Article Summary Line: In this study, we present a rapid antimicrobial susceptibility testing platform using RNA signatures for *Neisseria gonorrhoeae* upon minutes of azithromycin exposure.

Running title: RNA markers for AST in *Neisseria gonorrhoeae*

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A novel platform to accelerate antimicrobial susceptibility testing in *Neisseria gonorrhoeae* using RNA signatures

Marjan M. Hashemi, Nikhil Ram-Mohan, Nicholas R. Gessner, Karen C. Carroll, Tza-Huei Wang, Samuel Yang

Stanford University, Stanford, CA, USA (M.M. Hashemi, N, Ram-Mohan, N.R. Gessner, S. Yang); and Johns Hopkins University, Baltimore, MD, USA (K.C. Carroll, T-H. Wang)

Address for correspondence: Samuel Yang, Stanford Emergency Medicine Dept, 900 Welch Road, Suite 350, Palo Alto, CA 94304; Email: syang5@stanford.edu; Phone: (650) 725-9492; Fax: (650) 723-0121

Abstract

The rise of antimicrobial resistant pathogens can be attributed to the lack of a rapid pathogen identification (ID) or antimicrobial susceptibility testing (AST), resulting in delayed therapeutic decision at the point of care. Gonorrhea is usually empirically treated with no AST results available before treatment, thus contributing to rapid rise in drug resistance. Herein we present a rapid AST platform using RNA signatures for *Neisseria gonorrhoeae* (NG). RNA-seq followed by bioinformatic tools were applied to explore potential markers in transcriptome profile of NG upon minutes of azithromycin exposure. Validation of candidate markers using PCR showed that two markers (*arsR* (NGO1562) and *rpsO*) can deliver accurate AST results across 14 tested isolates. Further validation of our cutoff in comparison to MIC across 64 more isolates confirmed reliability of our platform. Our RNA markers combined with emerging molecular point-of-care systems has the potential to greatly accelerate both ID and AST to inform treatment.

Introduction

The use of conventional clinical methods for pathogen identification (ID) and antimicrobial susceptibility testing (AST) is a time-consuming process that contributes to the rise of antimicrobial resistance and may in turn increase mortality rates (*1*). Thus, expedition of diagnostic methods for timely and directed therapy is an urgent clinical need that is to be addressed.

46 *Neisseria gonorrhoeae* (NG), the causative agent of gonorrhea, is an increasingly common
47 sexually transmitted infection with more than 550, 000 cases reported annually in the United
48 States (*2*). Gonorrhea is usually seen in female cervicitis and male urethritis (*3*). Untreated
49 gonorrhea infections can lead to major complications such as heart and nervous system
50 infections, infertility, pelvic inflammatory disease, and newborn blindness (*4*, *5*). Gonorrhea is
51 often empirically treated based on a clinical syndromic diagnosis or after laboratory molecular
52 detection with nucleic acid amplification testing (NAAT). NAAT have largely replaced
53 diagnostic culture methods because they are faster, automated, with higher sensitivity to allow
54 for cost-effective diagnosis (*6*). Treatments often proceed without AST results. NG AST requires
55 laborious and time-consuming (at least 1-2 days) culture methods and is only undertaken in cases
56 of treatment failure (*7*, *8*).
57
58 NG can rapidly develop resistance to antimicrobial agents due to innate mechanisms for
59 acquiring resistance genes (*9*). Treatment is more challenging due to rapidly increasing
60 resistance to all of the most commonly used antimicrobials including sulfonamides, penicillin,
61 tetracyclines, and second-generation fluoroquinolones such as ciprofloxacin (*10*). Azithromycin
62 is a macrolide antimicrobial that binds to the 23S rRNA component of the 50S ribosome, thereby
63 inhibiting protein synthesis (*11*, *12*). Azithromycin has been shown an effective treatment against
64 gonococci with prolonged level in tissues and cells. Following oral administration, azithromycin
65 concentrates in tissues including genital sites (*12*). However high levels of azithromycin
66 resistance have been progressively reported worldwide (*13*, *14*). Some of the known mechanisms
67 of resistance to azithromycin include overexpression of the efflux pump (*mtrR*), 23S rRNA

