Genomic and transcriptomic variation in *Bordetella* spp. following induction of erythromycin resistance

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**Background:** The emergence of macrolide resistance in *Bordetella pertussis*, the causative agent of pertussis, due to mutations in the 23S rRNA gene has been recently recognized. However, resistance mechanisms to macrolides in *Bordetella parapertussis* and *Bordetella holmesii* remain unknown.

**Objectives:** This study investigated genomic changes induced by *in vitro* exposure to erythromycin in these three main pathogens responsible for pertussis-like disease.

**Methods:** A set of 10 clinical and reference strains of *B. pertussis*, *B. parapertussis* and *B. holmesii* was exposed to erythromycin for 15 weeks or 30 subculture passages. Antibiotic pressure was achieved by growth on the selective media with erythromycin Etest strips or impregnated discs. Genome polymorphisms and transcriptomic profiles were examined by short- and long-read sequencing of passaged isolates.

**Results:** *B. parapertussis* and *B. holmesii* isolates developed significant *in vitro* resistance to erythromycin (MIC > 256 mg/L) within 2 to 7 weeks and at 5 to 12 weeks, respectively. *B. pertussis* remained phenotypically susceptible to the antibiotic following 15 weeks of exposure, with the MIC between 0.032 to 0.38 mg/L. Genomic analysis revealed that *B. holmesii* developed resistance due to mutations in the 23S rRNA gene. The resistance mechanism in *B. parapertussis* was hypothesized as being due to upregulation of an efflux pump mechanism.

**Conclusions:** These findings indicate that both *B. holmesii* and *B. parapertussis* can be more prone to induced resistance following exposure to treatment with erythromycin than *B. pertussis*. The surveillance of macrolide resistance in *Bordetella* isolates recovered from patients with pertussis, especially persistent disease, is warranted.

**Introduction**

The *Bordetella* genus is comprised of several species and includes the mammalian pathogens *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. The human pathogens, *B. pertussis* and *B. parapertussis*, are the causative agents of pertussis, a highly infectious respiratory disease associated with prolonged coughing episodes. *B. pertussis* is the primary cause of pertussis, however it is estimated that *B. parapertussis* is responsible for approximately 1% of pertussis cases worldwide. In recent years, the emergence of a closely related species *Bordetella holmesii*, has impacted *B. pertussis* surveillance, as both species contain the PCR target used to diagnose *B. pertussis* infections. In Australia, *B. holmesii* has a prevalence of between 0%–16.8% and this reflects its prevalence in other developed countries.

The currently recommended treatment for pertussis infections and post-exposure prophylaxis are macrolide antibiotics. However, macrolide-resistant strains of *B. pertussis* have been reported for some years in the USA, France, China, and Vietnam. The resistance is due to a A2037G mutation in the 23S rRNA gene of *B. pertussis* in comparison with *B. pertussis Tohama I.* The increased prevalence of these strains in recent years has raised concerns for their global expansion. Given pertussis can...
also be caused by other Bordetella species, namely, B. parapertussis and B. holmesii, the ability to recognize and monitor macrolide resistance in clinical strains of all Bordetella spp. becomes crucial. This study examined the comparative ability of several strains of B. pertussis and other significant Bordetella spp. (B. parapertussis and B. holmesii) to develop induced phenotypic resistance following exposure to erythromycin in vitro. Further strains were sequenced and the genomes interrogated for any potential variation that may indicate erythromycin resistance.

Materials and Methods

Strain selection and culture conditions
A set of clinical and reference isolates was selected for antibiotic resistance induction—four B. pertussis strains (CIDM-BP1, CIDM-BP2, CIDM-BP3 and CIDM-BP4), three B. parapertussis strains (CIDM-BPP1, CIDM-BPP2 and CIDM-BPP3) and three B. holmesii strains (CIDM-BH1, CIDM-BH2 and CIDM-BH3). B. pertussis strains were chosen based on MLST, SNP and vaccine antigen types in order to represent currently co-circulating genotypes of the pathogen (Table 1). B. parapertussis and B. holmesii were chosen based on availability in the culture collection (Table 2).

