 5% CO₂ to the mid-log phase. Following incubation, donor and recipient were mixed at a 1:1 ratio. The mixture was centrifuged at 13,000 RPM for one minute, the supernatant was discarded, and the cell pellet was washed twice in 1 mL of broth. The supernatant was resuspended in 30 μL of broth. The mixture was placed on a sterile nitrocellulose filter (0.22 mm, Millipore) on CBA plates (Hardy diagnostics, Santa Maria, CA), except the conjugation with *H.
*influenzae* recipient cells, which was performed on chocolate agar plates (Hardy diagnostics, Santa Maria, CA). Mating plates were incubated at 37 °C with 5% CO₂ for 24 hours. Control cultures of recipient and donor strains alone were treated in the same manner.

The bacteria were collected from mating filters in 1 mL of broth, serially diluted, and plated on the suitable selection agar plates. Transconjugants, donors, and recipients were purified on agar plates containing 50 μg/mL rifampicin + 8 μg/mL tetracycline, 8 μg/mL tetracycline, 50 μg/mL rifampicin respectively. The transfer frequency was calculated as the number of transconjugants per donor. Three biological replicates were done for each conjugation assay. Colonies of resulting transconjugants were re-streaked twice for purification on suitable selection plates before the transconjugants were confirmed by susceptibility testing and PCR screening for resistance genes and ICE-related genes.

### 2.3 Antimicrobial susceptibility assay

Antimicrobial susceptibility of the donor, recipient, and transconjugants was assayed by the E- strip test (Oxoid, Hampshire, UK), and broth microdilution methods.

#### 2.3.1 Minimum inhibitory concentration evaluators (**M.I.C.E**TM)

**M.I.C.E**TM is a simple quantitative technique to determine the minimum inhibitory concentration (MIC) in μg/mL. It consists of a plastic strip which has a predefined gradient of 15 antibiotic concentrations in two-fold dilutions. When **M.I.C.E** strip is placed on the surface of an agar plate inoculated with the test strain, it releases the antimicrobials from the plastic carrier, establishing an antimicrobial gradient in the agar plate. After 18-24 hours incubation period, a drop-shaped inhibition zone intersects the graded test strip at the inhibitory concentration of the
antibiotic. Tetracycline M.I.C.E was used as a screening method for the donor, recipients, and transconjugants.

The tetracycline M.I.C.E was performed as per manufacturer instructions (Oxoid, Hampshire, UK). The inoculum was prepared by taking 3-5 separated colonies from an overnight culture on a chocolate agar plate. The colonies were emulsified in Mueller-Hinton broth (MHB) (Becton, Dickinson and company, MD, USA) as recommended by CLSI [194]. The turbidity of the suspension was standardized to 0.5 McFarland (~1x10⁸ CFU/mL) using the Sensititre® Nephelometer. Next, the inoculum was spread on chocolate Mueller-Hinton agar (CMHA), as recommended for H. somni by CLSI [194]. After allowing the plate to dry for 5 minutes, the M.I.C.E strip was placed on CMHA with the MIC scale facing upward. The plate was incubated at 37°C with 5 % CO₂ for 24 hours. The result was interpreted by reading the elliptical inhibition zone. The MIC was read at the point where ellipse intersects with the M.I.C.E. The detailed protocol for the M.I.C.E is described in Appendix 5. M.I.C.E for H. influenzae was done using Haemophilus Test Medium (HTM) (Becton, Dickinson and company, MD, USA) as recommended by CLSI.
Figure 2.1: Tetracycline antimicrobial susceptibility of *H. somni* KLM08 using the M.I.C.E method

The test strain from standardized broth culture was inoculated onto a chocolate Mueller-Hinton agar media, and a tetracycline M.I.C.E was applied. The plate was incubated at 37°C with 5% CO₂ for 24 hours. The inhibition zone appears as an ellipse shape. The MIC is read at the point where ellipse intersects with the M.I.C.E strip. An MIC value printed in the scale at the intersection of the zone of inhibition to the M.I.C.E strip (0.25 μg/mL) was recorded.
2.3.2 Broth microdilution method using Sensititre® system

The Sensititre susceptibility system is an advancement of the traditional broth dilution technique. The principle of the Sensititre® system is to define the minimum inhibitory concentration (MIC) by recognizing the growth of test organisms inoculated in a range of antimicrobial concentrations. The technique involves using Sensititre® susceptibility panels, which are 96 well microtiter plates. Each plate has the antimicrobial agents at appropriate dilutions. Sensititre plates can be inoculated by broth suspension manually or by using the Sensititre® Auto-inoculator™. Sensititre® Auto-inoculator™ is a device used to accurately dispense a specific volume of the suspension to the panels. The required volume and type of the plates can be adjusted using the touch screen of the Sensititre® Auto-inoculator™. After incubation, the plates can be read either automatically or manually. The plates can be read manually using the Sensititre® manual viewer or the sensiTorch for the computer-assisted reading, while the plates can be read automatically using the Sensititre OptiRead Automated Fluorometric Plate Reading System. The Sensititre® Auto-Reader™ or OptiRead™ can detect the bacterial growth by monitoring the amount of fluorescence liberated by bacterial enzymes that cleave fluorogenic substrates in the broth added to each well of an antibiotic panel. The Sensititre® Auto-Reader™ is connected to the Sensititre Windows® (SWIN) Software System that offers automated expert control for the whole process, and that includes scanning and reading the plates, interpreting the MIC results based on the program reference database, exporting data and printing the results.

Sensititre™ Bovine/Porcine BOPO6F Vet AST Plate (Trek Diagnostic Systems, Cleveland, Ohio, USA) that contains 18 different antimicrobials in two-fold dilution was used in the susceptibility assay. Inoculum preparation was done by taking 3-5 separated colonies from an overnight culture on a chocolate agar plate. The colonies were emulsified in CAMHB (cation
adjusted Mueller-Hinton broth), and the turbidity of the suspension was standardized to 0.5 McFarland (approximately = 1x10^8 CFU/mL) using the Sensititre® Nephelometer. As recommended for *H. somni*, 50 μL of the suspension was transferred to Sensititre Veterinary Fastidious Medium (VFM) (Oxoid, Ontario, Canada) or Mueller-Hinton Fastidious Medium with Yeast Extract (MHF-Y) (Oxoid, Ontario, Canada) to reach an inoculum of 5x10^5 CFU/mL. Afterwards, 50 μL of the suspension was transferred to each well using the Sensititre® Auto-inoculator™. The plates were covered by a perforated adhesive seal to prevent desiccation and incubated at 37°C in a CO2 incubator for 20 - 24 hours. Sensititre plates were read automatically using the Sensititre Auto-Reader™. The MIC report was printed, and the data were stored in the system. Control wells were read first, and if no growth detected, the results were rejected. If the colony count was not in the range from range 2x10^5 – 7x10^5 CFU/mL, the result should not be reported. The Senstititre detailed protocol and quality control procedures are illustrated in detail in appendix 6.

The Sensititre micro-broth dilution assay was used at the beginning of the experiments to assess the antimicrobial resistance profiles for the bacteria used in the experiments. However, the broth microdilution test was used instead afterwards as the whole panel of antimicrobials of the Sensititre® system was not needed. In addition, the Sensititre® system provides a narrow range of antimicrobial concentrations which was not broad enough to determine the exact MIC of some of the antimicrobials for each isolate.

2.3.3 Broth microdilution test

Broth microdilution assays are used to determine the MICs of antimicrobial agents by measuring the *in vitro* activity of an antimicrobial against a bacterial isolate. In this assay, a sterile 96 well plate allowed approximately 12 antibiotics to be tested in series of eight two-fold dilutions.
The wells were inoculated to reach a standardized bacterial suspension of 1-5x10^5 CFU/mL. After overnight incubation at 35-37°C, the MIC was measured by observing the lowest concentration of an antimicrobial agent (μg/mL) that prevents the appearance of visible growth of bacteria. Performance of the assays followed the recommendations given in documents VET01-5, VET01-A4 of the CLSI. The assay involved three steps 1) The preparation of the stock solution of antimicrobial agents, 2) The preparation of the 96 well plates, 3) The preparation of the inoculum [194], [197].

2.3.3.1 Preparation of the antimicrobial stock solutions

Antimicrobial stock solutions were prepared at a concentration of 10 mg/mL. The stock solution was prepared based on the equation \( W = \frac{C(\mu \text{g}/\text{mL}) \times V(\text{mL})}{P(\mu \text{g}/\text{mg})} \), where \( W \) = weight of antibiotic powder (mg), \( P \) = potency given by the manufacturer and is expressed as a percentage or in units of μg/mg, \( V \) = volume required (mL), and \( C \) = final concentration of solution (μg/mL) [194], [198].

The Antimicrobial powder was dissolved into a minimum amount of solvent, and the final stock concentration dilution was completed with water. The solvents and diluents used for the preparation of the stock solutions are described in table 3. The solutions were filter sterilized using 0.22-millipore membrane filters (VWR, Solon, OH, USA). Small volumes of the sterile stock were aliquoted in Eppendorf tubes, carefully sealed, and stored at – 20°C.

Preparation of a working solution, from the initial 10 mg/mL solution was done using the equation \( C_1V_1 = C_2V_2 \), where \( C_1 \) is the concentration of the initial stock solution, \( V_1 \) is the volume of the initial stock solution, \( C_2 \) is the concentration of the working solution, \( V_2 \) is the volume of the working solution.
Table 2.1: Solvents and diluents used for the preparation of stock solutions

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Solvent</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>Water or ETOH*</td>
<td>Water</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>DMSO**</td>
<td>Water</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
<td>Sulfadimethoxine</td>
<td>DMSO**</td>
<td>Water</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>DMSO**</td>
<td>Water</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Water</td>
<td>Water</td>
</tr>
</tbody>
</table>

*ETOH, Ethyl Alcohol; **DMSO, Dimethyl sulfoxide

2.3.3.2 Preparation of the 96 well microplates

CLSI recommends CAMHB for antimicrobial susceptibility testing (AST) because this medium contains fewer inhibitors that might affect the antimicrobials such as tetracycline, and it also supports the growth of most pathogens. However, this media cannot be used for fastidious organisms such as *H. somni* so alternative medium were used instead. The composition of these media is basically MHB, with the addition of some supplements such as lysed horse blood (LHB) and yeast extract to enhance growth of fastidious organisms. Thus, VFM broth (Oxoid, Ontario, Canada) or MHF-Y broth (Oxoid, Ontario, Canada) were used for culture of *H. somni* while CAMHB (Oxoid, Ontario, Canada) was used for *P. multocida* culture.

The microdilution trays were prepared by making two-fold dilutions of the antimicrobial agents using the broths. First, 100 μL of broth was dispensed from wells 1 to 12; Then 100 μL of
antimicrobial stock solution was added to the first well. Afterwards, two-fold dilutions of antimicrobials were done from wells 1 to 10, but no antibiotic was added to wells 11 (growth control) and 12 (sterility control). The filled trays were sealed in plastic bags and placed at -20°C until needed.

2.3.3.3 Preparation of the inoculum

The colony suspension method was used for inoculum preparation. This technique can be applied for most organisms and is recommended for fastidious organisms such as *H. somni*, and *Haemophilus spp* [194], [198]. Inoculum preparation was done by taking 3-5 separated colonies from an overnight culture on a chocolate agar plate. The colonies were emulsified in sterile CAMHB, and the turbidity of the suspension was standardized to 0.5 McFarland (approximately = 1x10⁸ CFU/mL) using the Sensititre® Nephelometer. Each well should contain approximately 5 x 10⁵ CFU/mL, so the 1x10⁸ CFU/mL was diluted 1:20 to yield 5 x 10⁶ CFU/mL, then wells 1 to 11 were inoculated by 10 μL of the diluted suspension to reach a final inoculum of 5 x 10⁵ CFU/mL. The trays were sealed in plastic bags to prevent dryness and incubated in at 37°C in a CO₂ incubator for 20-24 hours. Viable counts were done to make sure that the suspension contains 5 x 10⁵ CFU/mL. That was done by diluting 10 μL from the growth control well in 10 mL broth (1:1000 dilution). After mixing, 100 μL of the diluted suspension was spread on chocolate agar plate and incubated for 22-24 hours. After incubation the presence of ~50 colonies indicates the desired inoculum density.

2.3.3.4 Reading the plates and recording the MIC

The plates were read using the unaided eye. The MIC was defined as the lowest concentration of antibiotic that completely inhibited the bacterial growth. If no growth was noticed in the growth control well or any growth appeared in the sterility control well, the plate was
discarded, and the experiment was repeated. H. somni ATCC700025 was used for quality control as recommended by the CLSI VET08.

2.4 Copper tolerance assay

There are no established protocols for metal tolerance testing by the CLSI, however some studies used agar dilution [25], [199]–[201] or a broth dilution method [199] to measure the MIC of copper. In this study, Copper susceptibility was determined using a broth microdilution method following the same procedure and CLSI protocols described in the previous section.

2.4.1 Preparation of copper stock solution and working solution

The stock solution (640 mM) of copper sulfate pentahydrate (CuSO₄+5H₂O) (Sigma Aldrich, St. Louis, MO, USA) was prepared by dissolving 1.597 g of copper sulfate in 10 mL of sterile distilled water. The stock solution was then 10-fold diluted using sterile distilled water to prepare a working solution with a final metal concentration of 64 mM. Copper sulfate is known to cause a significant drop in the PH of the medium [202], so the solution should be adjusted to a PH of 7 to prevent confounding factors that might affect the growth of the bacteria. The PH of the media was adjusted to 7 using a 1M solution of TRIS base (PH 11) (VWR, Solon, OH, USA). The working solution was filter sterilized using 0.22 mm membrane filters (VWR, Solon, OH, USA).

2.4.2 Preparation of 96 well microplates and inoculum preparation

A ninety-six well microplate (Thermo Scientific, MA, USA) was prepared in two-fold serial dilutions using the recommended broth to get final concentrations of 32, 16, 8, 4, 2, 1, 0.5, 0.25 mM. The inoculum was prepared as previously described in section 2.4.3.3. Ten µl of the prepared inoculum was inoculated to each well so each well gets an inoculum of 1x10⁵ CFU/mL.
2.4.3 Incubation, reading the plates and recording the MIC

The trays were read by a microplate reader (Spectramax M2, Molecular Devices, USA) at an OD_{600} and were incubated at 37°C, 5% CO₂ for 24 hours. After incubation the absorbance of the trays was read a second time. The mean absorbance units (AU) obtained from three technical replicates were compared against the concentration of copper (mM) to determine the MIC. The detailed protocol for copper (Cu) tolerance assay is described in appendix 8.

2.5 Polymerase chain reaction (PCR)

Polymerase chain reaction assays were conducted to confirm the transfer of the ICEHs02. The transconjugants were screened for the presence of ICEHs02-associated core genes including integrase, relaxase, transposase (tnpA), and single-stranded DNA binding protein (SSDNA), ICEHs02-associated resistance genes including tetracycline resistance gene (tetH), copper tolerance gene (mco). (Table 2.2). P. multocida transconjugants were subjected to species-specific PCR to assure that transconjugants were not spontaneously mutated-donor cells. In all the PCR assays, the original recipient strain served as a negative control, while H. somni KLM014 served as a positive control.

Dream Taq Green PCR Master Mix (2X) was used for PCR reactions (ThermoFisher Scientific, CA). The master mix is composed of 0.05 U/μL Taq DNA polymerase, reaction buffer, 4 mM MgCl₂, 0.4 mM of each dNTPs. The master mix was gently vortexed and centrifuged after thawing. Each PCR reaction (25 μL) in final concentration contained 12.5 μL of PCR Master mix(2X), 0.1-1.0 μM of forward primer, 0.1-1.0 μM of reverse primer, 10 pg - 1 μg of DNA template. The PCR was performed using T100™ Thermal Cycler (Bio-Rad, CA, USA) following these conditions: initial denaturation at 94°C for 1-3 min; 30-34 cycles of: denaturation at 94°C for 30 seconds, annealing for 30 seconds and the temperature depends on the primer (Table 2.2.),
and extension at 72°C for 1 min; with a final extension at 72°C for 15 min. PCR target genes, primer names, PCR primer sequences, amplicon size, and annealing temperatures are illustrated in Table 2.2. Agarose gel electrophoresis was conducted following PCR to analyze the PCR product. Afterwards, a ChemiDoc™ MP Imaging System (Bio-Rad, CA, USA) was used to visualize the target amplicons of desired sizes. The detailed protocols for agarose gel electrophoresis and imaging are explained in detail in sections 2.5.3 and 2.5.4.

2.5.1 DNA extraction for PCR

Genomic DNA (gDNA) was isolated using the boiling method using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems™ Inc., ON. Canada). The protocol recommended by the user’s guide was used. In short, 100 μL of the reagent was dispensed into a microcentrifuge tube. A small loopful of an isolated colony from an overnight culture on CBA was emulsified into the reagent. The tube was then vigorously vortexed for 10-30 seconds. Afterwards, the suspension tube was placed in a water bath set to 95-100°C for 10 minutes. The tube was removed from the water bath and allowed to cool for 2 minutes at room temperature. The tube was centrifuged in a microcentrifuge at 13,000 RPM for 2 minutes. Finally, 50 μl of the supernatant was transferred into a clean microcentrifuge tube.