68 mutation in azithromycin binding sites (C2611T and A2059G) and ribosomal target modification
69 by methylase (*ermC* and *ermB*) (*3*).
70
71 CDC reported antimicrobial resistant NG as one of the three most urgent threats to public health
72 and currently recommends dual therapy with ceftriaxone and azithromycin for the treatment of
73 uncomplicated gonorrhea (*15*). Despite recommended dual therapy, azithromycin monotherapy
74 could be still used to treat uncomplicated gonorrhea in cephalosporin-allergic patients (*16*). In
75 this study azithromycin has been selected for our phenotypic AST development for gonorrhea as
76 a proof of concept; however our approach has potential to be extended to other antimicrobials.
77
78 Given the time-consuming conventional AST, increasing level of resistance, and the challenge of
79 slow growth rate (doubling time 60min) of NG (*17*), development of a novel AST that can guide
80 initial treatment at the point of care is critically needed. Recently, RNA-seq has been effectively
81 applied in discovery of reliable genomic biomarkers to develop clinical diagnosis (*18*). Previous
82 work in our lab described a novel and accelerated AST workflow based on RNA-seq in
83 *Klebsiella pneumoniae* upon exposure to ciprofloxacin (*19*). This method is independent of cell
84 division unlike other molecular phenotypic AST that measure growth kinetics of bacteria based
85 on DNA copies (*20*, *21*). As proof-of-concept, in this study we focused on developing an ultra-
86 rapid molecular phenotypic AST for NG. RNA-seq followed by bioinformatic analysis was used
87 to identify candidate diagnostic RNA markers to determine susceptibility upon a short exposure
88 to azithromycin. Further validation of selected markers was performed through qRT-PCR using
89 14 isolates followed by validation of our cutoff in comparison to reported MICs using 64 more
90 isolates to determine susceptibility.
91
92

93 Material and methods
94
95 Microorganisms and culturing

96 Reference strains of azithromycin susceptible and resistant NG, SPL-4 and SPJ-15, were
97 obtained from CDC. Clinical isolates of NG were obtained from John Hopkins Medical
98 Institutions, Department of Pathology, Division of Medical Microbiology (Appendix Table 1).
99 Isolates were cultured from glycerol stocks on GC agar (#BD 228950, Becton Dickinson,
100 Cockeysville, MD) supplemented with 1% IsoVitaleX (#BD 211876, Becton Dickinson,
101 Cockeysville, MD). Plates were incubated at 35 °C, in 5% CO_2 for 24 h. A single colony of each
102 isolate was re-suspended in GW broth (*22*) and incubated overnight at 37 °C, 5% CO_2, 200 rpm
103 to an optical density equal to that of a 0.5 MacFarland standard.
104

105 Antimicrobial susceptibility testing

106 The E-test, bioMérieux (Durham, NC, USA), or agar dilution method, was used to determine the
107 minimum inhibitory concentration (MIC) of azithromycin (Astatech, Inc, Bristol, PA) (*23*). Agar
108 dilution method was performed using GC agar supplemented with 1% IsoVitaleX described by
109 the Clinical and Laboratory Standards Institute (CLSI). Briefly, two-fold dilutions of
110 azithromycin (range 0.03-256 μg/mL) was added to CG medium (*24*). Each plate was inoculated
111 with a 0.5 McFarland standard of bacterial suspension. Inoculated plates were incubated at 35
112 °C, in 5% CO_2 for 24 h. Measurements were performed in triplicate and positive and negative
113 controls were included for each MIC test. Following incubation, MICs were determined as the
114 lowest concentrations of azithromycin that inhibited visible growth of bacteria. According to the
115 guidelines set forth by the CLSI, isolates with MIC ≤2 μg/mL are classified as azithromycin
116 susceptible (*25*).