B. pertussis and B. parapertussis isolates were cultured on Charcoal Blood Agar without cephalaxin (CBA) and B. holmesii on Horse Blood Agar (HBA) (Thermo Fisher Scientific, USA). The cultures were incubated at 37°C for 3–4 days aerobically. Bacterial growth for RNAseq
To obtain the transcriptomic profile of resistant isolates under antibiotic pressure, CIDM-BH3 and CIDM-BPP2 (and their respective resistant descendants) cultures were grown in LB broth in triplicates. A loopful of colonies was transferred to LB broth, homogenized and divided equally into three tubes, the MF standard was calculated, and was kept consistent across treatment conditions. Susceptible (BH3OG and BPP2OG) and resistant isolates were cultured overnight (stopped at 12 h) with aeration at 250 rpm, in liquid media either with erythromycin (256 mg/L) (BH3RAB and BPP2RAB) or without the antibiotic (BH3RES and BPP2RES).

Induction of in vitro resistance
The strains were subcultured every 3–4 days. Briefly, a suspension equivalent to a 0.5 McFarland (MF) standard was made from bacterial colonies at the edge of the inhibition zone. A fresh (either HBA or CBA) plate was inoculated with the suspension and either erythromycin Etests (BioMérieux, France) or erythromycin-impregnated discs (BioMérieux, France) were used to provide antibiotic pressure (Figure S1, available as Supplementary data at JAC Online). Resistance to erythromycin was defined by an MIC >0.125 mg/L and consistently recorded for two or more passages. A total of 30 passages were performed, however, once antibiotic resistance was observed, the strain was plated on CBA or HBA without antibiotics and DNA was extracted within 48 h after inoculation. In parallel, the initial isolate was passaged every 3–4 days on media without antibiotic exposure to act as a laboratory passage control. MICs for all resulting isolates were determined by Etest.

Table 1. Summary of B. pertussis strains, year of isolation, vaccine antigen alleles, MLST type and initial MIC of erythromycin

| Isolate   | Year | ptxP | ptxA | prn | fhaB | fim2 | fim3 | MLST | Initial MIC (mg/L) | Sequencing technology |
|-----------|------|------|------|-----|------|------|------|------|-------------------|----------------------|
| CIDM-BP1  | 1954 | 1    | 4    | 1   | 1    | 1    | 1    | 1    | 0.032             | Illumina             |
| CIDM-BP2  | 2011 | 3    | 1    | 2   | 2    | 1    | 3    | 2    | 0.032             | Illumina & Nanopore  |
| CIDM-BP3  | 2015 | 1    | 1    | 2   | 3    | 2    | 2    | 0.016           | Illumina & Nanopore  |
| CIDM-BP4  | 2015 | 3    | 1    | 2   | 2    | 1    | 1    | 2    | 0.064             | Illumina             |

Table 2. B. parapertussis and B. holmesii isolates used in the study with their year of isolation and initial MIC of erythromycin

| Strains               | Year of isolation | Initial MIC (mg/L) | Sequencing technology |
|-----------------------|-------------------|--------------------|----------------------|
| Bordetella parapertussis |                  |                    |                      |
| CIDM-BPP1             | Unknown           | 0.125–0.19         | Illumina             |
| CIDM-BPP2             | 1993              | 0.125–0.19         | Illumina & Nanopore  |
| CIDM-BPP3             | Unknown           | 1                  | Illumina             |
| Bordetella holmesii   |                   |                    |                      |
| CIDM-BH1              | 2000              | 0.125–0.19         | Illumina             |
| CIDM-BH2              | 2014              | 0.047–0.064        | Illumina             |
| CIDM-BH3              | 2016              | 0.25               | Illumina & Nanopore  |