The quantification of gDNA (genomic DNA) was done by ND-1000 UV/Vis Nanodrop spectrophotometer (ThermoScientific, Waltham, USA). The detailed procedure is outlined in appendix 11. Two ratios of absorbance 260/280, 260/230 were used to examine the purity of DNA. The ratio of absorbance at 260 nm and 280 nm (260/280) was the primary ratio considered to assess the purity of DNA. A ratio of approximately 1.8 was accepted as high purity DNA. The ratio of 260/230 was used as a secondary measure of DNA purity. The acceptable range is usually
between 2.0 - 2.2. If the ratio is lower than expected it might indicate the presence of contaminants that absorb UV at a wavelength of 230 nm or 280 nm, such as protein.

### 2.5.2 Designing primers for PCR assay

#### 2.5.2.1 Overview on designing primers

There are plenty of tools on the internet that facilitate designing high-quality primers such as Primer-BLAST, Primer3Plus, Eurofins Genomics' Primer Design Tools, and IDT Primer Quest Tool. Many features should be considered when designing primers. These features include primer length, primer length difference, the GC content, nucleotide composition in the three prime ends (3') of the primer, The melting temperature (Tm), and the melting temperature difference between forward and reverse primer [203]. A primer length of 16–28 nucleotides is considered optimum for PCR. The difference in length between the forward primer and reverse primer should not exceed three nucleotides. The GC content should be in the range of 40-60%. GC base pairs are more stable than AT base pairs; thus, the presence of GC base pairs at the three ends of the primer will help to tightly bind the primers to the template. Primers with melting temperature range of 50-62 °C generally produce the best results. The melting temperature difference between the primer pair should be zero, however, the PCRs can be successful if the melting temperature between the primer pairs does not exceed 5°C. Stable secondary structures such as hairpin and dimers should be avoided. Primer dimers are the result of annealing two primers while the hairpin is when a primer anneals to itself [203].

#### 2.5.2.2 Designing primer protocol

Primer pairs were designed for detecting genes correlated with ICE using Primer-3 Plus. Primer-3 Plus is an online tool available on the website:
The sequence of the target genes used for designing the primers was obtained from *H. somni* strain UOC-KLM-ATR-014, whole genome shotgun sequencing project on the GenBank (accession number NZ_CP042993) on the website: [https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP042993.1](https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP042993.1), and *H. somni* strain UOC-KLM-ATR-014, ICEHsKLM-014 partial sequence (accession number MN401320) on the website: [https://www.ncbi.nlm.nih.gov/nuccore/MN401320.1](https://www.ncbi.nlm.nih.gov/nuccore/MN401320.1).

Primer parameters were set as follows; primer length 18-22 nucleotides, the melting temperature 50-62, the GC content 40-60%, and maximum Tm difference is 3°C. After designing the primers, specificity was checked by Primer-BLAST. Primer-BLAST is a tool to design primers, and also it can be used to check the specificity of the designed primers. The specificity assures that the intended primer can recognize a specific sequence to limit the unreasonable PCR results [203]. Primer-BLAST is available on the National Center for Biotechnology Information website[204]: [https://www.ncbi.nlm.nih.gov/tools/primer-blast/](https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

The last step in designing primers was to check for hairpin and primer-dimer secondary structures using IDT's Primer Quest. IDT's Primer Quest is an online tool used for the detection of secondary structures, available on Integrated DNA Technologies, Inc. [US] website: [https://www.idtdna.com/UNAFold?](https://www.idtdna.com/UNAFold?)

### 2.5.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA fragments according to their size. The negatively charged DNA molecules migrate through the agarose gel towards the positive charge using a constant current. The fragment separation depends on the mass and the charge of the DNA.
A 1% agarose gel was prepared by dissolving 1 gram of agarose powder (Qiagen Inc, Toronto ON. Canada) in 100 mL of 1X Tris-acetate-EDTA (TAE) buffer (VWR, Solon, OH, USA) in a microwavable flask. The mixture was dissolved by heating in a microwave for 30 to 90 seconds until clear solution was observed. After cooling the flask to about 60°C, five μL of RedSafe™ (Intron Biotechnology, Inc. CA, USA) was added to the gel solution. The agarose gel was then poured to the gel chamber with a suitable comb in place. After the gel solidified the comb was removed, and the tray was placed in an electrophoresis tank filled with 1X TAE buffer. Amplified PCR products were run in the gel. The wells were loaded carefully by pipetting 5 to10 μL of the DNA. DNA ladder markers (Qiagen Inc., Toronto ON. Canada) were used to identify the approximate size of PCR amplicons in the gel. Ladders of size 100 bp or 1 Kb were used depending on the expected product size.

2.5.4 Imaging and visualizing of PCR products

ChemiDoc™ MP Imaging System (Bio-Rad, CA, USA) was applied to visualize the target amplicons of desired sizes. The ChemiDoc™ MP Imaging System is connected to a computer and is controlled by Image Lab™ software. The gel was inserted inside the drawer of the imager. Using the Image Lab™ software, a new protocol was selected for the nucleic acid stained with gel red. The protocol was run and the image was displayed on the computer screen. The image can be adjusted for clarity using options in the software. The image was saved in the laboratory file for record and then printed.

2.6 In-silico prediction of ICEHs02 host range

The Basic Local Alignment Search Tool Nucleotide (BLASTn) was used for the prediction of host range of ICEHs02 using the conserved ICE integration site tRNA-LeuCAA of H. somni
The full length of the tRNA-Leu gene (86 bp) was uploaded to the BLASTn.
The default megablast program was optimized for highly similar sequences. Program parameters were set as follows: maximum target size, 5000; word size, 28; match/mismatch scores 1, -2. Standard databases (nr) were used and the queries were adjusted for short input sequences. The results were filtered to match records with percent identity of 80 to 100% and query coverage of 100%. The resulted alignments were filtered for ones having ICEHs02-associated direct repeats (DRs) with a 100% identity using SnapGene® version 5.1.5. The taxonomy report is generated from the taxonomy reports link on the BLAST results page.

2.7 Detection of the circular intermediate structure and the chromosomal junction in ICEHs02

As described previously in section 1.7.3, ICEs are integrated into the host chromosome and upon excision they form a circular intermediate. In this experiment, ICE specific primers (Table 2.2) were designed to detect the circular intermediate (CI), chromosomal junction (CJ), the integration sites of the ICE on the chromosome (Figure 2.2).

2.7.1 PCR to detect circular intermediate, chromosomal junction, and ICE attachment sites

Genomic DNA (gDNA) was isolated by the boiling method using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems™ Inc., ON, Canada). PCR was conducted using the primer pair PCIF and PCIR to detect the extrachromosomal circular form of ICEHs02, the primer pair anneal to sequences inside ICEHs02 and amplify the circular intermediate generated upon excision. The primer pair PCJF and PCJR anneal to sequences surrounding ICEHs02 and
amplify the repaired chromosomal junction formed upon excision. The primer pairs PCIF, PCJR and PCJF, PCIR were used to detect the integration of the *ICEHs02* in the chromosome (Figure 2.2). PCR was performed using T100™ Thermal Cycler (Bio-Rad, CA, USA) following these conditions: initial denaturation at 94°C for 1-3 min; 30-60 cycles, denaturation at 94°C for 30 seconds, annealing for 30 seconds at 58°C for Circular intermediate and 63°C for chromosomal junction, and extension at 72°C for 1 min; with a final extension at 72°C for 15 min. PCR was performed using at least three independent biological replicates. The products of the PCR were purified and sent for sequencing.

![Diagram](image)

**Figure 2.2:** A schematic diagram illustrating the location of PCR primers used to detect the excision and integration of the *ICEHs02* sequence

Primers 1 and 4 (PCJF and PCJR) anneal to sequences surrounding *ICEHs02* and amplify the repaired chromosomal junction formed upon excision. Primers 2 and 3 (PCIF and PCIR) anneal to sequences inside *ICEHs02* and amplify the circular intermediate generated upon excision.
2.7.2 Purification of PCR products

Before sequencing, PCR-amplified products were purified using the GenepHlow™ Gel/PCR Kit (FroggaBio Inc, ON, Canada). The kit is designed to concentrate DNA fragments from PCR reactions and to get rid of enzymes, excess nucleotides, primers, and buffer components. Gel/PCR buffer (binding buffer) is used to facilitate DNA binding to the silica membrane in the DFH column. Contaminants were removed with wash buffer (WB) and the purified DNA fragments were eluted by elution buffer (EU). The purity of gDNA (genomic DNA) was assessed by ND-1000 UV/Vis Nanodrop spectrophotometer (ThermoScientific, Waltham, USA) as discussed previously in section 2.5.1. The detailed protocol of the purification of PCR products is described in appendix 12.

2.7.3 Sequencing of PCR amplicons

Fifty nanograms (ng) of purified PCR products were mixed with 3.2 picomoles (pmoles) of primers in 200 μL tubes, free nuclease water was added to the tubes to reach a total volume of 12 μL. Two tubes were used for the forward and reverse primers for each reaction. The tubes were sent for sequencing to the CHGI (Center for Health Genomics) at the UCDNA (University of Calgary Core DNA services). DNA sequencing was done using Applied Biosystems 3730xI 96 capillary DNA Analyzer. Sanger dideoxy sequencing with four colour fluorescence is performed using Applied Biosystems PRISM cycle sequencing kits: BigDye Terminators Version 3.1; or dGTP BigDye Terminators version 3.0 (https://ucalgary.ca/dnalab/sequencing/3730x1).

SnapGene ® version 5.1.5 was used for aligning the primer sequences and the amplicons to the WGS of *H. somni KLM014* (Accession number NZ_CP042993) and *ICEHsKLM-014* (Accession number MN401320).
Table 2.2: PCR primers used in the detection of ICE associated core genes, accessory genes, circular intermediate, and species- specific primers

<table>
<thead>
<tr>
<th>PCR target</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Amplicon size</th>
<th>Annealing temperature</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ICE associated resistance genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet(H)</td>
<td>F: ATACTGCTGATCACCCTGCT</td>
<td>1076</td>
<td>60</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>R:TCCCAATAAGCGACGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mco</td>
<td>F: CAAGGCAGTGCTGGGACATA</td>
<td>458</td>
<td>58</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>R: GTTCCTTCGTTTCAACCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ICE associated Core genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tnpA</td>
<td>F: CCTGTTTTCAATGCAAAGTTTCCT</td>
<td>170</td>
<td>58</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>R: GCTCCCTTCAACACCACAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSDNA</td>
<td>F: GTGGTCAGTACCTACATAAGG</td>
<td>256</td>
<td>61</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>R: CAGAACGGGATATCGTCATCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relaxase</td>
<td>F: TTGGAATGGAGAACCCTCTTG</td>
<td>174</td>
<td>58</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>R: CCGGACATTGCACATCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrate</td>
<td>F: ACGGAATCATAGACCTGCCCAC</td>
<td>735</td>
<td>58</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>R: TCTGTTGAGTGTATGTCGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Species- specific primers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>F: GGCTGGAAGCCAAATCAAAG</td>
<td>1432</td>
<td>61</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>R: CGAGGGACATTACATCTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Circular intermediate structure of ICE, chromosomal junction, and chromosomal attachment sites (PCR, qPCR)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCIF</td>
<td>F: CGTCTGGAATTCCGGACAGA</td>
<td>267</td>
<td>58</td>
<td>This work</td>
</tr>
<tr>
<td>PCIR</td>
<td>R: TCAAATGGTGCCCGAGGTCGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: CGTAAAGCACACCATGCCAG</td>
<td>R: GAATGGAAAATCGGATTTGGTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-------------------------</td>
<td>-----------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCJF</td>
<td></td>
<td></td>
<td>104</td>
<td>63</td>
</tr>
<tr>
<td>PCJR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>F: CGTAAAGCACACCATGCCAG</th>
<th>R: TCAAATGGTGCCCTGAGGTCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCJF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCIR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>F: CGTCTGGAATTCGGACAGA</th>
<th>R: GAATGGAAAATCGGATTTGGTGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCIF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCJR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Primers used for the quantification of chromosomal copy numbers (qPCR)**

<table>
<thead>
<tr>
<th></th>
<th>F: CACATGCAAGTCAACGGTA</th>
<th>R: CCACAGTTTCCCCTGGCTAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>16s rRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.8 Effect of antibiotic induction on the transfer frequency of ICEHs02

2.8.1 Effect of tetracycline and ciprofloxacin on the conjugation rate

*H. somni* KLM014 was grown either with or without sub-inhibitory concentration (½ MIC) of tetracycline (Mediatech, Inc., Manassas, USA) or ciprofloxacin (Sigma-Aldrich, St Louis, USA) with shaking (225 RPM) at 37°C with 5% CO₂ to the mid-log phase. The recipient strains *H. somni* ATCC700025, *H. somni* KLM08 and *P. multocida* DSM16031 were grown without antimicrobials to the mid-log phase. Antimicrobials were removed by a washing step, and conjugation assay was done as previously described in section 2.2. The transfer frequency was calculated as the number of transconjugants per donor. The conjugation experiments were performed in three biological replicates with two technical replicates each.

2.8.2 Real-Time PCR (qPCR) assay

The excision rate of ICEHs02 was quantified by measuring the presence of circular intermediate forms and chromosomal junctions. The excision rate was investigated under normal conditions (control) and upon induction with tetracycline and ciprofloxacin. Copy numbers of circular intermediate, and chromosomal junctions were detected using qPCR following absolute quantification protocol to calculate the copy numbers/chromosome (excision rate) and relative standard curve method to calculate the fold change. The genomic DNA (gDNA) from *H. somni* KLM014 was extracted 22 hours post-inoculation in BHITT either with or without ½ MIC of tetracycline (8 μg/mL) and ciprofloxacin (2 μg/mL). The gDNA was extracted by the boiling method using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems™ Inc., ON, Canada). The amount of the DNA in each sample was measured by Qubit® 4 Fluorometer (ThermoFisher Scientific, CA) using the Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific,
CA) following the protocol indicated by the manufacturer. Briefly, The Qubit working solution was prepared by diluting the Qubit dsDNA reagent 1:200 using the Qubit® dsDNA working buffer. The standards were prepared by adding 10 μL of the standards to 190 μL of Qubit® working solution in the assigned tubes. The samples were prepared by adding 1-20 μL to the corresponding volume of Qubit® working solution (180-199 μL). The tubes were vortexed for 2-3 seconds and incubated at room temperature for 2 minutes and were read by Qubit® 3 Fluorometer. A detailed procedure for using the Qubit® 3 Fluorometer is described in appendix 13.

Primers used for qPCR were the same primers used to detect the circular form of ICEHs02. Primer pair PCIF, PCIR used to detect the circular intermediate form, and the primer pair PCJF, PCJR used for the detection of the junction formed after the excision of the ICE. The qPCR reactions were performed in the Bio-Rad CFX96™ Real-Time PCR Detection System (Bio-Rad, CA) using the SYBR™ Green PCR Master Mix (ThermoFisher Scientific, CA). Each reaction was carried out in 25 μL volumes containing 1x SYBR Green Master Mix, 0.2 μM forward and reverse primers and 0.4 ng of gDNA. The samples were loaded into 96-well white plates (Bio-Rad, CA). The cycling parameters were set as follows: initial activation step (95°C, 10 min) and 40 cycles of denaturation (95°C, 15 sec) and combined annealing/extension (60°C, 1 min). The PCR products were subjected to a melting curve analysis between 50 and 95°C to ensure the absence of non-specific amplifications like primer dimers and contamination with gDNA. The specific amplification was confirmed by the presence of a single peak in the melting curve. The quantification cycles and the baselines were automatically determined, and results were further analyzed using Microsoft Excel 365.

For the creation of standard curves, PCR products obtained from conventional PCR using the same primers were purified using the GenepHlow® Gel/PCR Kit (FroggaBio Inc, ON, CA) as
previously described in section 2.6.2. The gDNA was measured by Qubit® dsDNA BR Assay Kit, as described previously. A 10-fold dilution series of known templates concentration (copy numbers) was used to establish standard curves. The threshold cycle (Ct) values are plotted against the log of each concentration. The standard curves were used to calculate the Correlation Coefficient (R²), slope, and reaction efficiency. The reaction efficiency was determined by the following equation:

\[ \text{Efficiency} = 10^{\frac{-1}{\text{slope}}} - 1. \]

The amounts of excised circular intermediate and chromosomal junction were normalized to the amount of chromosomal DNA in each sample by amplifying the 16s rRNA gene, a chromosomal gene unaffected by the ICEHs02 excision. A standard curve for the 16s rRNA gene was built as mentioned previously. The curve was used to quantify the chromosome copies in each sample. The copy numbers of the circular intermediate and chromosomal junction per chromosome were calculated as the (CI or CJ / 16s rRNA). The qPCR reactions were performed in triplicates, and a non-template control (NTC) was included with each triplicate. Each experiment was performed using three different samples. The presence of five functional copies of the 16s rRNA in the chromosome was considered in the calculations.

2.8.3 Statistical analysis

GraphPad Prism version 8.4.2 was used for the statistical analysis for both experiments. Conjugation rates and copy numbers/chromosome were expressed as the means ± standard deviation (SD) from at least three independent biological replicates. T-test with Welch’s correction was used to compare the conjugation frequencies with and without antibiotics (tetracycline and ciprofloxacin) and was also used to compare the copy numbers of the circular intermediate with
and without antibiotics (tetracycline and ciprofloxacin). P-values ≤ 0.05 were considered statistically significant.