117

118 RNA extraction and sequencing

119 Bacterial culture (2mL) was prepared as described previously. Samples were exposed to
120 azithromycin (2μg/mL) and incubated at 37 °C, in 5% CO_2 for 10 minutes and 60 minutes (see
121 workflow in Figure 1). Bacteria without exposure to azithromycin were also incubated in the
122 same condition as a control (water was added to the controls). Samples were collected and
123 preserved in 4mL RNA Protect Reagent (Qiagen, Valencia, CA, USA) at each time point. RNA
124 extraction was conducted using RNeasy Mini Kit (Qiagen,74524) according to the
125 manufacturer's instruction. Extracted RNA was subjected to DNase treatment (Turbo DNase
126 complete kit Life Technologies, AM1907). Concentration of RNA was measured using QIA
127 expert spectrophotometer (Qiagen, USA). To consider biological variability triplicate RNA
128 samples were prepared from susceptible and resistant isolates.

129

130 Sample preparation for sequencing was performed using ScriptSeq complete kit for bacteria with
131 rRNA depleted (Illumina Inc, BB1224, RSBC10948 and SSIP1202). Samples were bio-analyzed
132 at Stanford Functional Genomics Facility (SFGF) and libraries were sequenced at 75 pair-end
133 reads on Illumina MiSeq. Quality control on raw reads from the sequenced libraries was
134 conducted to remove the low-quality reads (using FastQC) (*26*), remove PCR duplicates (using
135 SuperDeduper) (*27*) and trim the adaptor sequences (using Trim Galore, version 0.4.5) (*28*).
136 Finally, reads were aligned to NG FA 1090 (NCBI Reference Sequence: NC_002946.2RNA-seq
137 data are archived in the GenBank SRA (BioProject ID: PRJNA627416).

138

139 Differential expression analysis

140 Quantification of transcript abundance and differential expression analyses were carried out
141 using Rockhopper (*29*). Spearman correlation between samples/replicates from the normalized
142 transcript counts obtained from Rockhopper were calculated using the cor function in R (*30*).
143 The multidimensional scaling (MDS) that show the dissimilarity of the replicates/samples by
144 projecting them into two dimensions was also done using the cmdscale function.
145 To further analyze the differentially expressed genes under azithromycin treatment, the list was
146 cut down to 568 differentially expressed genes with a p-value<0.05 in susceptible compared to
147 control (SvC) and resistant (SvR) strains. Heatmaps were generated using the heatmap.2 function
148 to represent the logFC between these comparisons.
149
150
151 Marker selection and validation
152 Candidate markers were selected from the RNA-seq dataset based on the following criteria: 1)
153 significant fold change in susceptible but not in resistant after 10min drug exposure; 2) high level
154 of expression after 10min drug exposure; 3) same trend of expression in 10&60 min drug
155 exposure. Selected candidate markers were further evaluated using qPCR.
156
157 Validation of final markers in clinical isolates was performed using quantitative real-time PCR
158 (qRT-PCR). Primer-blast was used to design primers for conserved regions of selected genes
159 (Appendix Table 2). Quantification of gene expression level was conducted with Rotor-Gene
160 SYBR Green RT-PCR kit (Qiagen, cat# 204174) on the Rotor-Gene-Q PCR cycler (Qiagen).
161 The concentration of component used in final 25µL PCR reaction mix were as follows: 1x SYBR
162 Green Master mix, 1mM forward primer, 1mM reverse primer, 0.05U/µL Rotor Gene RT mix
163 and 0.2U/µL SUPERase-in RNase inhibitor and 8% RNA template.

