**Supplementary materials - Table S2**. Primer binding other species.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Accession | Fcoverage | Fstart | Fend | Pcoverage | Pstart | Pend | Rcoverage | Rstart | Rend |
| CP047349.1 | 100 | 3188078 | 3188099 | 95.833 | 3188130 | 3188153 | 95.455 | 3188182 | 3188161 |
| CP034668.1 | 100 | 3395406 | 3395427 | 95.833 | 3395458 | 3395481 | 95.455 | 3395510 | 3395489 |
| CP045008.1 | 100 | 3373133 | 3373154 | 95.833 | 3373185 | 3373208 | 95.455 | 3373237 | 3373216 |
| MK134853.1 | 100 | 2781 | 2802 | 100 | 2833 | 2859 | 100 | 2885 | 2863 |
| MK110805.1 | 100 | 2853 | 2874 | 100 | 2905 | 2931 | 100 | 2957 | 2935 |
| CP026856.1 | 100 | 2611214 | 2611235 | 100 | 2611266 | 2611292 | 100 | 2611318 | 2611296 |
| CP026860.1 | 100 | 316468 | 316489 | 100 | 316520 | 316546 | 100 | 316572 | 316550 |
| CP024444.1 | 100 | 151028 | 151049 | 100 | 151080 | 151106 | 100 | 151132 | 151110 |
| CP011374.1 | 100 | 2160774 | 2160795 | 100 | 2160826 | 2160852 | 100 | 2160878 | 2160856 |
| KJ909292.1 | 100 | 4560 | 4581 | 100 | 4612 | 4638 | 100 | 4664 | 4642 |
| KC734562.1 | 100 | 3761 | 3782 | 100 | 3813 | 3839 | 100 | 3865 | 3843 |
| KC734560.1 | 100 | 3861 | 3882 | 100 | 3913 | 3939 | 100 | 3965 | 3943 |
| HF953351.1 | 100 | 10357 | 10378 | 100 | 10409 | 10435 | 100 | 10461 | 10439 |
| CP003745.1 | 100 | 83569 | 83590 | 100 | 83621 | 83647 | 100 | 83673 | 83651 |
| CP003745.1 | 100 | 83569 | 83590 | 100 | 83621 | 83647 | 100 | 107554 | 107532 |
| CP003745.1 | 100 | 83569 | 83590 | 100 | 107502 | 107528 | 100 | 83673 | 83651 |
| CP003745.1 | 100 | 83569 | 83590 | 100 | 107502 | 107528 | 100 | 107554 | 107532 |
| CP003745.1 | 100 | 107450 | 107471 | 100 | 83621 | 83647 | 100 | 83673 | 83651 |
| CP003745.1 | 100 | 107450 | 107471 | 100 | 83621 | 83647 | 100 | 107554 | 107532 |
| CP003745.1 | 100 | 107450 | 107471 | 100 | 107502 | 107528 | 100 | 83673 | 83651 |
| CP003745.1 | 100 | 107450 | 107471 | 100 | 107502 | 107528 | 100 | 107554 | 107532 |
| AM992204.1 | 100 | 4183 | 4204 | 100 | 4235 | 4261 | 100 | 4287 | 4265 |
| AY987962.1 | 100 | 658 | 679 | 100 | 710 | 736 | 100 | 762 | 740 |
| AJ245947.1 | 100 | 429 | 450 | 100 | 481 | 507 | 100 | 533 | 511 |
| AY362554.1 | 100 | 2898 | 2919 | 100 | 2950 | 2976 | 100 | 3002 | 2980 |
| CP053042.1 | 100 | 2567543 | 2567522 | 95.833 | 2567491 | 2567468 | 95.455 | 2567439 | 2567460 |
| CP053044.1 | 100 | 573063 | 573042 | 95.833 | 573011 | 572988 | 95.455 | 572959 | 572980 |
| CP047639.1 | 100 | 1269079 | 1269058 | 100 | 1269027 | 1269001 | 100 | 1268975 | 1268997 |
| CP047639.1 | 100 | 2711671 | 2711685 | 100 | 1269027 | 1269001 | 100 | 1268975 | 1268997 |
| CP047340.1 | 100 | 124594 | 124573 | 95.833 | 124542 | 124519 | 95.455 | 124490 | 124511 |