2.9 Growth curves, competition experiments, and serial passage experiments

2.9.1 Growth curves to evaluate the fitness cost of the ICEHs02 on the recipient strains

Growth curves of *H. somni* ATCC700025 and *P. multocida* DSM16031 transconjugants and their isogenic ancestors (recipients) were performed in triplicates. Growth curves were conducted as previously explained [190], with some modifications. Briefly, 200 μL of BHITT (*H. somni*) or BHI (*p. multocida*) broth was dispensed in a 96-well plate (Thermo Scientific, MA, USA); then, the broth was inoculated at an OD$_{600}$ of approximately 0.05. The plates were sealed with perforated adhesive seal, and were incubated in a CO$_2$ incubator with shaking (225 RPM) at 37°C. Bacterial growth was monitored continuously in a microplate-reader (Spectramax M2, Molecular Devices, USA) and reads were taken every 2 hours till the bacteria reach the stationary phase. Growth curves were obtained by plotting the OD values versus time. The exponential curves were displayed as a straight line by plotting the OD on a logarithmic scale (Figure 2.3). The exponential phase is used to determine the growth rate (μ) from the two data points, OD$_1$ and OD$_2$ that were chosen on the fitted line and their corresponding $t_1$ and $t_2$ (Figure 2.3) using the equation:

$$\mu = \frac{2.303 (\log OD_2 - \log OD_1)}{t_2 - t_1},$$

where OD$_1$ and OD$_2$ are the optical densities obtained from the best fitted line, and $t_1$ and $t_2$ are their corresponding time. The doubling time was calculated using the equation: $td = \frac{\ln 2}{\mu}$. The detailed protocol for growth curve and measuring the growth rate and doubling time is described in appendix 14.
Figure 2.3: A graph illustrating the mathematical points on the best fitted line used for calculating the growth rate of bacteria

The blue points are the measured points; the red points are the mathematical points calculated using the best fitted line equation. The asterisk refers to the best fitted line equation \( y = A e^{Bx} \). The growth rate was calculated using the equation: \( \mu = \frac{2.303 (\log OD_2 - \log OD_1)}{t_2 - t_1} \), where \( OD_1 \) and \( OD_2 \) are the optical densities obtained from the best fitted line, and \( t_1 \) and \( t_2 \) are their corresponding time. The doubling time was calculated using the equation: \( td = \frac{\ln 2}{\mu} \).

2.9.2 Competition experiments and measuring the relative fitness effect of ICEHs02

Pairwise competition experiments were used to evaluate the relative fitness effect of the newly acquired ICEHs02 in *H. somni ATCC700025* and *P. multocida DSM16031*. The competition experiments were done in triplicates with independent starting cultures with two technical replicates each. The competitions were conducted as previously described [185], [205], [206], with some modifications. Briefly, the transconjugants and their isogenic ancestors were cultured separately in 3 mL of BHI broth to an OD_{600} of \(~0.5\) for *H. somni ATCC700025* and \(~0.4\) for *P. multocida DSM16031*. The cultures were 10-fold diluted using 0.9% NaCl solution or broth. The competitors were then mixed 1:1 and transferred into 2.7 mL of prewarmed BHIT broth (*H. somni*) and BHI broth (*P. multocida*). The mixed cultures were then incubated for 24 hours at 37°C with shaking at 225 RPM. Initial \((N_0)\) and final \((N_{24})\) densities of the competing strains were
measured from CFU data by serial diluting and plating the cultures onto TSA blood agar with and without 8 μg/mL tetracycline. From these densities, the population growth of each competitor or the Malthusian parameter \((M)\) was calculated using the equation \(M = \ln \left( \frac{N_{24}}{N_0} \right)\). The relative fitness cost \((w)\) of each transconjugant was calculated as the ratio of the Malthusian parameter of the transconjugant to that of the recipient counterpart. By definition, a fitness of 1 indicates that the acquisition of the ICE has no fitness effect on its new host, while a ratio greater than or less than 1 indicates increased or decreased fitness, respectively. The detailed protocol of the competition experiment is described in appendix 15.

2.9.3 Serial passage experiments

Serial passage experiments were conducted using \(H. \text{somni ATCC700025}\) transconjugants as previously described [190] with few modifications. Three transconjugants were inoculated into 2mL of BHITT broth or BHITT broth supplemented with \(\frac{1}{2}\) MIC of tetracycline (0.125 μg/mL) in a sterile 12-well plate. The plate was incubated in a CO₂ incubator with shaking (225 RPM) at 37°C. The cultures were diluted 1:100 every ~ 3 days into fresh media having the same criteria for 45 days. Every 15 days, the CFU of each passage was counted simultaneously on CBA for the total count, and CBA supplemented with 8 μg/mL tetracycline (selective for \(ICEHs02\)). The presence of \(ICEHs02\) was confirmed by PCR. The experiment was done using three biological replicates and two technical replicates each.

2.9.4 Statistical analysis

Statistical analysis (means, standard deviations, one sample t-test, student t-test with Welch’s correction) was done using GraphPad Prism version 8.4.2. The relative fitness cost was expressed as the means ± standard deviation (SD) from three independent biological replicates with two technical replicates each. P-values ≤ 0.05 were considered statistically significant. The
Friedman Test, a non-parametric alternative to the repeated measures ANOVA (IBM SPSS, v25.0.0.1; IBM Corporation, Armonk, New York, USA) was used to analyze the association between log10 of the number of colony-forming units (CFU) enumerated from the four different treatment groups assessed for conjugation (no drug CBA, no drug TET, ½ MIC CBA, ½ MIC TET) over time. The significance level was set at p < 0.05. All pair-wise comparisons (6) within variables were performed using the Wilcoxon signed rank test with a Bonferroni correction to the significance level p < 0.05/6=0.008.
Chapter Three: Results

3.1 Conjugal transfer activity of ICEHs02

ICEHs02 was able to transfer from the donor strain *H. somni* KLM014 to the recipient strains *H. somni* ATCC700025, *H. somni* KLM08, and *P. multocida* DSM 16031 at transfer rates of $10^{-6}$ for *H. somni* recipients and a lower transfer frequency of $10^{-8}$ for *P. multocida* recipient. When *P. multocida* TG (*P. multocida* ICEHs02) was used as a donor, the transfer frequency to the NaI* H. somni* ATCC700025 was $10^{-6}$ while the transfer rate to the NaI* P. multocida* strain was $10^{-5}$. The transfer frequency rates of ICEHs02 are shown in Table 3.1. Conjugation assays between *H. somni* KLM014 and *M. haemolytica* h186, *H. influenzae* h211, h040, and *E. coli* K12 MG 15655 were not successful.

The transfer of ICE was confirmed by PCR and susceptibility testing. PCR confirmed the transfer of the ICE-associated core genes putatively encoding the relaxase, transposase, integrase, single-stranded DNA, as well as the resistance genes for tetracycline and multi-copper oxidase (Figure 3.1) to all the recipient strains. Susceptibility testing confirmed the activity of all the resistance genes after the transfer. Data of the MICs for the donor, recipients, and transconjugants are shown in Table 3.2.

The transconjugants of *H. somni* strains carrying ICEHs02 showed an increase in the MIC values of all the antimicrobials tested to reach the same MIC values of the donor. The MIC values for neomycin and gentamicin in *P. multocida* were two-fold higher than the MIC of these two antimicrobials in the donor strain. In addition, *H. somni* ATCC700025 transconjugants showed an increase in the MIC value of sulfadimethoxine (from 256 to 512 μg/mL). However, this antibiotic could not be investigated further due to the high MIC values in the other recipients. The MICs of
sulfadimethoxine in *H. somni* KLM014, *H. somni* KLM08, and *P. multocida* DSM16031 were 512 µg/mL, 512 µg/mL, and 1024 µg/mL respectively (Table A.1). The susceptibility testing for the *floR* gene in the *H. somni* KLM014 strain showed that the gene is not functional. According to the CLSI, the breakpoint for florfenicol in *H. somni* is ≥ 8 µg/mL. *H. somni* KLM014 was susceptible to florfenicol with MIC less than 0.25 µg/mL (Table A.1). This antimicrobial was not examined in the transconjugants.

The MIC values for copper were also elevated in all *H. somni* transconjugants to reach the same MIC level as the donor, indicating the activity of the multi-copper oxidase gene (*mco*) in the transconjugants. On the other hand, although the MIC of copper in *P. multocida* transconjugants increased to 2 mM in the transconjugant, but it did reach the same MIC as in the donor strain (Table 3.2).
Table 3.1: Conjugative transfer frequency of *ICEHs*02, and genotypic characteristics of donors (*H. somni* KLM014, *P. multocida* DSM16031<sup>ICEHs02</sup>), recipients (*Rif<sup>r</sup> *H. somni* KLM08, *Rif<sup>r</sup> *H. somni* ATCC700025, *Rif<sup>r</sup> *P. multocida* DSM16031, *Nal<sup>r</sup> *P. multocida* DSM16031, *Nal<sup>r</sup> *H. somni* ATCC700025) and transconjugant strains using PCR primers to detect four core genes (*traI*, *ssDNA*, *tnp*, and *int*) and two accessory genes (*tetH*, *mco*)

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Transfer frequency</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>tet(H)</td>
</tr>
<tr>
<td><em>H. somni</em> KLM014</td>
<td><em>Tet&lt;sup&gt;r&lt;/sup&gt;</em></td>
<td>Donor</td>
</tr>
<tr>
<td><em>H. somni</em> ATCC700025</td>
<td><em>Rif&lt;sup&gt;r&lt;/sup&gt;</em></td>
<td>Recipient</td>
</tr>
<tr>
<td><em>H. somni</em> KLM08</td>
<td><em>Rif&lt;sup&gt;r&lt;/sup&gt;</em></td>
<td>Recipient</td>
</tr>
<tr>
<td><em>P. multocida</em> DSM16031</td>
<td><em>Rif&lt;sup&gt;r&lt;/sup&gt;</em></td>
<td>Recipient</td>
</tr>
<tr>
<td><em>H. somni</em> KLM014 X</td>
<td>1.9 x 10&lt;sup&gt;6±&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>H. somni</em> ATCC700025</td>
<td>2.1 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>H. somni</em> KLM014 X</td>
<td>2.7 x 10&lt;sup&gt;6±&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>H. somni</em> KLM08</td>
<td>3.6 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>H. somni</em> KLM014 X</td>
<td>3.5 x 10&lt;sup&gt;8±&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>P. multocida</em> DSM16031</td>
<td>2.8 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>P. multocida</em> DSM16031&lt;sup&gt;ICEHs02&lt;/sup&gt;</td>
<td>Donor</td>
<td>+</td>
</tr>
<tr>
<td><em>P. multocida</em> DSM16031</td>
<td><em>Nal&lt;sup&gt;r&lt;/sup&gt;</em></td>
<td>Recipient</td>
</tr>
<tr>
<td><em>H. somni</em> ATCC700025</td>
<td><em>Nal&lt;sup&gt;r&lt;/sup&gt;</em></td>
<td>Recipient</td>
</tr>
<tr>
<td><em>P. multocida</em>&lt;sup&gt;ICEHs02&lt;/sup&gt; X</td>
<td>3.7 x 10&lt;sup&gt;5±&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>P. multocida</em> DSM16031</td>
<td>8.7 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>P. multocida</em>&lt;sup&gt;ICEHs02&lt;/sup&gt; X</td>
<td>1.8 x 10&lt;sup&gt;6±&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td><em>H. somni</em> ATCC700025</td>
<td>5.6 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

*Tet<sup>r</sup>*, tetracycline resistant; *Rif<sup>r</sup>*, rifampicin resistant; *Nal<sup>r</sup>*, nalidixic acid resistant; *tet(H)*, tetracycline; *mco*, multi-copper oxidase; *tral*, relaxase; *tnpA*, transposase; *int*, integrase; *ssDNA*, single-stranded DNA; *P. multocida* DSM16031<sup>ICEHs02</sup>, *P. multocida* DSM16031 transconjugant.
Table 3.2: Phenotypic characteristics of *ICEHs02*-carrying donor (*H. somni* KLM014), recipient strains (Rif\(^r\) *H. somni* KLM08, Rif\(^r\) *H. somni* ATCC70025, and Rif\(^r\) *P. multocida* DSM16031) and transconjugant strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Tetracycline MIC(µg/mL)</th>
<th>Streptomycin MIC(µg/mL)</th>
<th>Neomycin MIC(µg/mL)</th>
<th>Gentamycin MIC(µg/mL)</th>
<th>Copper MIC(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. somni</em> KLM014 tet(^r)</td>
<td>16</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td><em>H. somni</em> ATCC700025 Rif(^r)</td>
<td>0.25</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td><em>H. somni</em> KLM08 Rif(^r)</td>
<td>0.25</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td><em>P. multocida</em> DSM16031 Rif(^r)</td>
<td>0.5</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>H. somni</em> ATCC700025 <em>ICEHs02</em></td>
<td>16</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td><em>H. somni</em> KLM08 <em>ICEHs02</em></td>
<td>16</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td><em>P. multocida</em> DSM16031 <em>ICEHs02</em></td>
<td>16</td>
<td>64</td>
<td>64*</td>
<td>32*</td>
<td>2*</td>
</tr>
<tr>
<td><em>P. multocida</em> <em>ICEHs02</em> X</td>
<td>16</td>
<td>64</td>
<td>64</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td><em>P. multocida</em> DSM 16031</td>
<td>16</td>
<td>64</td>
<td>64</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td><em>H. somni</em> ATCC700025</td>
<td>16</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>4</td>
</tr>
</tbody>
</table>

MIC, minimum inhibitory concentration; tet\(^r\), tetracycline resistant; Rif\(^r\), rifampicin resistant; nal\(^r\), nalidixic acid resistant; *H. somni* ATCC700025 *ICEHs02*, *H. somni* ATCC700025 transconjugant; *H. somni* KLM08 *ICEHs02*, *H. somni* KLM08 transconjugant; *P. multocida* DSM16031 *ICEHs02*, *P. multocida* DSM16031 transconjugant. The asterisks indicate 2-fold change in the MIC of neomycin, gentamycin, and copper for *P. multocida* DSM16031 TGs. MIC of neomycin and gentamicin increased by 2-fold while copper MIC decreased by 2-fold.
Figure 3.1: Agarose gel electrophoresis visualizing products of PCR assays to confirm the transfer of *ICEHs02* from the donor strain *H. somni* KLM014 to the recipient strain *H. somni* ATCC700025

The first lane shows the DNA ladder (100bp, or 1Kb). Lane A: donor (*H. somni* KLM014), lane B: recipient (*H. somni* ATCC700025), lanes C&D: transconjugant 1 and transconjugant 2, 1. PCR products (174 bp) using primer pairs targeting *traI* (relaxase) gene, 2. PCR products (256 bp) using primer pairs targeting *SSD* (single-stranded DNA) gene, 3. PCR products (170 bp) using primer pairs targeting *tnpA* (transposase) gene, 4. PCR products (458 bp) using primer pairs targeting *mco* (multicopper oxidase) gene, 5. PCR products (735 bp) using primer pairs targeting integrase gene, 6. PCR products (1024 bp) using primer pairs targeting *tetH* (tetracycline resistance) gene.
3.2 Detection of the circular intermediate form of ICEHs02

Four primers were designed to investigate the excision and integration of the extrachromosomal circular intermediate form of ICEHs02 in *H. somni KLM014*. The primer pair PCIF and PCIR are located near the ends of the element, and anneal to sequences inside ICEHs02 and amplify the circular intermediate generated upon excision (Figure 3.2A). The primer pair PCJF and PCJR anneal to sequences surrounding ICEHs02 and amplify the repaired chromosomal junction formed upon excision (Figure 3.2A). The primer pairs PCJF, PCIR, and PCIF, PCJR were used to confirm the ICE's integration in the chromosome. The expected sizes of the PCR products of the circular intermediate, the chromosomal junction, and chromosomal attachment sites were 267 bp, 104 bp, 198 bp, and 173 bp, respectively. All four PCR products were obtained, confirming the existence of the integrated and the excised form of the element (Figure 3.2B). As expected, the circular form of ICEHs02 was also detected in *H. somni KLM08, H. somni ATCC700025, P. multocida DSM16031* transconjugants, while it was absent in their isogenic ancestors (Figure 3.3). Sequencing of the obtained PCR products confirmed the identity of all amplicons (Appendix 15).
Figure 3.2 A: Diagram illustrating the excision (formation of circular intermediate) and integration of ICEHs02 in *H. somni KLM014* chromosome

ICEHs02 is shown as a red line or circle; chromosomal DNA is shown as blue lines; attachment sites are shown as black boxes; the primers' location is shown as red and blue arrows. Primers PCJF and PCJR (shown in red arrows) anneal to sequences surrounding ICEHs02 and amplify the repaired chromosomal junction formed upon excision. Primers PCIF and PCIR (shown in blue arrows) anneal to sequences inside ICEHs02 and amplify the circular intermediate generated upon excision. Letters B, C, D, E represent the products of PCR shown in Figure B.

Figure 3.2 B: Agarose gel electrophoresis visualizing PCR products of the circular intermediate, chromosomal junction, and chromosomal attachment sites.