Genomic DNA was extracted with the DNeasy Blood and Tissue Mini Kit (QIAGEN, Germany) or DNeasy UltraClean Microbial Kit (QIAGEN, Germany) for Illumina and Nanopore sequencing, respectively. WGS was performed at the Microbial Genomics Reference Laboratory, NSW Health Pathology. All strains were short-read sequenced on the NextSeq platform (Illumina, USA). In addition, strains CIDM-BP2, CIDM-BP3, CIDM-BH3, CIDM-BPP2 and CIDM-BPP2R were also long-read sequenced on the MinION platform (Oxford Nanopore Technologies plc, UK). Sequencing libraries for Illumina sequencing were prepared using the Nextera XT DNA Library Prep Kit (Illumina) and sequenced on a NextSeq 500 using NextSeq 500/550 v2 mid output kits (Illumina). Sequencing libraries for Nanopore sequencing were prepared using the Rapid Barcoding kit (SQK-RBK004) and sequencing on a R9 flowcell. Total RNA was extracted from liquid cultures using the RNeasy Plus Universal Mini Kit (QIAGEN, Germany), following manufacturer’s protocol.
Total RNA sequencing was performed by the Australian Genomics Research Facility (AGRF) utilizing the Illumina Stranded Total RNA Prep with Ribo-Zero Plus on the NovaSeq.

**Genome analysis**

The short-read sequenced raw reads were quality controlled using FastQC (v 0.11.3), Trimmomatic (v 0.36)\(^{17}\) and Centrifuge (v 1.0.4),\(^{18}\) prior to further analysis. For the strains sequenced by short-read technology, trimmed reads were assembled with default parameters by SPAdes (v 3.12.0).\(^{19}\) All assemblies were then annotated with Prokka (v 1.12)\(^{20}\) and Barnap (v 0.6) (https://github.com/tseemann/barnap), then scanned for virulence factors (VFDB)\(^{21}\) and resistance markers (CARD)\(^{22}\) with Abricate (v 0.9.8; https://github.com/tseemann/abricate). For long-read sequencing, base calling was performed on high accuracy mode and demultiplexing was performed on Guppy (v 2.7b)\(^{23}\) with the ‘–asm-coverage’ parameter set to 30 and an expected genome size of 4.0 Mbp. Following long-read assembly, the sequence was corrected with Racon (v 1.3.1)\(^{24}\) four times, and Medaka (v 0.11.5)\(^{25}\) twice. The assembly was then polished with corresponding Illumina reads using Pilon (v 1.23)\(^{26}\) and repeated until there were no more changes.

Identification of SNPs in the resistant genomes from organisms that showed increased MICs post-erythromycin induction was performed using Snippy (v 4.3.5) (https://github.com/tseemann/snippy). Reference sequences used were *B. pertussis* Tohama I (NCBI GenBank accession number: NC_002929.2) and the long-read closed genomes or the short-read assembly from this study. Further comparisons of resistance genes between the *Bordetella* spp. were performed by the BLASTn and figures were drawn in EasyFig (v 2.2.2).\(^{26}\)

**Transcriptome analysis**

For RNAseq, the raw reads were also passed through the in-house quality control procedure consisting of FastQC (v 0.11.3), Trimmomatic (v 0.36)\(^{17}\) and Centrifuge (v 1.0.4).\(^{18}\) Mapping of RNAseq reads onto their corresponding long-read assembled genome was performed using BWA-MEM (v 0.7.17). Closed genomes were initially annotated with the NCBI Prokaryotic Genome Annotation Pipeline,\(^{27-29}\) and coding sequences were further corrected using EggNOG-mapper.\(^{30,31}\) HTSeq (v 0.11.2)\(^{32}\) was used to calculate the number of reads mapped to each gene feature.

**Statistical analysis**

All read counts were normalized using read counts per million (CPM) and transcripts per million (TPM). Statistical comparisons between treatment conditions were performed using unpaired t-tests on GraphPad Prism and plotted using BoxPlotR.\(^{33}\) Raw data has also been supplied in the Supplementary data.

**Data availability**

Closed genomes and sequencing reads of resistant isolates have been published in Bioproject: PRJNA224116.