| KX426227.1 | 100 | 83943 | 83922 | 100 | 83891 | 83865 | 100 | 83839 | 83861 |
| CP006956.1 | 100 | 241917 | 241896 | 100 | 241865 | 241839 | 100 | 241813 | 241835 |
| CP006955.1 | 100 | 2348525 | 2348504 | 100 | 2348473 | 2348447 | 100 | 2348421 | 2348443 |
| CP006955.1 | 100 | 2348525 | 2348504 | 100 | 2348473 | 2348447 | 100 | 2372260 | 2372282 |
| CP006955.1 | 100 | 2348525 | 2348504 | 100 | 2372312 | 2372286 | 100 | 2348421 | 2348443 |
| CP006955.1 | 100 | 2348525 | 2348504 | 100 | 2372312 | 2372286 | 100 | 2372260 | 2372282 |
| CP006955.1 | 100 | 2372364 | 2372343 | 100 | 2348473 | 2348447 | 100 | 2348421 | 2348443 |
| CP006955.1 | 100 | 2372364 | 2372343 | 100 | 2348473 | 2348447 | 100 | 2372260 | 2372282 |
| CP006955.1 | 100 | 2372364 | 2372343 | 100 | 2372312 | 2372286 | 100 | 2348421 | 2348443 |
| CP006955.1 | 100 | 2372364 | 2372343 | 100 | 2372312 | 2372286 | 100 | 2372260 | 2372282 |
| CP006942.1 | 100 | 975286 | 975265 | 100 | 975234 | 975208 | 100 | 975182 | 975204 |
| KC734561.1 | 100 | 4169 | 4148 | 100 | 4117 | 4091 | 100 | 4065 | 4087 |
| FJ012880.1 | 100 | 36507 | 36486 | 100 | 36455 | 36429 | 100 | 36403 | 36425 |

**Supplementary materials - Table S4**. Target and non-target isolates used to validate assay specificity, with respective cycle threshold (Ct) values obtained from multiplex qPCR and MIC values for antimicrobial resistance determination.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Isolate (Accession Number) | Species | Antibiotic Susceptibility1 | | MIC Values (µg/mL) | | Cycle threshold (Ct) Values2 | | | |
| Macrolide | Tetracycline | Tulathromycin | Tetracycline | *erm42* | *msrE* | *mphE* | ICE*tetR* |
| 2016003312 | *H. somni* | R | S | >64 | 4 |  |  |  | 16.72 |
| 2016003129 | *H. somni* | R | R | >64 | 8 | 15.47 |  |  | 17.21 |
| 2016005177 | *H. somni* | R | R | 64 | >8 | 13.54 | 12.6 | 13.37 | 17.48 |
| 2016004379 | *H. somni* | R | R | >64 | 8 | 14.19 | 12.71 | 13.59 | 15.81 |
| 2018004447 | *H. somni* | R | R | 64 | >8 | 14.56 | 13.24 | 14.05 | 16.21 |
| 2018003024 | *H. somni* | R | R | >64 | >8 |  |  |  | 16.71 |
| 2018005809 | *H. somni* | R | R | >64 | 8 | 14.66 | 13.38 | 14.2 | 16.29 |
| 2018011103 | *H. somni* | R | R | >64 | >8 |  | 11.91 | 12.42 | 17.99 |
| 2019001055 | *H. somni* | R | R | 32 | >8 | 15.51 | 14.29 | 15.22 | 17.33 |
| 2019001996 | *H. somni* | R | R | >64 | >8 | 15.23 | 13 | 13.95 | 16.74 |
| 2016000948 | *H. somni* | R | S | >64 | <=0.5 | 12.38 | 13.31 | 14.12 | 16.65 |
| 2015003760 | *H. somni* | R | S | >64 | 4 |  |  |  | 17.01 |
| 2019002695 | *H. somni* | R | R | >64 | >8 | 15.25 | 14.01 | 14.89 | 16.92 |
| 2016004313 | *H. somni* | R | S | >64 | <=0.5 |  |  |  | 15.76 |
| 2016003735 | *H. somni* | R | S | 64 | <=0.5 | 14.52 | 13.23 | 14.15 | 16.31 |