Lane A, 100 pb DNA ladder; Lane B, confirmation of circular intermediate (CI) using primer pair PCIF, PCIR (product size:267); Lane C, confirmation of chromosomal junction (CJ) using primer pair PCJF, PCIR (product size:104); Lane D, E, confirmation of ICE attachment sites on the chromosome using primer pair PCJF, PCIR (product size:198), and primer pairs PCIF, PCJR (product size:173).
Figure 3.3: Agarose gel electrophoresis visualizing the circular intermediate form of ICEHs02 in the donor strain *H. somni* KLM014 and the transconjugant strains *H. somni* KLM08, *H. somni* ATCC700025, and *P. multocida* DSM16031 using the primer pair (PCIF, PCIR)

Agarose gel electrophoresis showing: Lane A, 100 bp DNA ladder; Lane B, *H. somni* KLM014 (D); Lane C, *H. somni* KLM08 (R); Lane D, *H. somni* ATCC700025 (R); Lane E, *P. multocida* DSM16031 (R); Lane F, *H. somni* KLM014ICEHs02 (TG); Lane G, *H. somni* ATCC700025ICEHs02 (TG); Lane H, *P. multocida* DSM16031ICEHs02 (TG). The product size is 267 bp.
3.3 *In silico* prediction of *ICEHs02* host range

Pair-wise alignments of the tRNA-Leu$^{CAA}$ gene from *H. somni KLM014* to other bacteria were used to predict the potential host range of *ICEHs02*. Following retrieval of 386 alignments, the results were filtered to match records with percent identity between 80 to 100% and query coverage of 100%. The resulted alignments were filtered for the ones having *ICEHs02*-associated direct repeats (DRs) with a 100% identity.

One taxonomic class appeared in the BLAST search, which is the *Gammaproteobacteria*. As expected, the *Pasteurellaceae* family were the most abundant group noticed in the alignments. The results showed that *ICEHs02* would be likely to transfer between the three BRD pathogens (*H. somni, P. multocida, M. haemolytica*) more frequently than other *Pasteurellaceae* members (100% Blast identity). Other sheep, and pig-related pathogens, also represented and included *Glaesserella parasuis*, *Bibersteinia trehalose*, which also showed BLAST similarity ranged between 90-100% and a 100% identical DRs. The BLAST alignments also showed that *ICEHs02* would most likely transfer into the human pathogens *Haemophilus parainfluenzae* and *Haemophilus ducreyi*. The BLAST similarity for these species ranged between 87-93%, and the DRs were 100% identical. Alignments of the tRNA-Leu$^{CAA}$ gene of *H. somni KLM014* with the bacteria mentioned above are shown in appendix 17.

3.4 The effect of sub-inhibitory concentration of antimicrobials on conjugal transfer frequency of *ICEHs02*

The transfer frequency of *ICEHs02* was increased by antibiotic exposure in all the conjugation experiments conducted (Table 3.3). Pre-growth of donor strain with $\frac{1}{2}$ MIC of tetracycline significantly increased *ICEHs02* transfer from *H. somni KLM014* donor to the recipients *H. somni ATCC700025*, *H. somni KLM08*, and *P. multocida DSM16031* by 20.8-folds,
14.3-folds, and 19.1-folds respectively compared to conjugation experiments in which the donor was grown without tetracycline before the conjugation (P-value < 0.05).

Although the exposure to ½ MIC of ciprofloxacin increased the transfer frequency of \textit{ICEHs02} in all the conjugation experiments, the fold change was less than the change upon exposure to tetracycline. The transfer frequency increased by ~ 8 -folds in the conjugation between treated \textit{H. somni KLM014} and \textit{H. somni ATCC700025} compared to the untreated group while the conjugation rate increased 5-folds when \textit{P. multocida DSM16031} was used as a recipient. A comparison of conjugation frequencies of the control and treatment groups analyzed by the t-test showed a statistically significant difference between control and treatment groups (P-value ≤ 0.05). The exposure to ½ MIC of CIP did not significantly change the transfer rate in the conjugation between treated \textit{H. somni KLM014} and \textit{H. somni KLM08} compared to the untreated group (P-value > 0.05).
Table 3.3: Conjugal transfer frequency and fold change in the transfer frequency upon tetracycline and ciprofloxacin induction of the donor *H. somni* KLM014. *H. somni* ATCC700025, *H. somni* KLM08, *P. multocida* DSM16031 were used as recipient strains

<table>
<thead>
<tr>
<th>Donor X Recipient</th>
<th>Conjugation transfer frequency(^1) (Control)</th>
<th>Conjugation transfer Frequency(^1) (Tetracycline induction)</th>
<th>Fold Change(^2)</th>
<th>Conjugation transfer frequency(^1) (Control)</th>
<th>Conjugation transfer Frequency(^1) (Ciprofloxacin induction)</th>
<th>Fold Change(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. somni</em> KLM014 X</td>
<td>2.5 x 10(^6) ± 1.5 x 10(^7)</td>
<td>5.2 x 10(^5) ± 3.3 x 10(^6)</td>
<td>20.8*</td>
<td>2.6 x 10(^6) ± 2.1 x 10(^7)</td>
<td>2.1 x 10(^5) ± 6.8 x 10(^6)</td>
<td>8.1*</td>
</tr>
<tr>
<td><em>H. somni</em> ATCC700025</td>
<td>3.2 x 10(^6) ± 2.4 x 10(^7)</td>
<td>4.6 x 10(^5) ± 5.03 x 10(^6)</td>
<td>14.3*</td>
<td>3.1 x 10(^6) ± 1.05 x 10(^6)</td>
<td>2.4 x 10(^5) ± 9.2 x 10(^6)</td>
<td>7.7</td>
</tr>
<tr>
<td><em>H. somni</em> KLM08</td>
<td>3.7 x 10(^8) ± 4.97 x 10(^9)</td>
<td>7.1 x 10(^7) ± 1.15 x 10(^7)</td>
<td>19.1*</td>
<td>3.6 x 10(^8) ± 6.11 x 10(^9)</td>
<td>1.8 x 10(^7) ± 5.57 x 10(^8)</td>
<td>5*</td>
</tr>
<tr>
<td><em>P. multocida</em> DSM16031</td>
<td>The results shown are from three biological replicates with two technical replicates each, the data shown represent the mean ± standard deviation. The asterisks indicate statistical significance between antibiotic treated group and control group at level: p-value ≤ 0.05.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5 The effect of sub-inhibitory concentration of antimicrobials on the copy numbers of circular intermediate form of ICEHs02

The previous experiment showed an increase of the conjugation rates upon exposure to tetracycline and ciprofloxacin. The excision and the circular intermediate formation are the first steps of ICE transfer from the host strain to the recipients. To further investigate the antimicrobials' effect on ICEHs02, qPCR assays were done to quantify the circular intermediate (CI) and the chromosomal junction (CJ) copy numbers per chromosome. The quantification was done from cells harvested in exponential growth phase treated or not with ciprofloxacin or tetracycline at half of the minimal inhibitory concentration (½ MIC). The copy numbers per chromosome were calculated as the copy numbers of the circular intermediate or the chromosomal junction divided by the copy numbers of the 16s rRNA. The presence of five functional copies of the 16s rRNA in the chromosome was considered in the calculations.

Upon induction with ciprofloxacin, the circular intermediate forms' copy numbers increased significantly by ~4 folds to reach 1.87 x 10⁻⁴ ± (4.6 x 10⁻⁵) copies per chromosome (p-value ≤ 0.05). The same observation was obtained after the induction with tetracycline, where the increase in the copy numbers per chromosome was even higher, reaching ~37 fold more than the number of the circular forms without induction (p-value ≤ 0.05) (Figure 3.4).

Comparable results were obtained for the chromosomal junction copy numbers per chromosome. The chromosomal junction copy numbers per chromosome were significantly increased to reach 2.02 x 10⁻³ ± (4.19 x 10⁻⁴) upon tetracycline treatment (p-value ≤ 0.05) and 2.16 x 10⁻⁴ ± (2.33 x 10⁻⁵) upon ciprofloxacin treatment (p-value ≤ 0.05) when compared to the control with no treatment (Table 3.4). It was noticed from the experiment, after induction with both ciprofloxacin and tetracycline, that the copy numbers of the chromosomal junction/ chromosome
were ~ one-fold higher than the circular intermediate copy numbers/chromosome; however, there was no statistically significant difference between them. The qPCR reaction's efficiencies of the standard curves for circular intermediate form, chromosomal junction, and the 16s rRNA were 90%, 104%, and 93%, respectively. The R² was 0.99 for all the standard curves.

Table 3.4: Quantification of ICEHs02 circular form and chromosomal junction with tetracycline and ciprofloxacin induction and without induction using real-time PCR

<table>
<thead>
<tr>
<th></th>
<th>Copy number/ chromosome (Control)</th>
<th>Copy number/ chromosome (^1) (tet)</th>
<th>Copy number/ chromosome (^2) (cip)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circular intermediate</td>
<td>4.27 x 10⁻⁵ ± 1.56 x 10⁻⁵</td>
<td>1.59 x 10⁻³ ± 3 x 10⁻⁴</td>
<td>1.87 x 10⁻⁴ ± 4.6 x 10⁻⁵</td>
</tr>
<tr>
<td>Chromosomal junction</td>
<td>2.81 x 10⁻⁵ ± 2.76 x 10⁻⁶</td>
<td>2.02 x 10⁻³ ± 4.19 x 10⁻⁴</td>
<td>2.16 x 10⁻⁴ ± 2.33 x 10⁻⁵</td>
</tr>
</tbody>
</table>

\(^1\)tet, tetracycline; \(^2\)cip, ciprofloxacin; The results shown are from three biological replicates with three technical replicates each, the data shown represent the mean ± standard deviation. qPCR amplifications were performed on total DNA extracted from cells grown in BHITT medium, with or without treatment (ciprofloxacin and tetracycline). Cells were harvested at the mid-log phase. The circular form of ICEHs02 was detected using the primer pair (PCIF, PCIR) and the chromosomal junction was detected using the primer pair (PCJF, PCJR). Copy numbers per chromosome of the circular intermediate and chromosomal junction was calculated as (CI/16s rRNA), and (CJ/16srRNA) respectively.
Figure 3.4: Bar graph illustrating a comparison of the copy numbers of *ICEHs02* circular intermediate upon tetracycline and ciprofloxacin induction (treatment groups), and without induction (control group).

The effect of antimicrobial treatment on the copy numbers of the circular intermediate was measured by qPCR. qPCR amplifications were performed on total DNA extracted from cells grown in BHITT medium, with or without induction (ciprofloxacin and tetracycline). Cells were harvested at the mid-log phase. The circular form of *ICEHs02* was detected using the primer pair (PCIF, PCIR). The copy number is calculated as (CI/16srRNA). A, represents a pair-wise comparison of the circular intermediate copy numbers between the control (no induction with ciprofloxacin) and the test (induced with ciprofloxacin); B, represents a pair-wise comparison of the circular intermediate copy numbers between the control (no induction with tetracycline) and the test (induced with tetracycline). The exposure to tetracycline/ciprofloxacin significantly increased the formation of the circular intermediate form. The asterisks indicate statistical significance between the antibiotic-induced and control groups at the level: p-value ≤ 0.05. The results shown are from three biological replicates. Error bars represent the standard deviation.
3.6 The effect of ICEHs02 acquisition on the new hosts

OD-based growth curves were conducted to compare the growth parameters of *H. somni* ATCC700025 TG, *P. multocida* DSM16031 TG and their isogenic ancestors. Growth curves were performed to assess the effect of ICE acquisition on the growth rates and doubling time. The *H. somni* ATCC700025 transconjugant exhibited both extended lag phase and significant decrease in growth rate of ~ 21% compared to its isogenic ancestor (Figure 3.2). *H. somni* ATCC700025 TG showed a lower growth rate of 0.164 with a doubling time of 4.2 hours compared to its isogenic parent which showed a growth rate of 0.208 with a doubling time of 3.3 hours. On the other hand, *P. multocida* DSM16031 TG showed a non-significant decrease in the growth rate of ~ 6%, and about 6 minutes difference in doubling time (Figure 3.3) compared to *P. multocida* DSM16031 minus ICEHs02.

Head-to-head competition experiments were conducted to further investigate the fitness cost of ICEHs02 on its new host. *H. somni* ATCC700025 TG and *P. multocida* DSM16031 TG were competed against their isogenic parents. The acquisition of ICEHs02 reduced the relative fitness of *H. somni* ATCC700025 TG by 28 % (w = 0.72 ± 0.04, P ≤ 0.01) while, in *P. multocida* DSM16031 TG, the relative fitness was decreased by 15 % (w = 0.85 ± 0.0, P ≤ 0.01) (Table 3.4). The biological cost of ICEHs02 carriage in *H. somni* ATCC700025 TG and *P. multocida* DSM16031 TG was significantly different from 1 (P ≤ 0.01).
Figure 3.5 A: Comparison of the growth curves of *H. somni* ATCC700025 transconjugant (TG) and *H. somni* ATCC700025 isogenic ancestor

*H. somni* ATCC700025 TG shown as a black line and its isogenic ancestor *H. somni* ATCC700025 rif', shown as a red line. Optical density at 600 nm (OD\textsubscript{600}) of the cultures was measured every two hours up to 32 hours. The data shown represent the mean of three biological replicates. Error bars represent the standard deviation.

Figure 3.5 B & C: Exponential growth curves used for the calculation of the growth rate of *H. somni* ATCC700025 TG and *H. somni* ATCC700025 isogenic ancestor

X-axis represents the time in hours while Y-axis represents the OD in log scale. The black dotted line represents the best fitted line used for the calculation of the growth rate. The growth rate was calculated by equation: \( \mu = \frac{2.303 (\log \text{OD}_2 - \log \text{OD}_1)}{t_2 - t_1} \), where OD\textsubscript{1} and OD\textsubscript{2} are the optical densities obtained from the best fitted line, and \( t_1 \) and \( t_2 \) are their corresponding time. The doubling time was calculated using the equation: \( td = \frac{\ln 2}{\mu} \).
Figure 3.6 A: Comparison of the growth curves of *P. multocida DSM16031* transconjugant (TG) and *P. multocida DSM 16031* isogenic ancestor

*P. multocida DSM16031* TG shown as a black line and its isogenic ancestor *P. multocida DSM16031 rif*\(^r\), shown as a red line. Optical density at 600 nm (OD\(_{600}\)) of the cultures was measured every two hours up to 16 hours. The Data shown represent the mean of three biological replicates. Error bars represent the standard deviation.

Figure 3.6 B & C: Exponential growth curves used for the calculation of the growth rate of *P. multocida DSM16031 TG* and *P. multocida DSM16031* isogenic ancestor

X-axis represents the time in hours while Y-axis represents the OD in log scale. The black dotted line represents the best fitted line used for the calculation of the growth rate. The growth rate was calculated by equation: 

\[
\mu = \frac{2.303 (\log OD2 - \log OD1)}{t2 - t1},
\]

where OD\(_1\) and OD\(_2\) are the optical densities obtained from the best fitted line, and \(t1\) and \(t2\) are their corresponding time. The doubling time was calculated using the equation: 

\[
td = \frac{\ln 2}{\mu}.
\]
Table 3.4: Relative fitness effects of *ICEH02* on *H. somni ATCC700025* TG and *P. multocida DSM16031* TG

<table>
<thead>
<tr>
<th>Competing strains</th>
<th>Relative fitness&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. somni ATCC700025</em>&lt;sup&gt;ICEH02&lt;/sup&gt;/ <em>H. somni ATCC700025</em></td>
<td>0.72 ± 0.04</td>
</tr>
<tr>
<td><em>P. multocida DSM16031</em>&lt;sup&gt;ICEH02&lt;/sup&gt;/ <em>P. multocida DSM16031</em></td>
<td>0.85 ± 0.01</td>
</tr>
</tbody>
</table>

The relative fitness (*w*) was calculated as the M (transconjugants)/M (Recipient), where M is the Malthusian parameter of the competitors. The Malthusian parameter for each competitor is calculated by the equation $M = \ln \left( \frac{N_2}{N_0} \right)$. A relative fitness below 1 indicates a fitness burden associated with ICE carriage. <sup>1</sup>The results shown are from three biological replicates with two technical replicates each, the data shown represent the mean ± standard deviation. The biological cost of *ICEHs02* carriage is significantly different from 1 ($P \leq 0.01$).
Co-culture competition experiment were conducted between *H. somni* ATCC700025 TG and *P. multocida* DSM16031 TG (recipients with ICEHs02) and their isogenic parents (recipients without ICE). The relative fitness ($w$) was calculated as the $M$ (transconjugants)/$M$ (Recipient), where $M$ is the Malthusian parameter of the competitors. The Malthusian parameter for each competitor was calculated by the equation $M = \ln \left( \frac{N_t}{N_0} \right)$. A relative fitness below 1 indicates a fitness burden associated with ICE carriage. The results shown are from three biological replicates with two technical replicates each. Error bars represent the standard deviation.