**Results**

**MIC following induction of erythromycin resistance**

To induce resistance in four *B. pertussis*, three *B. parapertussis* and three *B. holmesii* strains, isolates were grown on media with an erythromycin Etest or a disc for 15 weeks. *B. parapertussis* isolates gradually increased their MIC levels to those corresponding to in vitro resistance (>256 mg/L) within 2 to 7 weeks (within 6–15 passages) (Figure 1 and Figure S2). *B. holmesii* isolates took slightly longer than *B. parapertussis* to develop resistance (>256 mg/L), at 5 to 12 weeks (13–25 passages) (Figure S3). However, after 15 weeks (30 passages), *B. pertussis* did not develop resistance and the MICs of all four isolates fluctuated between 0.032 and 0.38 mg/L (Figure S4).

**Genomic variability in isolates with elevated MIC to erythromycin**

Passaged isolates were sequenced every month (i.e. 4 weeks/8 passages) to monitor any intermediate genomic variation that may have contributed to phenotypic increase in MIC. For those that developed resistance, the majority of isolates developed the highest resistance within one passage (spontaneously) rather than slowly accumulating resistance and increasing MIC over several passages (progressively), which suggested that resistance was driven by SNPs. Despite some elevation of the MIC of erythromycin, the genomes of the *B. pertussis* and *B. parapertussis* isolates contained no mutations in the 23S rRNA gene sequence reported in macrolide-resistant *B. pertussis*.\(^{5}\) However, such mutations in the 23S rRNA gene were detected in all resistant *B. holmesii* (Table 3) with each having a distinct mutation in positions G2031A (strain CIDM-BH2), A2032G (CIDM-BH3), and C2585T (CIDM-BH1). Long-read sequencing allowed the differentiation of the three 23S rRNA gene copies, which cannot be resolved with short-read sequencing. Mapping the short-read CIDM-BH3 resistant (CIDM-BH3R) reads to the closed CIDM-BH3 genome confirmed that all three copies of the 23S rRNA carried the A to G mutation in position 2032. No mutations were observed in the 23S rRNA gene for either the CIDM-BPP2 or CIDM-BPP2 resistant strain (CIDM-BPP2R).

As the 23S rRNA gene of CIDM-BPP2R did not possess mutations that were implicated in macrolide resistance, the genome was screened for other genes of interest that could potentially confer resistance to macrolides (Table S1). None of the selected genes was present but analysis against the CARD and VFDB database yielded the presence of the *Pseudomonas aeruginosa* mexB gene. This gene is part of a tripartite efflux pump mechanism described in *P. aeruginosa* and known to confer macrolide resistance.\(^{34}\) The mexB gene was present in both susceptible and resistant strains and BLASTn alignments of the entire mexAB-oprM operon yielded 75.6% identity to 88% coverage in both CIDM-BPP2 and CIDM-BPP2R (Figure 2). A BLASTn search of the mexAB-oprM operon from the CIDM-BPP2 genome to the entire NCBI nucleotide database, showed that all other *B. parapertussis* strains carried this operon, as did *Bordetella bronchiseptica* with a 99.7% similarity. Thus, presence of this operon in the primary pathogens from the *Bordetella* spp. was determined and is shown in Figure 2.

**Transcriptome analysis**

With the presence of the *mexAB-oprM* orthologue in *B. parapertussis*, we investigated the changes in transcriptional regulation that could result in macrolide resistance. The whole transcriptome was captured with RNAseq for isolates CIDM-BH3, CIDM-BPP2 and their resistant derivatives. In both cases, three
growth conditions were applied, the susceptible isolate (CIDM-BH3/BH3OG and CIDM-BPP2/BPP2OG), the resistant isolate (CIDM-BH3R/BH3RES and CIDM-BPP2R/BPP2RES) and the resistant isolate grown under macrolide pressure (BH3RAB and BPP2RAB). The raw TPM data and condition comparisons are provided in the Supplementary data.