164 Expression of each candidate marker was quantified in the control and treated samples.
165 Threshold of 0.1 was set to obtain accurate CT values for each isolate. This threshold is a point at
166 which amplification plot reaches a fluorescent intensity above background level (*31*). To
167 calculate ΔCT value, a housekeeping gene was used to normalize potential differences between
168 the control and treated samples. From our RNA-seq results, *atpA* gene was selected as an internal
169 control as its expression level did not affect significantly upon antimicrobial treatment for the
170 tested isolates in this study. Finally, fold change (FC) in gene expression between the control and
171 treated samples was calculated as follows: $FC=2^{(-\Delta\Delta CT)}$ (*18*).
172
173 **Results**
174 Shift in transcriptome response following azithromycin exposure
175 To identify markers that significantly differentiate susceptible and resistant isolates of *N.*
176 *gonorrhoeae*, RNA-seq was used to compare their transcriptome response after 10 and 60 min of
177 azithromycin exposure. A summary of workflow is shown in Figure 1.
178 Multidimensional scaling analysis of the expression profile of the biological replicates, treatment
179 conditions, and the two strains revealed strong reproducibility amongst replicates and
180 dissimilarity between the conditions and strains (Figure 2). MDS plot was also used to provide
181 insights into the association between transcriptional profile and exposure time (10 and 60 min) to
182 azithromycin. Biological replicates of control and treated samples clustered very closely
183 indicating high correlation among replicates and high reproducibility of library preparation.
184 MDS plot demonstrated four distinct transcriptional clusters within the first two dimensions
185 (dim1 and dim2), an indication of remarkable transcriptional changes upon antimicrobial
186 exposure. Well separation of the control and treated samples in the first dimension showed that
187 the sequencing data were qualified for identification of differentially expressed genes.

188 Additionally, a significant diversity between the gene expression of 10min and 60 min treated
189 samples was observed suggesting that distinct gene expression profiles are triggered by
190 azithromycin in a time-dependent manner.
191 Subsequently, statistical analysis of differentially expressed genes following exposure to
192 azithromycin was calculated using logFC (gene expression ratio of treated compared to the
193 control) against their p-values <0.05 (Figure 3A). Consistent with MDS results, a global shift in
194 SvC and SvR was observed as early as 10 min of azithromycin exposure, although distribution of
195 gene expression profile illustrated a higher magnitude of fold change at 60 min.
196
197 Marker selection and validation of candidate markers in *NG* clinical isolates
198 Differential expression analyses of the susceptible strain revealed more down than up regulated
199 genes at 10 minutes (185:129). At 60 minutes however, there are 386 and 212 genes up and
200 down regulated, respectively (Appendix Table 3). Since we are interested in markers that will
201 successfully determine susceptibility, we further screened for genes that were differentially
202 expressed between SvC and SvR. A p-value cutoff of 0.05 resulted in 568 genes (Appendix
203 Figure 1) which narrows down to 48 when screening for abs(logFC) ≥1 under both conditions
204 (Figure 3B).
205
206 Among the differentially expressed genes with significant logFC, two more criteria including
207 high level of expression and same trend of expression in 10&60 min (see methods) were applied
208 to select the candidate markers. The initially selected markers include NGO0373, NGO1920,
209 NGO1562, NGO0191, NGO0405 and NGO1078, hereafter referred to as *ABC*, *bolA*, *arsR*, *rpsO*,
210 *dinD* and *acoT*, respectively (Appendix Table 2). These were tested in one susceptible and one