Figure 3.7: Bar graph illustrating a comparison of the relative fitness cost of the newly acquired ICEHs02 on the recipient strains *H. somni* ATCC700025 and *P. multocida* DSM16031
3.7 Stability of *ICEHs02* in its host in non-selective environments

Serial passage experiments were conducted to assess if the *ICEHs02* would be lost from its host in a non-selective environment. The bacterial cultures were repeatedly passaged for 45 days in a non-selective medium and medium having a low concentration of tetracycline. The CFU/mL were enumerated every 15 days on CBA to assess the total count, and CBA supplemented with tetracycline at a concentration selective for *ICEHs02*. The experiment showed no statistically significant difference in total bacterial counts and resistant bacterial counts (ICE-carriers) in selective and non-selective environments. There was no statistically significant difference in the Log$_{10}$ of CFU enumerated between groups $\chi^2$= 3.900, df=3, p=0.324. There was a statistically significant difference in the Log$_{10}$ of CFU enumerated between the different time periods, $\chi^2$= 12.000, df=3, p<0.001. The pair-wise comparisons did not identify any pairs that were statistically different $w$ = -1.826, p = 0.68). The assay pointed out that repeated passage of *ICEHs02*-carrying transconjugants did not result in any considerable loss of *ICEHs02* over repeated generations in non-selective environments (Figure 3.8).
Figure 3.8: Long-term serial passage experiment to detect the change of *ICEHs02* carriage in *H. somni ATCC700025* transconjugants (TGs) grown on BHITT with and without tetracycline selection

The bacteria were enumerated every 15 days on CBA for total count and CBA with tetracycline (8 μg/mL) for *ICEHs02*-carrying TGs. The results shown are from three biological replicates. Error bars represent the standard deviation.
Chapter Four: Discussion

4.1 *ICEHs02* is a dynamic mobile genetic element

Mobile genetic elements play a significant role in the evolutionary changes in bacteria [149], [207]. Bacteria usually live in polymicrobial communities, and their genomes not only develop due to mutation and natural selection but also as an outcome of horizontal gene transfer between different individuals in the community [149], [207]. ICEs are a distinct group of mobile genetic elements known to contribute to the evolution and diversification of the genomic pool of bacterial communities. ICEs are able to confer many beneficial traits, such as antimicrobial resistance and metal tolerance between bacteria [21][149]. Many studies showed that antimicrobial resistance is increasing over time among BRD pathogens [21], [25], [115] and a reason for that might be the exchange of genes between bacteria. Many ICEs that carry multidrug resistance have been recently identified in some members of the *Pasteurellaceae* family that contribute to the BRD disease in cattle [25], [120], [156], [157].

This study aimed to investigate the host range of a new ICE (*ICEHs02*) identified in a *H. somni* strain isolated from feedlot cattle in Alberta. ICEs are usually found integrated into the host chromosome, and upon excision, they form a circular intermediate, which is the first step of the transfer of ICEs via conjugation [134]. This study showed that *ICEHs02* is a functional integrative and conjugative element that can actively excise from the chromosome, form a circular intermediate, and transfer into other bacteria. The chromosome is repaired after the *ICEHs02* excision, forming an unoccupied chromosomal junction. ICEs integrate and excise from the chromosome using ICE-encoded integrases (recombinases) [134]. Many ICEs usually rely on tyrosine recombinases, however, some ICEs utilize serine or DDE recombinases [131], [133], [134], [142], [150], [151]. *ICEHs02* integrates into a specific attachment site on the chromosome.
which is tRNA-Leu$^{CAA}$ [193] and uses tyrosine recombinase for site-specific recombination [193]. Tyrosine recombinases mediate site-specific recombination between dsDNA molecules (ICE and chromosome) at similar short sequences. When these sequences are on a circular ICE and the chromosome, recombination results in the ICE's integration into the chromosome resulting in two att sites flanking the ICE from the right (attR) and the left (attL). When recombination is between two att sites flanking the ICE (attL, attR), the result is excision of the ICE and forming the unoccupied chromosomal junction (attB site)[134].

Based on previous studies, ICEs can vary in their host range. Some ICEs have a wide host range while others have a limited host range. The host range of ICEHs02 has not been explored before this study. ICEHs02 was able to transfer into H. somni and P. multocida; however, the conjugal transfer of ICEHs02 into M. haemolytica, H. influenzae, and E. coli was not successful. Similarly, ICEHs1 identified by Krishna et al. (2016) in H. somni AVII was shown to conjugatively transfer to H. somni and P. multocida, but conjugal transfer to E. coli was not successful [25]. On the other hand, ICEPmu1 identified in P. multocida 36950 strain may have a broader host range than ICEHs02 as it was transferred to P. multocida, M. haemolytica, and E. coli [121], while the M. haemolytica 42548 strain ICEMh1 was shown to transfer to P. multocida [120]. Interestingly, ICEPmu1 could not transfer directly from H. somni, or M. haemolytica into E. coli, showing that P. multocida might be a potential intermediate for ICE transfer among the three bacterial species [21]. In this study, we were able to transfer ICEHs02 from P. multocida transconjugant back into P. multocida strain and H. somni strain; however, the transfer of ICEHs02 from P. multocida transconjugants into E. coli was not successful, indicating that many factors might contribute to the ability of the ICE to transfer to other bacteria. These factors will be discussed in the following section.
ICEs from different bacterial species have shown to integrate into various tRNAs [131], [134], [148], [208]. The tRNAs are considered a perfect site for the integration of ICEs. A reason for that might be that the tRNA molecules are the most conserved RNA molecule found in nature as they can be found in all living organisms and are involved in the translation machinery. In addition, organisms encode multiple tRNAs for particular amino acids. This phenomenon is called redundancy or degeneracy [190], [209], and it is important to the genetic code as it minimizes the lethal effect of the interruption of the tRNA. This factor might facilitate disseminating the ICEs without exerting a damaging effect on its host [190]. As mentioned earlier, ICEHs02 integrates into a specific target site on the chromosome (tRNA-Leu gene). Owing to the highly conserved nature of tRNA genes, they were used to predict the potential host range of ICEHs02. In this study, the in-silico investigation of the host range of ICEHs02 revealed that the ICE would transfer more frequently into H. somni, P. multocida and M. haemolytica. Additionally, the alignments showed that ICEHs02 would transfer into Glaesserella parasuis, and Bibersteinia trehalose, and the human pathogens H. parainfluenzae and H. ducreyi. A recent study by Cameron et al. (2019) investigated the potential host range of ICEMh1-like elements from M. haemolytica based on the specific integration site tRNA-Leu [165]. The latter study indicated that ICEMh1 could transfer with high frequency to the BRD pathogens H. somni, P. multocida, M. haemolytica, and to other bacterial pathogens including Haemophilus spp, E. coli, Salmonella, Burkholderia spp., Bordetella spp, Campylobacter, and Clostridia [165].

Although the host range investigation using bioinformatic methods is more convenient and less laborious, the method cannot be fully trusted to predict the potential host range of ICEs. In our study, in vitro conjugation of ICEHs02 was not successful with M. haemolytica. However, it was noticed from the tRNA-Leu genes' alignments that the probability of ICE's transfer to this
strain is high according to the perfect identity of the tRNA-leu and DRs. This result could indicate that although tRNA-Leu might be an essential requirement for a successful conjugation, many other factors might also significantly affect conjugation events and conjugation rates. These factors include the presence of other mobile genetic elements that might be incompatible with the ICE, the insertion region could be occupied by another ICE, the incompatibility of the secretion system of the ICE with the cell envelope of the recipient, or the presence of some cytoplasmic factors like restriction modification or CRISPR [134].

Historically, the identification of an ICE typically resulted from an interest in resistance to antibiotics, metals and their dissemination among organisms [134]. Since the discovery of ICEs, they were tied to the selective advantages and phenotypes they confer to their hosts [134]. Essentially, specific phenotypes, and the selective advantages they confer to their hosts, are usually the driving factor in identifying and defining ICEs. Defining the phenotypes conferred by the ICEs will help understand their role in disseminating AMR in the environment.

In this study, we characterized the phenotypes conferred by ICEHs02. H. somni KLM014 harbouring ICEHs02 showed resistance to tetracycline (16 μg/mL) and neomycin (32 μg/mL). Moreover, high MICs to streptomycin (64 μg/mL), gentamicin (16 μg/mL), and sulfadimethoxine (512 μg/mL) were detected, but surprisingly, although the floR gene was present on the ICE, the bacteria were susceptible to the florfenicol (MIC ≤ 0.25 μg/mL). A recent study by Mohammad et al. (2020) investigated the inactivity of the floR gene in ICEHs02 indicated that the floR gene inactivity might be due to a deletion or frameshift, which resulted in a different protein [193]. Pairwise alignment of the ICEHs02-floR gene with reference floR gene showed various gaps and misplaced amino acids indicating that the gene might not encode for a functional protein [193]. A similar observation was discussed before in ICEPmu1, where a complete blaoxa-2 gene was
identified, which seemed to be functionally inactive in *P. multocida*. However, the study has not investigated the reason for the gene inactivity [121], [157].

In this study, only tetracycline and florfenicol susceptibility results were interpreted because not all the antimicrobial compounds have validated interpretive breakpoints for *H. somni* in CLSI. There was no established breakpoint for neomycin, but the breakpoint established in a previous study was used to interpret the data [114].

As expected, *ICEHs02* was able to confer resistance phenotypes of tetracycline, streptomycin, neomycin, gentamicin, sulfadimethoxine, and copper to the new hosts (transconjugants). Similar results were previously obtained in the BRD bacteria *P. multocida, M. haemolytica*, and *H. somni*. It was noticed that *ICEPmu1* conferred eleven out of twelve antimicrobial resistances identified in the donor [121]. In addition, *ICEMh1* was also able to confer resistance to neomycin, streptomycin, and tetracycline [120], and *ICEHs1* conferred tetracycline resistance, copper and zinc tolerance [25]. In this study, the MIC for neomycin and gentamicin in *P. multocida* transconjugants carrying-*ICEHs02* was higher than the MIC of these two antimicrobials in the donor strain, indicating the better activity of *APH (3’)-Ia* gene in *P. multocida*. This is similar to the previous finding in *ICEPmu1* that the MIC for chloramphenicol, florfenicol, and ampicillin in *E. coli* transconjugants was higher than the MIC of these antimicrobials in *P. multocida* (donor), indicating the better activity of *floR* and *blaOXA-2* genes in *E. coli* [121], [157]. The study indicated that both genes are not indigenous *Pasteurellaceae* genes but have been detected in many *Enterobacteriaceae* members, and that might explain the higher activity in *E. coli* [121]. The variable activity of resistance genes in different strains might be due to "codon usage bias," which refers to the difference in synonymous codons’ occurrence frequency. Synonymous codons are various codons that encode the same amino acid [210]. Some codons are
regularly used, while others are unusually used in a particular organism [210]. Horizontally gained genes that employ codons that better match their new host genome's codon usage will be better expressed within that genome's setting and will confer less of a fitness cost on their new host [210]. In addition, the upregulation of gene expression might also result from mutations in resistance genes [211]. A single mutation can cause multiple phenotypic variations that concurrently change the resistance and susceptibility to multiple antimicrobials [211].

ICEHs02 is the first well-defined ICE in *H. somni* that carries multi-drug resistance and metal tolerance genes. All the previously identified ICEs in the BRD pathogens (ICEPmul identified in *P. multocida*, and ICEMh1 identified in *M. haemolytica*) were shown to harbour multiple drug resistance genes [120], [121]. Also, ICEHs1, identified in *H. somni*, was shown to carry tetracycline resistance gene(*tetH*), a multi-drug efflux pump (*ebrB*) and two metal tolerance genes (copper and zinc) [25]. The presence of ICEs that harbour multi-drug resistance genes might contribute to increasing the AMR. Recent studies showed increasing resistance to multiple antimicrobial classes in the BRD pathogens [21], [117]. In *H. somni*, the resistance to oxytetracycline, neomycin, penicillin, and tylosin was shown to increase over time, as reported in a study done by Krishna et al. (2016) [25]. A ten-year study in American and Canadian feedlots showed that *M. haemolytica*, *P. multocida*, and *H. somni* exhibit resistance to some important antimicrobials used to treat BRD such as enrofloxacin, tilmicosin, and tulathromycin [115]. Additionally, a recent study by E. Timsit et al. (2017) showed that *P. multocida* and *M. haemolytica* exhibit increased resistance to tulathromycin and oxytetracycline, while *H. somni* showed a high frequency of resistance to oxytetracycline and penicillin [116]. There might be a possibility that the escalating resistance to multiple antimicrobial classes is due to the presence of multi-resistant ICEs in the BRD pathogens. The three BRD pathogens *H. somni*, *P. multocida*, and
*M. haemolytica* are known to live together in biofilms [22], [212]. It was discussed earlier that biofilms are hotspots for interactions in microbial communities and thus might be associated with increasing HGT between bacteria [142], [216]. Formation of biofilms among the BRD bacteria might allow the transfer of ICEs carrying different gene cassettes and consequently increase AMR.

The presence and dissemination of ICEs carrying multi-resistance among the BRD pathogens might limit therapeutic choices, creating the need to use multiple or more potent drugs. Increasing resistance might increase illness and death, raise the cost of and extend the duration of treatments. In addition, increasing the length of treatment might lead to the development of more resistant bacteria. The advancement in developing new antibiotics is limited, so there is a need to maintain the effectiveness of the current antimicrobials.

Copper tolerance genes have previously been identified in *H. somni* strains [25], [213]. A multi copper oxidase gene (*mco*) was identified in *ICEHs02* [193]. This study characterized the phenotypes associated with the multi-copper oxidase gene (*mco*) in the transconjugants. We have noticed that the MIC of copper was elevated in all *H. somni* transconjugants to reach the same MIC level of the donor strain, showing the gene's activity in the transconjugants. Interestingly, the MIC of copper in *P. multocida* transconjugants is also increased to 2 mM in the transconjugant but has not reached the same MIC of copper in the donor strain (4 mM), indicating increased activity of the *mco* gene in *H. somni*. Comparable results were obtained from a recent study done by Krishna et al. (2016), where *ICEHs1* conferred copper tolerance to all the transconjugants to reach the same MIC level in donor strain [25]. There is no established breakpoint for Cu in CLSI, so the result was interpreted based on the reference strain *H. somni ATCC700025* (MIC = 0.5 mM), as described elsewhere [25].
Copper (Cu) is a vital element for a variety of biochemical activities [200]. In feedlot cattle, copper requirements are met by including copper sulphate supplement in diet [200]. The recommended concentration of Cu by the National Research Council (NRC) in cattle feed is 10 mg/kg [214]. However, the actual amount of Cu used in feedlot cattle exceeds the recommended concentrations [215]. Although copper is essential as an enzymatic cofactor, the excess amount of copper can lead to toxicity due to the copper redox activity [216]. The excess amount of copper usually excreted into the environment creating selection pressure for the bacteria. Thus, to survive, bacteria may acquire metal tolerance genes via horizontal gene transfer [200], and this might explain the presence and the activity of the mco gene in ICEHs02. In addition, the presence of copper in the environment might play a significant role in the co-selection of other genes present on the same mobile element like antimicrobial resistance genes. It has been known for decades ago that metal and antimicrobial resistance are linked, especially on plasmids [217]. The genetic linkage (co-resistance) between copper, zinc and tetracycline was demonstrated when copper and zinc resistances were transferred by conjugation in H. somni and P. multocida [25]. Copper residues present in the environment might create a selection pressure and help propagate the ICE carrying multi-drug resistances into broader environments. Educating the farmers and close monitoring to the amount of copper incorporated infeed might help decrease the dissemination of antimicrobials into the broader environment.

The mechanisms by which bacteria acquire AMR are complicated and involves many factors. The process is not only limited to the selection pressure of resistant bacteria due to the constant exposure to drugs or metals, but also involves other mechanisms like mutation, evolution and acquiring resistance genes via HGT. The presence of environmental stressors like sub-inhibitory concentrations of antibiotics may significantly increase the horizontal transfer rate of
many types of MGEs [183], [185]–[188]. Considering all these factors, might help us to understand the problem to discover a solution to limit the dissemination of AMR into a broader environment. The role of sub-inhibitory concentration on antimicrobials in the dissemination of *ICEHs02* is discussed in the following section.

### 4.2 Tetracycline and ciprofloxacin induce the conjugal transfer activity of *ICEHs02*

Antimicrobials are used for the treatment and control of many infections; however, there is an increasing concern that the misuse of antimicrobials might contribute to increasing AMR dissemination. The presence of a sub-inhibitory concentration of antimicrobials in the environment can enhance the transfer frequency of ICEs both *in vitro* and *in vivo* [129]. This study investigated the effects of sub-inhibitory concentrations of tetracycline and ciprofloxacin on the conjugal transfer frequency rate and the excision rate of *ICEHs02*.

Many antimicrobials like tetracyclines are used in animal feed; thus, they can easily contaminate the natural environment [61]. In addition, antimicrobials are only partially metabolized in animal bodies [218], [219]. Consequently, a considerable percentage of antibiotics (17% - 90%) are excreted in urine and feces to the external environment [218], [220]. Antimicrobial compounds could be transformed into active compounds [221]. Antimicrobials can also persist in the environment for a long time. The antimicrobial degradation rate relies on many factors including the physicochemical properties of the antibiotic, soil, pH, and temperature [222]. Thus, the antimicrobials' half-life time in the environment might vary for different antibiotics and different environments. The antimicrobials with a high risk to the environment usually have a low degradation rate, high water solubility, high KD value (sorption coefficient to the soil particles), or are used in high amounts in feedlots. Tetracycline was included in this study as it is commonly used in cattle [61], [83], [84]. In addition, the half-life time or the degradation rate for tetracycline
is considered medium and ranges from 10-50 days; however, its water solubility and the sorption coefficient are very high [222]. On the other hand, ciprofloxacin has a longer persistence in soil (30-80 days) [220]. Although not labelled for veterinary use in Canada, it is a metabolite of enrofloxacin, a commonly used antimicrobial in Canadian feedlots [61], [223]. Therefore, both antimicrobials are considered high risk for the ecosystem and this guided their inclusion in this study. We assumed that if these antimicrobials persist in the environment for a long time, they might act as stressors and play a significant role in disseminating ICE into the broader environment. Thus, we investigated the effect of these two drugs on the conjugation rate of \textit{ICEHs02}.