The transcriptome profile over the mexAB-oprM in CIDM-BPP2 revealed average expression between BPP2OG and BPP2RES also had an average 2.2 ± 0.6-fold upregulation (Figure 3a). However, it was also observed that an average 2.3 ± 0.7-fold upregulation occurred in expression of BPP2RAB compared with BPP2OG (Figure 3b). Further investigations of the transcriptome of B. parapertussis, revealed another efflux pump that was highly expressed (i.e. 5.1 ± 1.2-fold increase). This efflux pump was identified to be another acr-like pump, named acr/bepE, which is closely related to the mexI/mexW family of genes in P. aeruginosa (Figure S5). The entire gene locus showed a 3.9 ± 3.5-fold increase in expression when BPP2OG was compared with BPP2RAB. However, compared with BPP2RES, it was a 6.3 ± 8.2-fold increase in expression. In addition, the gene within this locus with the

Table 3. Summary of all strains of Bordetella spp. enrolled in this study including the initial and final MIC for erythromycin, and presence and absence of the 23S rRNA gene mutation resulting in phenotypic resistance

| Sample                  | Initial MIC (mg/L) | Final MIC (mg/L) | 23S rRNA mutations                  |
|-------------------------|--------------------|------------------|-------------------------------------|
| Bordetella pertussis    |                    |                  |                                     |
| CIDM-BP1                | 0.032              | 0.125            | Not detected                        |
| CIDM-BP2                | 0.032–0.047        | 0.125            | Not detected                        |
| CIDM-BP3                | 0.016              | 0.125            | Not detected                        |
| CIDM-BP4                | 0.064              | 0.125            | Not detected                        |
| Bordetella parapertussis|                    |                  |                                     |
| CIDM-BPP1               | 0.125–0.19         | >256             | Not detected                        |
| CIDM-BPP2              | 1                  | >256             | Not detected                        |
| CIDM-BPP3               | 0.125–0.19         | >256             | Not detected                        |
| Bordetella holmesii    |                    |                  |                                     |
| CIDM-BH1                | 0.125–0.19         | >256             | C to T (Position 2585)              |
| CIDM-BH2                | 0.047–0.064        | >256             | G to A (Position 2031)              |
| CIDM-BH3                | 0.25               | >256             | A to G (Position 2032)              |

aThe resistant isolate of CIDM-BPP2 was named CIDM-BPP2R.
highest increased fold-change (7.4-fold in BPP2RAB and 14.5-fold in BPP2RES) was the efflux transporter outer membrane subunit (oprN). Examination upstream and downstream of this operon demonstrated that the first TR (adjacent to oprN) is an ArsR family transcriptional regulator, which are repressors of di- and multi-valent heavy metal ions. Adjacent to the TR is a highly upregulated azurin (azn) gene, hence the TR and azurin are likely linked (Figure S6).

The transcriptome of B. holmesii under antibiotic pressure behaved similarly to that of B. parapertussis. The expression profile over the mexAB-oprM equivalent locus, revealed an average 1.6 ± 0.6-fold increase in BH3OG versus BH3RAB, and a 1.1 ± 0.3-fold change in upregulation for BH3OG versus BH3RES (Figure 4). Further, the acr-like operon present in B. parapertussis was not detected in B. holmesii.

### Resistance mechanism and housekeeping gene expression

To further clarify the changes in expression of housekeeping genes, a set as selected by the Bordetella spp. MLST scheme and the bgvAS locus were investigated and compared alongside the mexAB-oprM operon. Of the seven housekeeping genes, three (tyrB, pepA and pgm) in B. parapertussis remained consistently expressed across all normalized conditions (fold-change between 0.9–1.1). However, three were downregulated ~2-fold (adk, fumC and glyA), and one was upregulated 1.54 ± 0.06-fold (icd) (Supplementary data).

### Discussion

This study demonstrated that repeated exposure to erythromycin induced in vitro resistance in B. parapertussis and B. holmesii but not in B. pertussis for the duration of our study. While exposure decreased susceptibility to erythromycin in B. pertussis, the MICs did not reach levels defined as in vitro resistance. The predicted mechanisms of resistance varied between species, with B. holmesii containing a 23S rRNA gene mutation and B. parapertussis having no obvious mutations relating to macrolide resistance in that gene. The B. holmesii strains each acquired unique 23S rRNA mutations in different nucleotide positions, all of which have conferred resistance to macrolides in previous reports. As induced erythromycin-resistant B. parapertussis did not possess mutations in the 23S rRNA gene, other possible resistance mechanisms such as the presence of erm, mef, mex or ere were investigated.