211 resistant strain to confirm the diagnostic potential of markers to characterize azithromycin
212 susceptibility using RT-qPCR. All six candidate RNA markers are highlighted in Appendix
213 Table 4, among which *ABC*, *bolA*, *arsR*, and *rpsO* are upregulated while *dinD* and *acoT* are
214 downregulated. Further validation of markers was conducted in eleven susceptible and one
215 resistant clinical isolates of NG to confirm that RNA-seq nominated markers can be applied in
216 different strains (Figure 4). We calculated -ΔΔCT values for samples following 10 min and 60
217 min exposure to azithromycin and compared it between resistant and susceptible isolates for each
218 marker. Overall -ΔΔCT of all six selected candidate markers was significantly different across
219 resistant and susceptible isolates indicating qualification of these candidate markers to be applied
220 in our new AST platform. Among six tested markers *arsR* and *rpsO* demonstrated consistent
221 upregulation trends across all tested isolates. Although *ABC* and *bolA* were also validated as
222 significantly upregulated RNA markers in most tested isolates, down regulation of these two
223 markers was observed in a few tested susceptible isolates. On the other hand, *dinD* and *acoT*
224 were validated as significantly downregulated RNA markers, however inconsistency of results
225 from a few isolates was also found. According to the validation results of six selected markers,
226 further analysis was conducted using *arsR* and *rpsO* as our final markers.
227
228 We examined the reliability of our method compared to the gold standard using relationship
229 between $2^{-\Delta\Delta CT}$ values of final markers and MIC in susceptible and resistant strains after 10min
230 exposure to azithromycin (Figure 5A). For both *arsR* and *rpsO*, $2^{-\Delta\Delta CT}$ values significantly
231 changed between susceptible and resistant and notably no overlap in $2^{-\Delta\Delta CT}$ values was observed
232 between two groups of susceptible and resistant strains. Additionally, a $2^{-\Delta\Delta CT} = 2$ was defined as
233 threshold susceptibility for susceptible and non-susceptible isolates ($2^{-\Delta\Delta CT} \geq 2$ susceptible, 2^{-}

234 $^{\Delta\Delta CT}$< 2 non-susceptible). We also used a linear fitting to show how transcriptional response of
235 the final markers are associated to the MIC in susceptible and resistant strains. A strong negative
236 relationship was observed between $\log_2 2^{-\Delta\Delta CT}$ and $\log_2$ MIC values, (R^2 values 0.64 and 0.63 for
237 *arsR* and *rpsO* respectively), meaning $2^{-\Delta\Delta CT}$ value in susceptible strains is significantly higher
238 than that in resistant strains (Figure 5B).
239
240 Finally, to validate our susceptibility threshold, we used MIC information of two panels of NG
241 including 64 clinical isolates from CDC (Antibiotic Resistance Isolate Bank) on the equation for
242 the line of best fit for our markers. Notably, *arsR* supported the delineated $2^{-\Delta\Delta CT}$ value by
243 correctly classifying susceptibility of all 64 CDC isolates based on our $2^{-\Delta\Delta CT}$ threshold as 51
244 susceptible strains and 13 resistant strains that was in agreement with CLSI susceptibility
245 category (MIC breakpoint of ≥2μg/ml) (Appendix Table 5). *rpsO* also accurately identified
246 susceptibility of most tested isolates with a broad range of MIC, although susceptible isolates
247 with MIC at 1μg/ml were classified as non-susceptible isolates; however, this category is in
248 agreement with EUCAST breakpoint values (MIC breakpoint of ≥1μg/ml).
249
250 In summary, these observations confirmed the accurate application of $2^{-\Delta\Delta CT}$ values to categorize
251 susceptibility of isolates as a replacement of MIC in traditional AST.
252
253 **Discussion**
254 To stem the tide of multi-drug resistant NG, a rapid NAAT-based diagnostic with compatible
255 molecular phenotypic AST capability is critically needed to inform initial treatment decisions. In