| 2018010157 | *H. somni* | R | S | >64 | 1 | 13.11 | 14.27 | 15.11 | 17.29 |
| 2019001152 | *H. somni* | R | R | 32 | >8 | 14.61 | 13.29 | 14.19 | 16.27 |
| 2019005019 | *H. somni* | R | R | 32 | >8 | 12.32 |  | 17.54 | 26.59 |
| 2019003774 | *H. somni* | R | R | >64 | >8 |  |  |  | 21.15 |
| 2018007471 | *H. somni* | R | R | 64 | >8 | 14.61 |  | 17.11 | 25.96 |
| 2019005004 | *H. somni* | R | R | >64 | >8 |  |  |  | 20.15 |
| 2018004588 | *H. somni* | R | R | 64 | >8 | 18.68 |  | 16.97 | 25.65 |
| 2018008918 | *H. somni* | S | S | 8 | 1 |  |  |  |  |
| 2018006051 | *H. somni* | S | S | 2 | <=0.5 |  |  |  |  |
| 2019000908 | *H. somni* | S | S | 4 | 1 |  |  |  |  |
| 2019003011 | *H. somni* | S | R | 4 | >8 |  |  |  | 16.36 |
| 2019000681 | *H. somni* | S | S | 2 | 1 |  |  |  |  |
| 2017003091 | *H. somni* | S | S | 4 | <=0.5 |  |  |  |  |
| 2015004975 | *H. somni* | S | S | <=1 | <=0.5 |  |  |  |  |
| 2014010339 | *H. somni* | S | R | 4 | >8 |  |  |  | 16.77 |
| 2015004145 | *H. somni* | S | S | <=1 | <=0.5 |  | 39.24 |  |  |
| UNL 2018000848 | *H. somni* | R | S | >64 | 2 |  |  |  | 15.65 |
| UNL 2018001358 | *H. somni* | R | R | >64 | >8 |  |  |  | 21.94 |
| UNL 2018001311 | *H. somni* | S | S | 2 | <0.5 |  |  |  |  |
| UNL 2018001868 | *H. somni* | S | S | <1 | <0.5 |  |  |  |  |
| UNL 2018001110 | *P. multocida* | S | S | <=1 | <=0.5 |  |  |  |  |
| UNL 2018001311 | *P. multocida* | S | S | <=1 | <=0.50 |  |  |  |  |
| UNL 2018001309 | *P. multocida* | R | R | 32 | >8 | 16.48 | 15.08 | 15.41 | 16.43 |
| UNL 2018001126 | *P. multocida* | S | S | 2 | 1 |  |  |  |  |
| UNL 2018001132 | *P. multocida* | S | S | 4 | <=0.5 |  |  |  |  |
| UNL 2018001126 | *Bibersteinia trehalosi* | S | S | 2 | 1 |  |  |  |  |
| UNL 2018003753 | *Bibersteinia trehalosi* | R | R | 64 | >8 |  | 13.23 | 13.71 | 14.67 |
| ATCC 49244 | *Mannheimia granulomatis* | NA | NA | NA | NA |  |  |  |  |
| ATCC 27090 | *Actinobacillus pleuropneumoniae* | NA | NA | NA | NA |  |  |  |  |
| ATCC 29703 | *Bibersteinia trehalosi* | NA | NA | NA | NA |  |  |  |  |
| ATCC 43625 | *H. somni* | NA | NA | NA | NA |  |  |  |  |
| ATCC 700025 | *H. somni* | NA | NA | NA | NA |  |  |  |  |

1Susceptibility to macrolide and tetracycline antibiotics (S = susceptible; R = resistant). MIC breakpoints for macrolide (tulathromycin): 64 = R; <64 = S. MIC breakpoints for tetracycline: 8 = R; <8 = S. Tulathromycin represents macrolide antibiotics.

2Template cell lysate was prepared using a 1 McFarland Standard of pure culture growth. Ct values for each target are reported using the Rotor-gene Q instrument using threshold values of 0.1. Dynamic tube normalization was used for all analysis. ATCC reference controls were used for specificity evaluation and thus do not have MIC values availability.