In this study, we found that tetracycline treatment significantly increased the \textit{ICEHs02} conjugal transfer rate from \textit{H. somni} KLM014 donor to the recipients \textit{H. somni} ATCC700025, \textit{H. somni} KLM08, and \textit{P. multocida} DSM16031 by 20.8-fold, 14.3-fold, and 19.1-fold respectively compared to conjugation experiments in which the donor was grown without treatment. Similar results were obtained when the bacteria were induced by ciprofloxacin, but the magnitude of the effect was smaller. The transfer frequency increased by ~ 8-fold in the conjugation between ciprofloxacin-treated \textit{H. somni} KLM014 and \textit{H. somni} ATCC700025 compared to the untreated group while the conjugation rate increased by 5-fold when \textit{P. multocida} DSM16031 was used as a recipient. The variable fold changes might be due to the different recipients used in conjugation. A recent study supported this theory, as they denoted that antimicrobials contribute to the increased HGT, but the level of HGT depends on both the donors and the recipients involved in conjugation [224].

To investigate whether subinhibitory concentration have an effect on the excision rate (copy numbers/chromosome), we conducted a qPCR to compare the copy numbers of the circular
intermediate and the chromosomal junction formed after the ICE excision upon induction with tetracycline, induction with ciprofloxacin and without induction. We found that the copy numbers of the circular intermediate/chromosome and chromosomal junction/chromosome were significantly higher upon induction with both antimicrobials.

Tetracycline was previously known to increase the transfer rate of plasmids and ICEs [174], [177], [178]. In 2002, Whittle et al. observed that the presence of tetracycline enhanced the transfer of ICE CTnDOT and showed that the conjugal transfer decreased significantly in the absence of tetracycline, indicating the role of tetracycline in the regulation of the conjugation [179]. The study explained the mechanism behind this effect by the presence of the tetQ gene on the same operon with rteA, rteB, and rteC. In this regulatory series, sub-inhibitory concentration of tetracycline activates the tetQ gene's transcription and the downstream genes rteA, rteB, and rteC. In the presence of tetracycline, the tetQ, rteA, and rteB are transcribed, and the product of rteB transcription activates the transcription of rteC, which controls the excision and the transfer of the ICE [179].

Ciprofloxacin is a DNA damaging agent known to induce the SOS response in bacteria [225], [226]. Beaber. et al. (2004) showed that the SOS response, induced by ciprofloxacin or mitomycin C (MMC), increased the expression of genes necessary for SXT transfer (ICE in Vibrio cholerae) and they observed that the frequency of transfer increased by 300-fold [180]. In this model, it is suggested that the SOS response stimulates SXT transfer by increasing the transcription of setD and setC genes required for the element transfer. The SOS response activates RecA protein, which facilitates the autocleavage of setR (repressor) and alleviates the repression increasing the expression of setC and setD genes and consequently, the activation of tra and int genes [180].
It was noted from the previous studies and in our study that subinhibitory concentrations of antimicrobials might contribute to the rapid dissemination of MGEs. However, this concept remains controversial. A recent report by A. J. Lopatkin et al. (2016) suggested that the standard experimental settings do not allow observers to differentiate between enhanced transfer efficiency and selective growth advantage for transconjugants in the presence of antibiotics [227]. The report concluded that antimicrobials neither affect the conjugation rate nor the transconjugant selection. In addition it was claimed that the effect of antimicrobials on the HGT may have been exaggerated [227]. Later in 2017 a study addressed the concerns in the first study by creating a new experimental design, pre-growing the donor in antimicrobials and performing mating assays in an antimicrobial free medium to separate conjugation events from the subsequent growth of transconjugants. The study confirmed the positive effect of cefotaxime (CTX) on plasmid transfer (PT) and indicated that ciprofloxacin (CIP) and ampicillin (AMP) have the same effect on PT. The study indicated that the transfer genes (tra) were up-regulated in the presence of the three antibiotics, while SOS-related genes (recA, sfiA) were only up-regulated in the presence of CIP, which is a DNA damaging antimicrobial [175]. Our study results agree with those from the second study, as we showed that tetracycline and ciprofloxacin enhance the transfer rate and excision rate of ICEHs02; however, the specific mechanisms behind this effect have not yet been explored.

Sub-inhibitory concentrations of antimicrobials appear to play a significant role in disseminating MGEs; however, the effect might differ from one mobile genetic element to another or from one antimicrobial to another. Further experiments should be conducted to investigate the mechanisms by which the tetracycline and ciprofloxacin enhance the excision rate and the transfer frequency of ICEHs02.
4.3 **ICEHs02 is costly to its new host**

The fitness cost of MGEs is usually represented as a reduced growth rate, virulence, or competitive ability [233]. The fitness cost of the MGEs is not only due to the backbone of the MGE, but also due to resistance genes they carry [194], [195]. The resistance determinants might differ from one MGE to the other, which might explain the variability in the fitness cost imposed by the MGE [233]. However, it is still unclear if specific resistance mechanisms are more costly than others [233]. The fitness cost of ICEs is highly variable among different ICEs. Some studies indicated that the acquisition of ICE is costly to the host [198], [199], while others have found that ICE imposes little if any cost [200], [201]. Despite the importance of ICEs to the progression of antimicrobial resistance, there is limited data on the ICE’s fitness costs and none associated with compensatory mutations that may alleviate their cost [197].

In this study, we have estimated the fitness effects of the newly acquired *ICEHs02* in *H. somni* ATCC700025 and *P. multocida DSM16031* using two methods. First, we assessed the fitness cost using growth curves to compare the growth rates and the doubling time in *H. somni* ATCC700025 and *P. multocida DSM16031* transconjugants versus their isogenic ancestors. Second, the fitness costs were estimated by competing *H. somni* ATCC700025 and *P. multocida DSM16031* with their isogenic parents to measure the relative fitness imposed by *ICEHs02* on the two strains.

The growth curve experiment result revealed that the *H. somni* ATCC700025 transconjugant exhibited both an extended lag phase, an approximately 21% decrease in growth rate, and 54 minutes difference in the doubling time compared to its isogenic ancestor. The *P. multocida DSM16031* TG showed 6 mins delay in doubling time but did not exhibit a significant change in growth rate. Moreover, it was noted in the co-competition experiments that the acquisition
of ICEHs02 reduced *H. somni* ATCC700025 TG's relative fitness by 28%, and for *P. multocida* DSM16031 TG, the relative fitness was decreased by 15%.

ICEHs02 is a large element of a size ~73 kb, whereas *H. somni* and *p. multocida* chromosomal sizes range between ~ 2200 kb and ~ 2500 kb. The ICE adds about 3% extra to the total chromosomal size of the transconjugants, and that might explain the fitness cost imposed by the ICE on the TGs compared to the isogenic ancestors. The ICE's fitness cost might also be due to the structural and accessory genes that the ICE carries [185], [186]. A recent review by A. San Millan et al. (2017) indicated that the expression of plasmid-encoded genes might explain the fitness cost imposed by the plasmid. The review also highlighted that plasmids usually bring new proteins to the host bacteria, and it is difficult to predict the impact of these proteins on the host strain [184]. In addition the interaction between MGEs might impose a fitness cost; an example of this is the interaction between Tn5386 and Tn916 ICEs which resulted in genomic deletions in the host genome in *E. faecium* [184].

To our knowledge, this study is the first to investigate the fitness cost of acquisition of ICEs in *H. somni*. A recent study by Cameron et al. (2019) investigated the acquisition of ICEMh1PM22 on *M. haemolytica* and *P. multocida*. The study revealed that the acquisition of ICEMh1PM22 created an extended lag phase in both strains. In addition, a reduced growth rate was observed in *P. multocida* bearing the ICE, however, no change in growth rate was observed in *M. haemolytica*. Competition experiments performed on these strains suggested that ICEMh1PM22 exhibits a fitness cost on the host [190]. Our study and the latter study revealed that acquiring new ICE might impose a fitness cost on the host.

In our study, we assumed that if ICEHs02 is costly to the new host it might be lost over time in the absence of selective environments. However, we observed that despite the measured
fitness cost imposed by ICEs02, the repeated passage of ICEs02-carrying transconjugants did not result in any significant loss of ICEs02 over repeated generations in non-selective environments. A similar observation was detected in P. multocida and M. haemolytica transconjugants, where ICEMh1PM22 was maintained in the host over a serial passage for 150 days in the absence of antimicrobial selection [190].

As described previously, despite the potential benefits conferred by ICEs, they also have a fitness cost for their hosts which is manifested as a reduced growth rate and inability to compete in non-selective environments. Therefore, it is not easy to understand why ICEs persist in the host chromosome in the absence of environmental stressors. There is scarce information to answer this question, however, there is a hypothesis that compensatory evolution might play a role in relieving the fitness cost of plasmids. The theory suggests that although the plasmid imposes fitness cost to the host upon arrival, the cost might be mitigated over time by mutations in the plasmid, the host chromosome, or both [184], [185], [188]. A recent study by Eaton et al. (2017) evaluated the evolutionary mechanisms by RP4 plasmid in Pseudomonas sp. and showed that the plasmid's fitness cost eased after only 600 generations [228]. Another study on R1 and RP4 plasmids in E. coli showed that the plasmids infer initial cost to their host; however, this cost was alleviated after 1100 generations through genetic changes in both the plasmid and the host [186]. Based on the similarity of ICEs and plasmids, we can assume that the ICEs have some compensatory mutations that alleviate the cost of fitness similar to plasmids. However, there are currently no studies in the literature which evaluated strategies to decrease or eliminate ICE carriage fitness costs and the presumed development of compensatory mutations in ICEs.

Another hypothesis that explains the persistence of ICEs might be the involvement of maintenance systems carried by the ICEs. These systems play a significant role in keeping ICEs
in their hosts. One system is the toxin-antitoxin system [197]. This system consists of a restriction enzyme (toxin) and a modification enzyme (antitoxin) [233]; when this system is carried on ICEs, it assures that only daughter cells that inherit the ICE survive after the cell division. If the ICE is lost in a daughter cell, the unstable antitoxin is degraded, and the stable toxic protein destroys the new host cell [197]. Cell death induced by loss of the genetic element is called post-segregational killing or genetic addiction [197], [233]. It has been reported that the toxin-antitoxin system plays a role in maintaining the SXT/R391 ICE family isolated from Providencia rettgeri and Vibrio cholerae [234].

Another system that may assist the ICE maintenance in the host might be the partition system, a well-known mechanism that ensures the stable inheritance of plasmids [233]. Partition is a dynamic process in which plasmids are moved inside the cell, so that cell division separates one copy into each cell. Usually, the process involves one partition site (par), which functions as the bacterial equivalent of centromere, and two proteins. One protein is a site-specific DNA binding protein that recognizes the par sites, and the other is ATPase or GTPase, which is responsible for the plasmid movement inside the cell [235]. Recently, a partitioning system was reported in SXT/R391 and ICECic and was shown to enhance the stability of the excised form of the ICE [234], [235].

In addition to the systems mentioned previously, the stability and propagation of some ICEs have been demonstrated to be reliant upon replication. Autonomous replication was observed in ICECic, SXT, and ICEBsl [236].

Previous research by Mohammad et al. (2020) identified ParA, ParB genes in ICEHs02 [193], however, the effect of these genes on the maintenance of the ICE have not been investigated yet. In our study, we have only investigated the fitness cost imposed by ICEHs02 on the host
strains, and we observed the stability of the ICE in non-selective environments. However, we have not investigated the ICE's compensatory mutations or identified any other genes related to the systems mentioned previously.
Chapter Five: Conclusion and Future directions

This study investigated the host range of *ICEHs02* and characterized the phenotype transferred with the ICE. *ICEHs02* was able to transfer to *H. somni* and *P. multocida* strains, conferring all resistance genes, the multi-copper oxidase gene, and associated phenotypes to all the transconjugants. *ICEHs02* could not transfer to *M. haemolytica* however, we only attempted transfer to one strain. *In silico* prediction of the host range indicated that *ICEHs02* might transfer to *H. somni*, *P. multocida*, *M. haemolytica*, *G. parasuis*, *B. trehalose*, *H. ducreyi*, and *H. parainfluenzae*. It was not possible to try all of these recipient species in this study; however, the *In silico* prediction of the host range, in combination with conjugation assays and susceptibility assays, confirmed the ability of the ICE to disseminate to other bacteria, conferring multidrug-resistance to new hosts. We have also to consider that the WGS data available in the databases like gene bank is still limited. In light of the available information, we hypothesize that ICEs play an important role in the emergence of the antimicrobial resistance among the BRD pathogens. BRD is considered the primary cause of death and illness in feedlot cattle. The transfer of *ICEHs02* from *H. somni* to other *H. somni* strains and *P. multocida* conferring multi-drug resistance in a single HGT event raise the concern that this ICE contributes to the increasing AMR among BRD pathogens. The rapid spread of AMR will decrease therapeutic options for BRD and in the long term will increase resistance to the conventional treatments and will create a need to use more powerful antimicrobials and, consequently, create more resistance to similar antimicrobials used for human medicine.

Moreover, this study investigated the role of antimicrobial stressors in the dissemination of *ICEHs02*. We observed that sub-lethal concentrations of tetracycline and ciprofloxacin significantly increased the conjugation frequency and the excision rate of *ICEHs02*. The regulatory
role of antimicrobials in disseminating ICEs in the BRD pathogens has not been investigated yet. Understanding the antimicrobials' regulatory role might provide insight into what might happen in the feedlot environment and how AMR spreads among the BRD pathogens. There is an urgent need to investigate the mechanisms by which antimicrobials increase the transfer rate of the ICEs and to investigate the role of other antimicrobials and other environmental factors like UV and heavy metals on the transfer of ICEs since increased conjugation frequency will have an adverse effect on treating BRD.

Antibiotics are the pillar of modern veterinary medicine and intensive agriculture. As mentioned earlier, antibiotics are used for the treatment of infectious diseases in both humans and animals. Without safe and efficient antibiotics, major surgeries, immunosuppressive medications for autoimmune diseases, and cancer chemotherapies become too dangerous because the threat of infection becomes too high. Usually, when people address AMR, the main focus is on human pathogens and public health. However, antimicrobials are used to treat sick animals, and increasing resistance in animal pathogens may lead to therapy failure, which might lead to mortalities and economic losses.

Many solutions have been proposed to limit AMR’s dissemination such as limiting the use of antimicrobials as growth promotors, banning antimicrobials as prophylactic agents and treating infected animals individually rather than administering antimicrobials in feed. Research should be conducted to find alternatives to antimicrobials, understand the role of ICEs and other MGEs in disseminating AMR, and to identify the antimicrobials in manure, water, and soil in feedlot cattle, and investigate the relation between the presence of the antimicrobials in feedlots and the carriage of MGEs.
This study also observed that the newly acquired ICE is costly to its new host. We verified that the acquisition of *ICEHs02* is an irreversible process. Once the ICE is acquired, although it has a fitness cost, it will be maintained in the host chromosome even in the absence of selective pressure. Further experiments are required to evaluate the strategies used to decrease or even eliminate the ICE carriage fitness costs and the presumed development of compensatory mutations in ICEs or the host chromosomes. Repeated passage experiments of transconjugants and isogenic parents, followed by fitness measurements, should measure the emergence of compensatory mechanisms associated with ICE carriage. WGS of the recipient strains that newly acquired ICE and the compensated ICE-carrying recipient strains can be used to identify the compensatory mutations in both the ICE and the host chromosome. Using transcriptomics will help investigate variations in the recipient host's genes expression due to harbouring ICE and help understand the nature of compensatory adaptation.

So little is known about ICEs that research should be conducted to understand more details about ICEs, such as the evolution of ICEs, the relation between ICEs and conjugative plasmids, and ICE interactions with different MGEs. A better understanding of ICEs will help to inform the complex problem of increasing antimicrobial resistance.
References


[9] D. Joakim Larsson *et al.*, “Critical knowledge gaps and research needs related to the
environmental dimensions of antibiotic resistance,” 2018.


[18] C. L. Klima *et al.*, “Genetic characterization and antimicrobial susceptibility of Mannheimia haemolytica isolated from the nasopharynx of feedlot cattle.”


http://www.bioagrimix.com/compendium.”


C. A. Madampage *et al.*, “Reverse vaccinology as an approach for developing Histophilus somni vaccine candidates,” *Biologicals*, vol. 43, no. 6, pp. 444–451, Nov. 2015.


[84] K. M. Benedict et al., “Antimicrobial Resistance in Escherichia coli Recovered from


29-Mar-2020).


1632, 01-Apr-2016.


[141] J. E. Galán and G. Waksman, “Protein-Injection Machines in Bacteria.,” *Cell*, vol. 172,
no. 6, pp. 1306–1318, 2018.


[158] K. Hegstad, H. Mylvaganam, J. Janice, E. Josefsen, A. Sivertsen, and D. Skaare, “Role of


[181] A. R. Johnsen and N. Kroer, “Effects of stress and other environmental factors on horizontal plasmid transfer assessed by direct quantification of discrete transfer events.”