256 this study, we have discovered azithromycin-susceptibility RNA markers and demonstrated their
257 early promise for ultra-rapid AST in NG.
258
259 Transcriptome profiling of susceptible and resistant NG upon exposure to azithromycin revealed
260 a significantly altered response as early as 10 min in SvC and SvR (Figures 2 & 3). While
261 significantly different transcriptional response among NG was previously reported after 60 min
262 azithromycin exposure (*32*), our main objective was to discover the earliest susceptibility RNA
263 markers to minimize AST time. Therefore, two time points of 10min and 60min were included in
264 azithromycin treatment to be able to identify consistent and reliable RNA markers as part of
265 early cellular stress responses to antimicrobials. Unlike the previous reported studies at which
266 sublethal concentration of drugs was used (*8*, *32*), we studied bacterial transcriptomic profile
267 following a high concentration of azithromycin (2μg/mL) to enhance the discovery of
268 differentially expressed genes (*19*). Six candidate markers were initially identified through RNA-
269 seq and subsequently tested in 14 isolates with qRT-PCR. *arsR* and *rpsO* were chosen as our two
270 final markers as they were able to consistently determine susceptibility based on CT value only
271 after 10min antimicrobial exposure (Figure 4).
272
273 *arsR* gene (NGO1562) encodes a transcriptional regulatory protein that has been shown to
274 respond to environmental stimuli, such as iron. Upregulation of *arsR* may also play a role in
275 anaerobic growth of NG (*33-35*). Our study is the first to associate *arsR* with antimicrobial
276 susceptibility. Transcript *rpsO*, encoding 30S ribosomal protein S15, also forms a bridge to the
277 50S subunit to contact the 23S rRNA (*36*). Upregulation of ribosomal protein-encoding
278 transcripts such as *rpsO* upon antimicrobial exposure has been shown previously (*32*). Notably,

279 previously reported RNA markers for NG upon antimicrobial exposure included a different panel
280 of markers (except *rpsO* which is in common in both studies) suggesting that drug concentration,
281 antimicrobial exposure time, path of marker selection and bioinformatic pipelines could result in
282 different outcomes and should be considered in further application of this approach.
283
284
285 In order to use any novel AST platform in clinical decision making, it is necessary to compare it
286 to the gold standard AST method, MIC, which has been in used for decades (*37*). However,
287 current phenotypic assays still mistakenly classify some resistant strains as susceptible resulting
288 in failed clinical antimicrobial therapy (*38*). In this study, the measured changes in transcription
289 level, $2^{-\Delta\Delta CT}$, was correlated to MIC and translated to the susceptibility category resulted in a
290 threshold of a $2^{-\Delta\Delta CT} \geq 2$ for susceptibility (Figure 5). This threshold is further supported by the
291 clustering of 64 susceptible and resistant CDC isolates with the 14 experimentally tested strains
292 using their known MICs and calculated ΔΔCT values (Appendix Table 5). However, when
293 isolates with MIC=1 μg/mL, categorized as susceptible based on CLSI breakpoint, were tested
294 using *rpsO* linear fitting, it was categorized as non-susceptible based on our $2^{-\Delta\Delta CT}$ threshold. Of
295 note, this classification is in agreement with EUCAST susceptibility breakpoint where an isolate
296 with MIC≥1μg/ml is called resistant. This result highlights the difficulties underlying
297 azithromycin susceptibility across NG isolates due to lack of a universal breakpoint
298 interpretation in conventional AST confirming the critical need for a new AST with a reliable
299 susceptibility breakpoint (*24*).
300
301 Overall, we have demonstrated the potential for antimicrobial susceptibility RNA marker
302 discovery through combined application of NGS, bioinformatics, and validation assays. A larger

303 scale experimental validation of these putative RNA markers by testing additional NG strains
304 with different resistance mechanisms to azithromycin is still needed to better assess test accuracy
305 and reproducibility. We envision translation of our ultra-rapid susceptibility markers into an
306 NAAT-based diagnostic with combined ID and AST capability for point-of-care use (Figure 6).
307 We have previously developed a palm-sized magnetofluidic platform with combined bacterial ID
308 and AST directly from swab samples in less than 2.5 hours, of which AST required 2 hours of
309 antimicrobial incubation to assess for bacterial doubling (*39*). Integrating our growth-
310 independent, ultra-rapid AST markers on this platform would compress total assay time down to
311 40 minutes, drastically shifting the current paradigm for diagnosing and treating NG infections.
312