**Supplementary materials - Table S5.** Assessment of sufficiency of sample size for determining the optimal cycle threshold value.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| BRD Pathogen | Sample | Class | Antibiotics | No. of Total Samples | No. of Samples with1 | | Optimal Cycle Threshold (Ct) | Required Sample Size considering PPV2 | | | Required Sample Size considering NPV2 | | | Valid3 |
| R+I | S | Sample Size | Proportion of Cases | Number of Cases | Sample Size | Proportion of Cases | Number of Cases |
| *M.haemolytica* | Lung | Tetracycline | Oxytetracycline | 191 | 81 | 110 | 31.00 | 27 | 10% | 3 | 24 | 28% | 7 | YES |
| Macrolide | Tilmicosin | 191 | 74 | 117 | 33.04 | 27 | 14% | 4 | 17 | 30% | 5 | YES |
| Macrolide | Tulathromycin | 191 | 67 | 124 | 32.89 | 24 | 13% | 3 | 13 | 34% | 4 | YES |
| Macrolide | Tilmicosin or tulathromycin | 191 | 75 | 116 | 33.04 | 27 | 14% | 4 | 18 | 30% | 5 | YES |
| Nasal | Tetracycline | Oxytetracycline | 72 | 5 | 67 | 32.26 | 17 | 5% | 1 | 12 | 80% | 10 | NO |
| Macrolide | Tilmicosin | 72 | 2 | 70 | 21.42 | 44 | 1% | 0 | 13 | 50% | 6 | NO |
| Macrolide | Tulathromycin | 72 | 6 | 66 | 30.73 | 71 | 28% | 20 | 2 | 26% | 1 | NO |
| Macrolide | Tilmicosin or tulathromycin | 72 | 6 | 66 | 30.73 | 71 | 28% | 20 | 2 | 26% | 1 | NO |
| *P. multocida* | Lung | Tetracycline | Oxytetracycline | 96 | 30 | 66 | 36.10 | 23 | 15% | 3 | 10 | 43% | 4 | YES |
| Macrolide | Tilmicosin | 95 | 16 | 79 | 32.91 | 44 | 23% | 10 | 3 | 25% | 1 | YES |
| Macrolide | Tulathromycin | 96 | 10 | 86 | 32.91 | 25 | 15% | 4 | 3 | 38% | 1 | YES |
| Macrolide | Tilmicosin or tulathromycin | 95 | 16 | 79 | 32.91 | 44 | 23% | 10 | 3 | 25% | 1 | YES |
| Nasal | Tetracycline | Oxytetracycline | 78 | 15 | 63 | 29.35 | 32 | 17% | 6 | 4 | 28% | 1 | YES |
| Macrolide | Tilmicosin | 80 | 6 | 74 | 36.40 | 168 | 36% | 60 | 2 | 41% | 1 | NO |
| Macrolide | Tulathromycin | 78 | 4 | 74 | 31.47 | 157 | 34% | 54 | 1 | 29% | 0 | NO |
| Macrolide | Tilmicosin or tulathromycin | 80 | 7 | 73 | 32.22 | 288 | 39% | 112 | 2 | 27% | 1 | NO |
| *H. somni* | Lung | Tetracycline | Oxytetracycline | 93 | 49 | 44 | 36.28 | 32 | 23% | 7 | 62 | 48% | 30 | YES |
| Macrolide | Tilmicosin | 93 | 21 | 72 | 33.08 | 56 | 29% | 16 | 5 | 34% | 2 | YES |
| Macrolide | Tulathromycin | 93 | 28 | 65 | 31.67 | 40 | 23% | 9 | 8 | 29% | 2 | YES |
| Macrolide | Tilmicosin or tulathromycin | 93 | 32 | 61 | 33.08 | 39 | 23% | 9 | 11 | 30% | 3 | YES |
| Nasal | Tetracycline | Oxytetracycline | 29 | 13 | 16 | 32.85 | 32 | 20% | 6 | 30 | 63% | 19 | NO |
| Macrolide | Tilmicosin | 29 | 3 | 26 | 30.88 | 112 | 34% | 38 | 2 | 40% | 1 | NO |
| Macrolide | Tulathromycin | 28 | 7 | 21 | 27.19 | 36 | 11% | 4 | 31 | 83% | 26 | NO |
| Macrolide | Tilmicosin or tulathromycin | 28 | 9 | 19 | 26.83 | 36 | 12% | 4 | 40 | 83% | 33 | NO |
| At least one BRD pathogen | Lung | Tetracycline | Oxytetracycline | 296 | 132 | 164 | 36.06 | 25 | 18% | 4 | 23 | 44% | 10 | YES |