134


APPENDICES

Appendix 1: Preparation of culture media

Media used: Brain Heart Infusion (BHI) Broth (Becton Dickson and Company, MD, USA), Lauria-Bertani (LB) Broth (Becton Dickson and Company, MD, USA), Mueller-Hinton Agar (Hardy Diagnostics, SM, CA), Columbia Blood Agar (CBA) (Hardy Diagnostics, SM, CA), sBHI (supplemented Brain Heart Infusion), BHITT (Brain Heart Infusion with trizma base and thiamine monophosphate).

Preparation of broth and agar plates

1. Calculate the required amount of media for making the working stock. Use MHA=38 gm, LB=20 gm and BHI=37 gm per one litre of distilled water.
2. Mix the media powder with distilled water and boil in a conical flask on a magnetic stirrer heat plate.
3. After a clear solution is observed, autoclave at 121°C and 17 psi for 15 minutes and allow cooling at room temperature.
4. For making broth tubes, pour one to three mL media on sterile tubes and store at 40°C.
5. For making agar plates, after autoclaving, cool the media to approximately 50°C.
6. Pour 25 mL on 100 mm plates and 60 mL on 150 mm plates.
7. Allow the agar to solidify at room temperature for 2-3 hours and store at 4 °C.

Preparation of Columbia Blood Agar supplemented with 5% Sheep blood

1. Dissolve 42 g of CBA base in 1 litre of distilled water.
2. Stir to fully dissolve all the powder.
3. Sterilize the mixture by autoclaving at 121°C for 15 minutes.
4. Cool the mixture to 50°C, add 50 mL of sterile, defibrinated sheep blood (VWR, ON, CA).
5. Mix thoroughly and pour into sterile Petri dishes.

**Preparation of BHITT**

1. Suspend 37.0 g of BHI in 1 litre of distilled water.

2. To supplement the media with (0.1% trizma base and 0.01% thiamine monophosphate), add 1 gm and 0.1 gram of trizma base (Sigma-Aldrich Canada Co, ON, CA) and thiamine monophosphate (Sigma-Aldrich Canada Co, ON, CA), respectively.

3. Heat, if necessary, to dissolve the medium completely.

4. Dispense into bottles or tubes as desired.

5. Sterilize by autoclaving at 121°C for 15 minutes.

**Preparation of sBHI**

1. Suspend 7.4 g of BHI in 200 mL of distilled water.

2. Sterilize by autoclaving at 121°C for 15 minutes.

3. Hemin stock preparation (1mg/mL): put 4 mL triethanolamine, 96 mL of distilled water, and 100 mg Hemin in a glass bottle. Cap the bottle loosely and heat at 65°C for 30 minutes. Do not autoclave. The stock is sterile and stable for 6 months at 4°C.

4. Nicotinamide adenine dinucleotide (NAD⁺) stock preparation (10 mg/mL): add 0.1 gm to 10 mL of dH2o, mix, filter-sterilized using 0.22 mm membrane filters. The stock is stable for six months at -20°C.

5. Add 3.32 μg/mL of NAD⁺ and 10 μg/mL of hemin to the BHI (calculate using the equation $C_1V_1 = C_2V_2$, where $C_1$ is the concentration of the initial stock solution, $V_1$ is the volume of the initial stock solution, $C_2$ is the desired concentration, $V_2$ is the volume of the desired concentration.

6. Prepare as needed; use within 24 hours.
Appendix 2: Growth curve protocol for *H. somni, P. multocida, and M. haemolytica*

Day 1:
1. From the frozen stock, pick up a loopful of the bacteria and streak onto CBA agar plates to obtain single colony isolates.
2. Incubate the plates at 37 °C for 24 hours. For *H. somni*, the plates were incubated in 5% CO₂.

Day 2:
1. With a sterile inoculation loop, select a single colony from each strain from the CBA agar plates and inoculate it into a test tube containing 10 mL of autoclaved BHITT for *H. somni* and BHI for *P. multocida* and *M. haemolytica*.
2. Incubate the tubes at 37 °C with 5% CO₂ for 24 hours with shaking.

Day 3:
1. Put 250 mL of a sterile broth in a sterile 500 mL conical flask.
2. Inoculate 5 mL of the overnight culture in the flask and swirl the media to distribute the bacteria evenly.
3. Power on the spectrophotometer and set the wavelength to 600 nm. Add 1 mL of uninoculated sterile BHI broth to a clean cuvette and blank the device.
4. Measure the Optical density (OD) at zero hours. Incubate the flask at the same conditions.
5. Take 1 mL of the culture suspension at an interval of every 1 hour for *p. multocida* and *M. haemolytica* and every 2 hours for *H. somni* and read the OD at the same wavelength using the spectrophotometer till the reading becomes static.
6. At the end of the experiment, plot a graph of time in hours on the X-axis and OD₆₀₀ on the Y-axis to obtain the growth curve of the bacteria.
Appendix 3: Growth curves of *P. multocida, M. haemolytica, H. somni*

Figure A.1: Growth curves of *P. multocida DSM16031* and *M. haemolytica h186* showing the mid-log phase

The blue line represents the growth curve of *P. multocida DSM16031*, the black line represents the growth curve of *M. haemolytica h186*. *P. multocida DSM16031* and *M. haemolytica h186* were cultured separately in BHI broth, the cultures were incubated at 37 °C for 12 hours. The growth was monitored and the optical density was measured at 600 nm (OD$_{600}$) every one hour till the reads are static. Data for the approximate mid-log phase of the bacteria are shown in red.
Figure A.2: Growth curve of *H. somni* KLM014 showing the mid-log phase used in the conjugation experiment

*H. somni* KLM014 was cultured in BHITT broth, the culture was incubated at 37 °C, 5 % CO₂ for 34 hours. The growth was monitored and the optical density was measured at 600 nm (OD₆₀₀) every two hours till the reads are static. Data for the approximate mid-log phase of the bacteria are shown in red.
Appendix 4: Filter mating assay protocol

1. Donor and recipient strains are grown separately in broth to the mid-log phase (growth conditions and broth varies according to the strain).

2. Mix 500 µL of donor and 500 µL of the recipient in Eppendorf tube, centrifuge at 13.000 RPM for 2 minutes to pellet the bacteria. Do the same for the donor and recipient.

3. Aspirate and discard the supernatant, resuspend the bacterial pellet in fresh broth.

4. Centrifuge again and discard the supernatant, resuspend the bacterial pellet in 30 µL of fresh BHI. (Do the same for each strain on its own as a control).

5. Place filters on non-selective plates (CBA plates without antibiotics). Use three filters, one for the conjugation and two for the controls (donor and recipient)

6. Place the suspended bacteria on the filters, incubate the plate upside down at 37°C with 5% CO₂ for 24 hours.

7. Take out the plates from the incubator, use an aseptic technique to pick the filters from the plate, and place them in a 1.5 mL tube containing 1 mL of broth.

8. Vortex the tubes to free the bacteria from the filters, remove and discard the filters from the tubes using the aseptic technique.

9. Do serial dilutions and plate desired volumes of the bacterial suspension on selective agar plates (CBA plates with antibiotics) using a bacterial spreader.

10. Incubate overnight, count the CFU of donors/recipient and transconjugants from the dilution plates.

11. Calculate the frequency of transfer as the number of transconjugants per recipient or donor.
Appendix 5: Minimum inhibitory concentration evaluators (M.I.C.E.™)

1. Remove the M.I.C.E. test package from the freezer (-20°C), and keep it at room temperature for at least 30 minutes.

2. Preparation of inoculum:
   i) Streak the bacteria on chocolate agar plates and incubate them at 37 °C for 24 hours (conditions might vary for different bacteria).
   ii) Pick 3-5 colonies from the agar plate and emulsify in 5 mL of Cation Adjusted Muller-Hinton Broth (CAMHB)® (Oxoid, Ontario, Canada). Vortex the suspension or invert the tube 8-10 times. If particles are still visible, the tube should be vortexed again.
   iii) Standardize the turbidity of suspension to 0.5 McFarland (10^8 CFU/mL) visually or using the Sensititre® Nephelometer. For the calibration of the nephelometer, use the Sensititre® 0.5 McFarland Polymer Turbidity Standard. Invert the turbidity standard from 8 to 10 times and place it in the nephelometer. Press the calibrate button on the nephelometer. The green light will indicate a successful calibration.

3. Soak a sterile cotton swab into the inoculum suspension and remove excess fluid by pressing the swab against the wall of the tube.

4. Streak the swab uniformly all over the chocolate Mueller-Hinton Agar (CMHA) surface. Swab the entire surface in one direction, then rotate the plate 60 degrees, then swab the whole plate again and repeat several times to ensure that the whole plate is uniformly covered with suspension.

5. Let the plate to dry for 5 -15 minutes.

6. Using an aseptic technique, get the strip out of its package, hold the strip with sterile forceps, and apply gently on the plate.
7. Incubate the plate at 37°C for 24 hours (conditions might vary for different bacteria).

8. Result interpretation:
   
i) Read the MIC value at the point of intersection of the ellipse and the M.I.C.E strip.
   
ii) If the MIC was between two values, record the higher value.
   
iii) If the inhibition zone is not symmetrical at the two sides, read the MIC at the greater value.
   
iv) If a heavy growth or light growth is found, the test is not valid and should be repeated.
   
v) The results of MIC should be compared to the CLSI breakpoints if available.

Appendix 6: Broth microdilution method using Sensititre® system

Preparation of inoculum for Sensititre plates

1. Streak the bacteria on chocolate agar plates and incubate them at 37 °C for 24 hours (conditions might vary for different bacteria).

2. Pick 3-5 colonies from the agar plate and emulsify in 5 mL of Cation Adjusted Muller-Hinton Broth (CAMHB)® (Oxoid, Ontario, Canada). Vortex the suspension or invert the tube 8-10 times. If particles are still visible, the tube should be vortexed again.

3. Standardize the turbidity of suspension at 0.5 McFarland (10^8 CFU/mL) visually or use Sensititre® Nephelometer. Use Sensititre® 0.5 McFarland Polymer Turbidity Standard for the calibration of the nephelometer. Invert the turbidity standard from 8 to 10 times and place it in the nephelometer. Press the calibrate button on the nephelometer. The green light will indicate a successful calibration.

4. Transfer 50 μL of the suspension into Veterinary Fastidious Medium (VFM) broth® (Oxoid, Ontario, Canada) to give an inoculum of 5x10^5 CFU/mL (range 1x10^5 – 1x10^6).
5. Mix properly by vortex mixing.

6. These steps must be completed within 30 minutes to avoid excess growth.

**Preparation of Sensititre plates**

1. Transfer 50 μL to each well by either:
   - Sensititre® AutoInoculator® / AIM®.
   - Manual pipette.

2. For using Sensititre® Auto Inoculator® / AIM®
   a) Using an aseptic technique, replace the tube cap with a Sensititre® single-use dose head.
   b) Adjust the auto inoculator to dispense 50 μL of VFM into 96 well bovine/porcine antibiotic plate.
   c) Cover the plate with a perforated seal as recommended by the manufacturer. The adhesive seal provided with the plate can be used; make sure that all the wells are covered and sealed.
   d) Incubate at 35-37°C in a CO₂ incubator or a gas jar with a CO₂ gas pack for 20-24 hours (conditions might vary for different bacteria). It is essential to keep the moisture in the incubator to minimize evaporation. A moistened towel should be placed with the plates in the incubator, or some CO₂ incubators might have containers of water that will provide the moisture.

3. For using a manual pipette, pour the broth into a sterile boat and inoculate the plate using an appropriate pipette. Inoculate the plate with the Sensititre® label facing the user.

4. Sensititre plates can be read automatically on the Sensititre auto reader.
5. It is recommended to check the control well periodically. This can be done by performing a colony count. The growth of *H. somni* can vary depending on the conditions of incubation of the overnight agar plate culture.

6. Colony count procedure for Sensititre plates:

   i. Immediately following the inoculation of the plate, take a sample from the positive growth control well and streak it onto blood agar using a 1μL loop.

   ii. Take another sample from the same growth well using a 1μL loop and mix with 50 μL sterile deionized water. Streak a loop (1 μL) of this dilution onto a blood agar plate.

   iii. Incubate both plates at 35–37°C overnight under appropriate conditions.

   iv. Interpret the count as follows:

<table>
<thead>
<tr>
<th>Colony Count</th>
<th>1 ul plate</th>
<th>1/50 dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5 x 10^4 =</td>
<td>50 colonies</td>
<td>Ng</td>
</tr>
<tr>
<td>5 x 10^4</td>
<td>50 – 100</td>
<td>Ng – 2</td>
</tr>
<tr>
<td>1 x 10^5 – 5 x 10^5</td>
<td>&gt;100</td>
<td>&lt;10</td>
</tr>
<tr>
<td>&gt; 5 x 10^5</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

Ng, No growth. Do not report the results if the colony count is not in the range of 2 x10^5 – 7 x10^5 CFU/mL (5 x 10^5 CFU/mL)
Appendix 7: Antimicrobial resistance profile of *H. somni* KLM014, *H. somni* KLM08, *H. somni* ATCC700025, and *P. multocida* DSM16031 using broth microdilution assay

Table A.1: AMR profile of *H. somni* KLM014, *H. somni* KLM08, *H. somni* ATCC700025, and *P. multocida* DSM16031 using broth microdilution assay (sensititre ®)

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th><em>H. somni</em> KLM014</th>
<th><em>H. somni</em> KLM08</th>
<th><em>H. somni</em> ATCC700025</th>
<th><em>P. multocida</em> DSM16031</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimicrobials</td>
<td>MIC(μg/mL)</td>
<td>MIC(μg/mL)</td>
<td>MIC(μg/mL)</td>
<td>MIC(μg/mL)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.5</td>
<td>≤ 0.25</td>
<td>≤ 0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Ceftifour</td>
<td>≤ 0.25</td>
<td>≤ 0.25</td>
<td>≤ 0.25</td>
<td>≤ 0.25</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>2</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>Danofloxacin</td>
<td>≤ 0.12</td>
<td>≤ 0.12</td>
<td>≤ 0.12</td>
<td>≤ 0.12</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>≤ 0.12</td>
<td>≤ 0.12</td>
<td>≤ 0.12</td>
<td>≤ 0.12</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>≤ 0.25</td>
<td>≤ 0.25</td>
<td>≤ 0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Neomycin</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>≤ 4</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>&gt;8</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>Penicillin</td>
<td>1</td>
<td>≤ 0.12</td>
<td>≤ 0.12</td>
<td>≤ 0.12</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>16</td>
<td>16</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>Sulphadimethoxine</td>
<td>&gt;256</td>
<td>≤ 256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>≤ 4</td>
<td>≤ 4</td>
<td>≤ 4</td>
<td>≤ 4</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole</td>
<td>≤ 2</td>
<td>≤ 2</td>
<td>≤ 2</td>
<td>≤ 2</td>
</tr>
<tr>
<td>Tulathromycin</td>
<td>4</td>
<td>16</td>
<td>≤ 1</td>
<td>≤ 1</td>
</tr>
<tr>
<td>Tylosin</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>32</td>
</tr>
</tbody>
</table>

AMR profile and MIC values in μg/mL for *H. somni* KLM014, *H. somni* KLM08, *H. somni* ATCC700025, and *P. multocida* DSM1603 using Sensititre™ Bovine/Porcine BOPO6F Vet AST Plate. 1, Ampicillin; 2, Penicillin MIC values was used to confirm that the transconjugants of *H. somni* ATCC700025, and *H. somni* KLM08 were not spontaneous mutants from *H. somni* KLM014. 3, Florfenicol MIC values indicates the inactivity of floR gene in *H. somni* KLM014. 4, Sulphadimethoxine MIC values.
Appendix 8: Copper tolerance assay protocol

Preparation of copper stock solution and working solution

1. The stock solution (640 mM) of copper sulfate pentahydrate (CuSO₄·5H₂O) (Sigma Aldrich, St. Louis, MO, USA) was prepared by dissolving 1.597 g of copper sulfate in 10 mL of sterile distilled water.

2. First, to prepare a 64 mM working solution, mix 1 mL of the 640 mM stock solution with 7 mL of sterilized distilled water. Adjust the media's PH to 7.0 using 1M solution of TRIS base solution (VWR, Solon, OH, USA) using a dropper and complete the volume to 10 mL using water.

3. A ninety-six well microplate was prepared in two-fold serial dilutions. Add 100 μL of the recommended broth from well 1 to well 10. Two-fold dilutions of copper solution were prepared from well 1 to 8, but no copper was added to wells 9 (growth control) and 10 (sterility control). To prepare of two-fold dilutions, add 100 μL of the prepared working solution to the first well. Mix very well and transfer 100 μL of the solution to the second well, mix very well and transfer 100 μL from the second well to the third well. Do the same for all the wells from 3 to 8 to get final concentrations of 32, 16, 8, 4, 2, 1, 0.5, 0.25 mM.

- **Note:** CAMHB is used for *P. multocida*, and VFM broth is used for *H.somni*.

Preparation of the inoculum

1. Take 3-5 separated colonies from an overnight culture grown on a chocolate agar plate and emulsify them in sterile Cation-Adjusted Muller-Hinton Broth (CAMHB), mix the suspension using a vortex mixer.
2. Standardize the turbidity of the suspension to 0.5 McFarland (approximately = 1x10^8 CFU/mL) using the Sensititre® Nephelometer. The nephelometer should be calibrated before use.