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316

317 **Author Bio**
318 Dr. Marjan M.Hashemi is a postdoctoral fellow in the Department of Emergency Medicine at
319 Stanford University, Palo Alto. Her primary research interests include developing molecular
320 diagnostic assays for pathogen identification and antimicrobial susceptibility testing.
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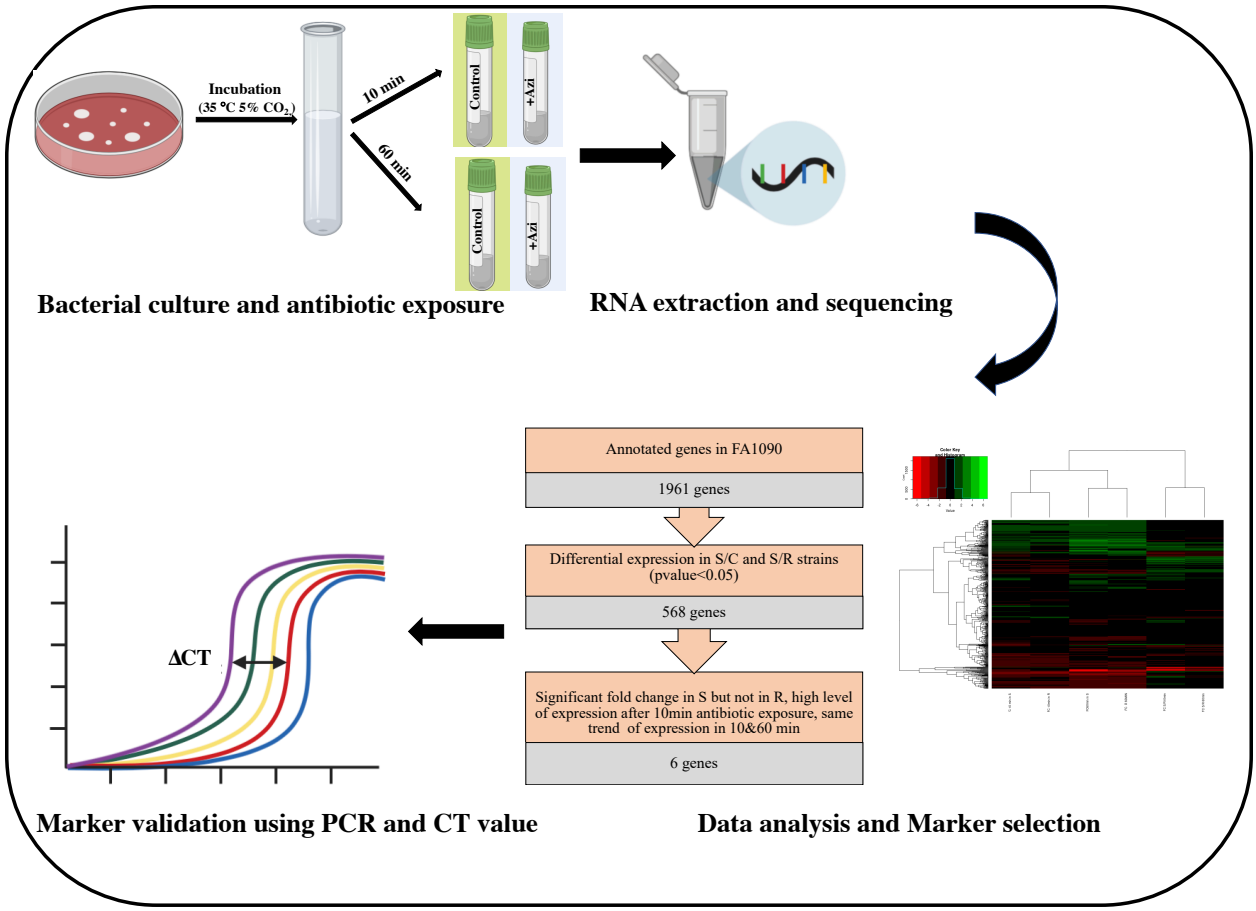


Figure 1: Summarized workflow for generation of candidate RNA markers for the proposed molecular AST. NG was cultured and exposed to azithromycin for 10 min and 60 min along with a control without azithromycin. Samples were collected for RNA-seq library and sequencing. Data analysis was conducted to find differentially expressed genes followed by marker selection steps. Selected markers were validated by qRT-PCR and ΔCT calculation.
This figure is created with BioRender.com and PPT.