| Macrolide | Tilmicosin | 295 | 101 | 194 | 33.08 | 30 | 18% | 6 | 11 | 31% | 3 | YES |
| Macrolide | Tulathromycin | 296 | 95 | 201 | 32.89 | 28 | 17% | 5 | 10 | 32% | 3 | YES |
| Macrolide | Tilmicosin or tulathromycin | 295 | 109 | 186 | 33.08 | 30 | 17% | 5 | 14 | 29% | 4 | YES |
| Nasal | Tetracycline | Oxytetracycline | 108 | 25 | 83 | 32.81 | 22 | 16% | 3 | 7 | 52% | 4 | YES |
| Macrolide | Tilmicosin | 108 | 11 | 97 | 31.82 | 48 | 26% | 12 | 2 | 33% | 1 | NO |
| Macrolide | Tulathromycin | 108 | 16 | 92 | 31.47 | 110 | 33% | 37 | 2 | 25% | 0 | NO |
| Macrolide | Tilmicosin or tulathromycin | 108 | 21 | 87 | 31.82 | 123 | 35% | 42 | 3 | 25% | 1 | NO |

1S, R, I denotes to susceptible, resistant, and intermediate resistant to the drug classified based on MIC test and CLSI breakpoints, and the categories of R and I are combined into a new category of “resistant”, or R+I. 2Minimum required sample size and the proportion of resistant samples (R+I) calculated considering PPV and NPV of importance, respectively. 3Valid takes value “YES” if the total sample size and number of resistant samples are both sufficient when considering both PPV and NPV of importance to calculate the optimal threshold based on sensitivity, specificity, and prevalence.

**Supplementary materials - Table S6.** Comparison of 5-fold cross-validation with the optimal cycle threshold (Ct) cutoff value obtained using ROC curves on the overall data.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Class | Antibiotics | No. of Samples with1 | | Overall Data | | 5-Fold Cross Validation | | |
| Optimal Cycle Threshold (Ct) | Kappa (κ) | Average Optimal Cycle Threshold (Ct) (95% CI) | Kappa (κ) on Train Data –  Average (Standard Deviation) | Kappa (κ) on Test Data –  Average (Standard Deviation) |
| R+I | S |
| Lung | Tetracycline | Oxytetracycline | 132 | 164 | 36.06 | 0.64 | 35.66  (34.8 – 36.5) | 0.64 (0.04) | 0.61 (0.17) |
| Macrolide | Tilmicosin | 101 | 194 | 33.08 | 0.61 | 33.12  (33.0 – 33.2) | 0.61 (0.04) | 0.60 (0.14) |
| Macrolide | Tulathromycin | 95 | 201 | 32.89 | 0.64 | 32.64  (32.2 – 33.1) | 0.65 (0.03) | 0.62 (0.12) |
| Macrolide | Tilmicosin or tulathromycin | 109 | 186 | 33.08 | 0.62 | 33.12  (33.0 – 33.2) | 0.61 (0.02) | 0.61 (0.09) |
| Nasal | Tetracycline | Oxytetracycline | 25 | 83 | 32.81 | 0.56 | 33.27  (32.4 – 34.2) | 0.56 (0.04) | 0.46 (0.19) |
| Macrolide | Tilmicosin | 11 | 97 | 31.82 | 0.30 | 31.83  (31.6 – 32.1) | 0.31 (0.04) | 0.26 (0.16) |
| Macrolide | Tulathromycin | 16 | 92 | 31.47 | 0.24 | 36.10  (32.4 – 39.8) | 0.10 (0.18) | 0.01 (0.09) |
| Macrolide | Tilmicosin or tulathromycin | 21 | 87 | 31.82 | 0.24 | 33.30  (30.4 – 36.2) | 0.20 (0.13) | 0.09 (0.12) |

1S, R, I denotes to susceptible, resistant, and intermediate resistant to the drug classified based on MIC test and CLSI breakpoints, and the categories of R and I are combined into a new category of “resistant”, or R+I.