3. The suspension (1x10^8 CFU/mL) is then diluted 1:20 to yield 5 x 10^6 CFU/mL, then 10 μL of the diluted suspension is transferred to each of the wells from 1 to 9 to reach a final inoculum of 5 x 10^5 CFU/mL.

**Incubation, reading the plates, recording the MIC**

1. The trays are read by a microplate reader (Spectramax M2, Molecular Devices, USA) at an OD_{600}.

2. The trays are covered by a plastic cover to prevent dryness and incubated at 37°C in a CO_2 incubator for 24 hours (conditions might vary for different bacteria).

3. After incubation, the absorbance of the trays is read a second time.

4. The readings before incubation are subtracted from the readings after incubation, and mean absorbance units (AU) obtained from three technical replicates were compared against the concentration of copper (μg/mL) to determine the MIC.

**Appendix 9: Agarose gel electrophoresis**

1. Measure 1 gm of agarose and mix it with 100 mL 1x TAE buffer in a microwavable flask to prepare 1% agarose.

   - **Note:** Agarose gel can be prepared in different concentrations according to the size of the bands.

2. Use the microwave to heat the mixture for 1-2 minutes until the agarose is dissolved. Make sure to check it every 30 seconds, swirl the flask to avoid superheating.
3. Let agarose solution cool down to about 50°C, add approximately 5μg of “Red safe” stain, and swirl the flask.

4. Pour the agarose slowly into the gel tray with the well-comb in place, let it solidify.

5. For running the samples in the agarose gel:
   - Mix 10 μL of the DNA with 1-2 μL of loading dye (This step can be skipped if you are using Dream Taq Green PCR Master Mix, which is premixed with a specific dye).
   - Load the mixture carefully into the wells

6. Run the gel at 80 -150 V. The typical time to run the gel is about 20 to 30 minutes, depending on the concentration of the gel and the voltage.

Appendix 10: Visualizing the DNA using ChemiDoc™ MP Imaging System

1. Turn on the device, open the lower drawer of the device and place the gel.

2. The device is connected to a computer. Open the Image Lab™ software, and choose a “new protocol.”

3. Choose “nucleic acid” then choose “gel red.”

4. Position the gel; if it was not in the right position, open the drawer, adjust the gel and close it again.

5. Run the protocol.

6. The image of the gel will be displayed on the computer; the image can be adjusted to see the fine details.

7. The image can be saved on the computer and can be printed.
Appendix 11: Quantification of gDNA (genomic DNA) using ND-1000 UV/Vis Nanodrop spectrophotometer

1. The device is connected to a computer screen. Choose “Nucleic acid” then “DNA.”
2. Clean the upper and lower optical lids of the device.
3. Place two μL of water or TE buffer on the lower lid of the device using a ten μL pipette.
4. Press the “blank” icon on the computer screen; all the values will appear to be zero on the screen.
5. Re-blank can be done using the “re-blank” icon on the computer software.
6. Clean both the upper and lower optical lid of the device.
7. Place two μL of the samples needed to be measured, then press the “measure icon” on the computer software.
8. Make sure to clean the upper and the lower lid before each sample.
9. The reads will appear on the computer screen

Appendix 12: Purification of PCR-amplified products using GenepHlow™ Gel/PCR Kit

1. Adjust the Volume of PCR product to 50 μL by adding ddH₂O.
2. Mix one volume of the PCR product to 5 volumes of Gel/PCR buffer.
3. Place the DFH column in a 2 mL collection tube.
4. Transfer the sample to the DFH column, centrifuge at 16000 x g for 30 seconds.
5. Discard the flow-through and place the column back in the collection tube.
6. Add 600 μL of wash buffer into DFH column, and let stand for one minute at room temperature.
7. Centrifuge at 16000 x g for 30 seconds and discard the flow-through.
8. Place the DFH column back in the collection tube and centrifuge at 16000 x g for 3 minutes to dry the column completely.

9. Transfer the dried column in a new microcentrifuge tube.

10. Add 50 μL of preheated (60 - 70°C) Elution buffer (EU) into the DFH column.

11. Let stand for 1 minute and then centrifuge for 2 minutes at 16000 x g to elute the purified DNA.

**Appendix 13: Quantification of gDNA using Qubit® Fluorometer**

1. Press “DNA” on the home screen of the Qubit® Fluorometer, then select “dsDNA”.

2. Open the measuring chamber lid, insert standard one into the chamber, close the lid, and then press “Read Standard” when the reading is complete and remove the first standard.

3. Insert standard 2 into the chamber, close the lid, then press “Read standard” when the reading is complete, remove the standard.

4. Press “Run Samples,” select the sample volume, adjust the units.

5. Insert sample tubes into the chamber, close the lid, press “Read Tube”, when the reading is complete, remove the sample from the chamber and insert the next sample. Repeat the steps for all the samples.

6. Record the result for each sample. The result can be recalled by pressing “Data.”

**Appendix 14: Growth curves and calculation of growth rate and doubling time**

1. From frozen stock, pick up a loopful of the bacteria and inoculate it in 5 mL of BHITT or BHI broth.
2. Incubate the plates at 37°C with 5% CO₂ for 22-24 hours (conditions might vary for different bacteria).

3. Measure the OD of the culture using the spectrophotometer (Spectramax M2, Molecular Devices, USA).

4. Adjust the OD of the inoculum to the standard value (0.05) by using the following formula: \( OD_1V_1 = OD_2V_2 \). Where,
   \( OD_1 = \) OD of the broth culture, inoculated the previous day.
   \( OD_2 = \) OD of the inoculum (as a standard, this value is adjusted to 0.05)
   \( V_1 = \) volume of this broth culture to be added to the inoculums
   \( V_2 = \) volume of the inoculums (in this experiment, 200 μL)

5. Seal the plates with a perforated adhesive seal and incubate it in a CO₂ incubator with shaking (225 RPM) at 37°C (conditions might vary for different bacteria).

6. Monitor the plate using the microplate reader every one- or two-hour intervals (depending on the bacteria) and record the data (till the bacteria reach the stationary phase).

7. Use the OD values for plotting the growth curve (absorbance versus time) in Excel.

8. Convert the Y-axis scale (OD) into a logarithmic scale to easily determine the exponential phase.

9. Delete the other values from the growth curve or extract them from the growth curve and draw another curve for the exponential stage between the OD (logarithmic scale) and time.

10. Draw the best-fitted line using Excel (right-click on the values and chose format trendline and then exponential from the trendline options).
11. Choose to insert the fitted-line equation and the R² value.
   - The fitted-line equation will be used in the calculation of the growth rate.
   - The R² value will give an idea about how close the fitted-line to the measured values.
   - Higher R² value indicates that the fitted-line is close to the measured values.

12. The exponential phase is used to determine the growth rate (μ) from the two data points, OD₁ and OD₂, that were chosen on the fitted line and their corresponding t₁ and t₂ (Figure 2.2) using the equation: \[ \mu = \frac{2.303 \times (\log OD₂ - \log OD₁)}{t₂ - t₁} \], where OD₁ and OD₂ are the optical densities obtained from the best-fitted line, and t₁ and t₂ are their corresponding time.

13. To determine the OD's mathematical points, use the fitted-line equation by putting X values (time) in the equation to detect Y values (OD).

14. The doubling time was calculated using the equation: \[ td = \frac{\ln 2}{\mu} \].

**Appendix 15: Competition experiment and measuring the relative fitness cost**

1. Take the transconjugant and the isogenic ancestor from the -80 C Freezer and inoculate them separately in 3 mL of BHI to an OD₆₀₀ of ~ 0.5 for *H. somni* and ~ 0.4 for *P. multocida*.

2. Dilute the cultures 1:10 using 0.9% NaCl Solution or broth.

3. Mix 150 μL of each competitor (1:1) and transfer it to 2.7 mL of prewarmed BHI broth.

4. Take 100 μL of the sample immediately to determine the initial cell density of the two competitors (N₀).

   For the determination of cell density:
   - Make a 10-fold serial dilution series.
• Culture the bacteria on selective (tetracycline 8 μL) and non-selective TSA blood plates.

5. Incubate the mixed culture for 24 hrs at 37 °C in a CO₂ incubator with shaking (225 RPM) (conditions might vary for different bacteria).

6. After incubation, dilute the culture, as mentioned in step number 4 and plate on selective and non-selective plates to estimate the final cell density (N₂₄).

7. The population growth of each competitor, known as the Malthusian parameter, can then be calculated using the equation \( M = \ln \left( \frac{N_{24}}{N_0} \right) \).

8. The relative fitness \( w \) is the ratio of the Malthusian parameter of the transconjugant to the isogenic strain.
Appendix 16: DNA sequence alignments of PCR sequenced products for circular intermediate, chromosomal junction, and chromosome attachment sites to the sequence of *ICEHsKLM014* (*ICEHs02*) and the whole genome sequence of *H. somni KLM014*

Figure A.3: DNA sequence alignment of the circular intermediate sequenced PCR amplicons to the sequence of *ICEHs02* using SnapGene® version 5.1.5

The alignment confirmed the identity of circular intermediate form obtained by the primer pair PCIF and PCIR (shown in violet arrows). Direct repeats are shown in red colour. The blue box represents the aligned parts of the sequenced products to the query sequence (*ICEHs02* sequence). The letter N highlighted in yellow refers to unknown nucleotide.
Figure A.4: DNA sequence alignment of the chromosomal junction sequenced PCR amplicon to the whole genome sequence of *H. somni KLM014* using snapGene® version 5.1.5

The alignment confirmed the identity of the chromosomal junction obtained by the primer pair PCJF and PCJR (shown in violet arrows). Direct repeats are shown in red colour. The blue box represents the aligned parts of the sequenced products to the query sequence.
Figure A.5: DNA sequence alignment of the left chromosomal attachment site sequenced PCR amplicon to the whole genome sequence of H. somni KLM014 using snapGene ® version 5.1.5

The alignment confirmed the identity of the left attachment site obtained by the primer pair PCJF and PCIR (shown in violet arrows). Direct repeats are shown in red colour. The blue box represents the aligned parts of the sequenced products to the query sequence.
Figure A.6: DNA sequence alignment of the right chromosomal attachment site sequenced PCR amplicon to the whole genome sequence of *H. somni KLM014* using snapGene® version 5.1.5

The alignment confirmed the identity of the right attachment site obtained by the primer pair PCiF and PCJR (shown in violet arrows). Direct repeats are shown in red colour. The blue box represents the aligned parts of the sequenced products to the query sequence.
Appendix 17: Pair-wise sequence alignments of tRNA-Leu gene from *H. somni KLM014* to other bacterial species to predict the host range of *ICEHs02*

a)

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>159 bits(86)</td>
<td>1e-35</td>
<td>86/86(100%)</td>
<td>0/86(0%)</td>
<td>Plus/Plus</td>
</tr>
<tr>
<td>Query 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sbjct 687439</td>
<td>CCGCTGAGTCATGAAATTGTTAGACATGCAAGATTTCAGAATCCTGTTGTGAATAACCGTGT</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Query 61</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sbjct 687499</td>
<td>CGGGTCGAGTCGCACTCAGGACCA</td>
<td>687498</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sequence alignment of tRNA-Leu gene from *H. somni KLM014* to the published sequence from the whole genome sequence of *Histophilus somni* strain UOC-KLM-ATR-04 (Accession number: CP043001) using BLASTn. The alignment shows 100% query coverage, 100% identity with E-value of 1e^{-35}. The highlighted part represents the direct repeats (100% identity).

b)

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>159 bits(86)</td>
<td>1e-35</td>
<td>86/86(100%)</td>
<td>0/86(0%)</td>
<td>Plus/Minus</td>
</tr>
<tr>
<td>Query 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sbjct 351273</td>
<td>CCGCTGAGTCATGAAATTGTTAGACATGCAAGATTTCAGAATCCTGTTGTGAATAACCGTGT</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Query 61</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sbjct 351213</td>
<td>CGGGTCGAGTCGCACTCAGGACCA</td>
<td>351214</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sequence alignment of tRNA-Leu gene from *H. somni KLM014* to the published sequence from the whole genome sequence of *Mannheimia haemolytica* strain 32635(Accession number: CP017504) using BLASTn. The alignment shows 100% query coverage, 100% identity with E-value of 1e^{-35}. The highlighted part represents the direct repeats (100% identity).
c)

Sequence alignment of tRNA-Leu gene from *H. somni KLM014* to the published sequence from the whole genome sequence of *Pasteurella multocida* strain PM22 (Accession number: CP045724) using BLASTn. The alignment shows 100% query coverage, 100% identity with E-value of 1e^{-35}. The highlighted part represents the direct repeats (100% identity).

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>159 bits(86)</td>
<td>1e-35</td>
<td>86/86(100%)</td>
<td>0/86(0%)</td>
<td>Plus/Minus</td>
</tr>
<tr>
<td>Query 1</td>
<td>GCCTGAGTGTGAAATTGGTAGACATGACGGATTCAAAATCCGGTTGGTGAATAACCCTGTT</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sbjct 1648270</td>
<td>GCCTGAGTGTGAAATTGGTAGACATGACGGATTCAAAATCCGGTTGGTGAATAACCCTGTT</td>
<td>1648211</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

d)

Sequence alignment of tRNA-Leu gene from *H. somni KLM014* to the published sequence from the whole genome sequence of *Glaesserella parasuis* strain YHP170504 (Accession number: CP054198) using BLASTn. The alignment shows 100% query coverage, 100% identity with E-value of 1e^{-35}. The highlighted part represents the direct repeats (100% identity).

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>159 bits(86)</td>
<td>1e-35</td>
<td>86/86(100%)</td>
<td>0/86(0%)</td>
<td>Plus/Minus</td>
</tr>
<tr>
<td>Query 61</td>
<td>CGGTTCGAGTCCGACCTCAGGCACCA</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sbjct 1648210</td>
<td>CGGTTCGAGTCCGACCTCAGGCACCA</td>
<td>1648185</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Query 61  | CGGTTCGAGTCCGACCTCAGGCACCA | 86 |
| Sbjct 1608820 | CGGTTCGAGTCCGACCTCAGGCACCA | 1608820 |

| Query 61  | CGGTTCGAGTCCGACCTCAGGCACCA | 86 |
| Sbjct 1608819 | CGGTTCGAGTCCGACCTCAGGCACCA | 1608794 |
Sequence alignment of tRNA-Leu gene from *H. somni KLM014* to the published sequence from the whole genome sequence of *Bibersteinia trehalosi* USDA-ARS-USMARC-190 (Accession number: CP006956) using BLASTn. The alignment shows 100% query coverage, 100% identity with E-value of $10^{-35}$. The highlighted part represents the direct repeats (100% identity).

Sequence alignment of tRNA-Leu gene from *H. somni KLM014* to the published sequence from the whole genome sequence of *Haemophilus ducreyi* strain GHA3 (Accession number: CP015431) using BLASTn. The alignment shows 100% query coverage, 93% identity with E-value of $10^{-25}$. The highlighted part represents the direct repeats (100% identity).
Sequence alignment of tRNA-Leu gene from *H. somni* KLM014 to the published sequence from the whole genome sequence of *Haemophilus parainfluenzae strain NCTC10665* (Accession number: LR134481.1) using BLASTn. The alignment shows 100% query coverage, 87.36% identity with E-value of 5e^{-17}. The highlighted part represents the direct repeats (100% identity).
Appendix 18: Copy-right agreement

Annual Reviews, Inc. - License Terms and Conditions

This is a License Agreement between Mai Farghaly ("You") and Annual Reviews, Inc. ("Publisher") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Annual Reviews, Inc., and the CCC terms and conditions.

All payments must be made in full to CCC.

Order Date
23-Jul-2020

Order license ID
1050400-1

ISSN
1545-2948

Type of Use
Republish in a thesis/dissertation

Publisher
ANNUAL REVIEWS

Portion
Image/photo/illustration

LICENSED CONTENT

Publication Title
Annual review of genetics

Date
12/31/1966

Language
English

Country
United States of America

Rightsholder
Annual Reviews, Inc.

Publication Type
e-Journal

URL
http://arjournals.annualreviews.org/loi/genet

REQUEST DETAILS

Portion Type
Image/photo/illustration

Number of images/photos/illustrations
1

Format (select all that apply)
Print, Electronic

Who will republish the content?
Academic institution

Duration of Use
Life of current edition

Lifetime Unit Quantity
NEW WORK DETAILS

Title
Examination of the horizontal gene transfer dynamics of an integrative conjugative element encoding multi-drug resistance in Histophilus somni (Thesis)

Instructor name
Karen Liljebjelke

Institution name
University of Calgary

Expected presentation date
2020-10-31

ADDITIONAL DETAILS

Order reference number
N/A

The requesting person / organization to appear on the license
Mai Farghaly

REUSE CONTENT DETAILS

Title, description or numeric reference of the portion(s)
Figure 1 Life cycle of integrative and conjugative element

Editor of portion(s)
N/A

Volume of serial or monograph
N/A

Page or page range of portion
581

Title of the article/chapter the portion is from
Integrative and conjugative elements (ICEs): what they do and how they work

Author of portion(s)
Christopher M Johnson, Alan D Grossman

Issue, if republishing an article from a serial
N/A

Publication date of portion
2015-10-14