While the 23S rRNA mutation was the most likely explanation for induced resistance in B. holmesii, isolates of B. parapertussis did not contain mutations in the same region. We found that none of the erm, mef or ere genes were present in the
Figure 3. Expression profile of the mexAB-oprM orthologue in B. parapertussis CIDM-BPP2 calculated based on genomic position and CPM. (a and b) Comparison of BPP2OG (erythromycin susceptible) with BPP2RES (resistant without antibiotic pressure), demonstrating a large proportion of reads encompassing the mexA gene. (a and c) Comparison of BPP2OG (susceptible) with BPP2RAB (resistant with antibiotic pressure), shows a similar outcome as BPP2RES. (d) Box plot of gene expression between conditions. For genes within mexAB-oprM and its transcriptional regulator (dmlR) all were significantly upregulated in the RES and RAB conditions. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Figure 4. Expression profile of the mexAB-oprM equivalent efflux pump in B. holmesii. (a and b) Comparison of BH3OG (susceptible) with BH3RES (resistant without antibiotic pressure), demonstrating relatively even distribution of reads across the gene locus. (a and c) Comparison of BPP2OG (susceptible) with BPP2RAB (resistant with antibiotic pressure), showed a similar outcome as BPP2RES. (d) Box plot of gene expression in isolates under different conditions. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
**Bordetella** species, however, an orthologue of the mexAB-oprM operon with >71% homology was detected in the genomes of all mammalian **Bordetella** spp. (*B. pertussis*, *B. parapertussis*, *B. holmesii*, and *B. bronchiseptica*). Previous genomic annotation predictions have called the mexAB-oprM system the acrAB-cusC operon, with the latter conferring resistance to acriflavine, other hydrophobic molecules and fatty acids. As erythromycin is a hydrophobic molecule, the mexAB-oprM system could facilitate the excretion of this molecule by the efflux pump system and this may explain the acquisition of induced resistance in this study. The mexAB-oprM operon is present in *Pseudomonas aeruginosa* and encodes an efflux pump that confers macrolide resistance. The mexAB-oprM operon in *B. parapertussis* had high sequence similarity (99.7%) to *B. bronchiseptica*, and the function of mexAB-oprM is predicted to be the same in both species. *B. bronchiseptica*, the common ancestor of *B. parapertussis* and *B. pertussis*, is inherently macrole resistance with an MIC beyond the time limits of this experiment and whether induced resistance is maintained without antibiotic pressure could help elucidate this point.

In conclusion, our findings have indicated that *B. holmesii* and *B. parapertussis* can readily develop phenotypic resistance to erythromycin under antibiotic pressure, while *B. pertussis* did not in the conditions described here. The predicted mechanisms of resistance varied between species, with *B. holmesii* containing a recognized 23S rRNA gene mutation and *B. parapertussis* having no obvious mutations relating to macrolide resistance. The presence of the mexAB-oprM orthologue (acrAB-cusC operon) has the potential to confer macrolide resistance in *B. parapertussis*. Genomic data and isolates with induced resistance can serve as reference points for development of diagnostic assays and surveillance of macrolide resistance in **Bordetella** recovered from patients with clinical pertussis.

These findings have significant implications for the development of antibiotic guidelines on treatment and prophylaxis of pertussis caused by these pathogens as infection with *B. parapertussis* or *B. holmesii* can be misdiagnosed as *B. pertussis*. The understanding of mechanisms of macrolide resistance and the ability to detect resistance in a timely fashion can improve patient outcomes and reduce the spread of the disease. The ability of *B. parapertussis* and *B. holmesii* to rapidly acquire macrolide resistance highlights the need for better surveillance and antibiotic stewardship in the management and control of pertussis cases and outbreaks.

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**Transparency declarations**

None to declare.

**Supplementary data**

Table S1, Figures S1 to S6 and TPM data are available as Supplementary data at JAC Online.
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