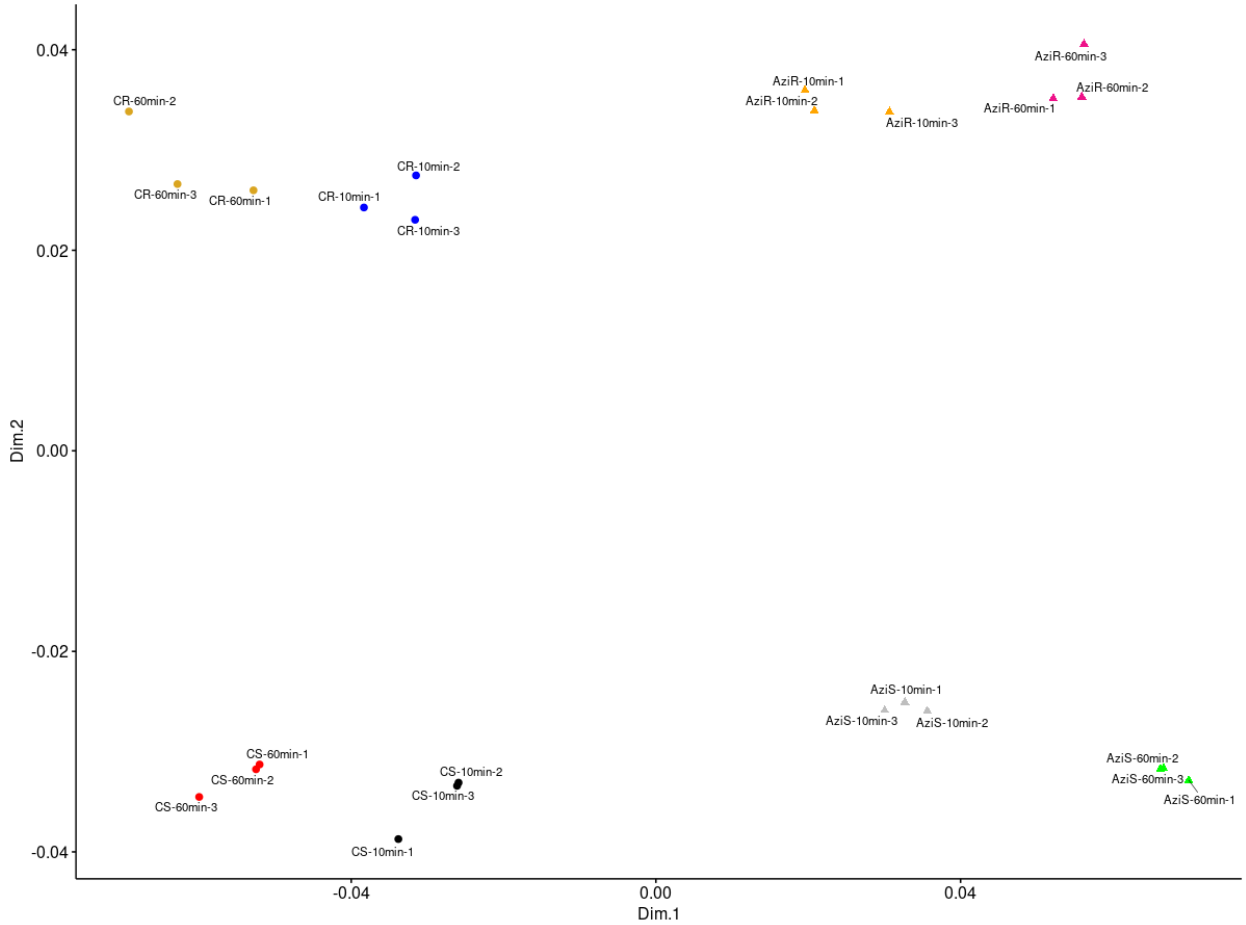
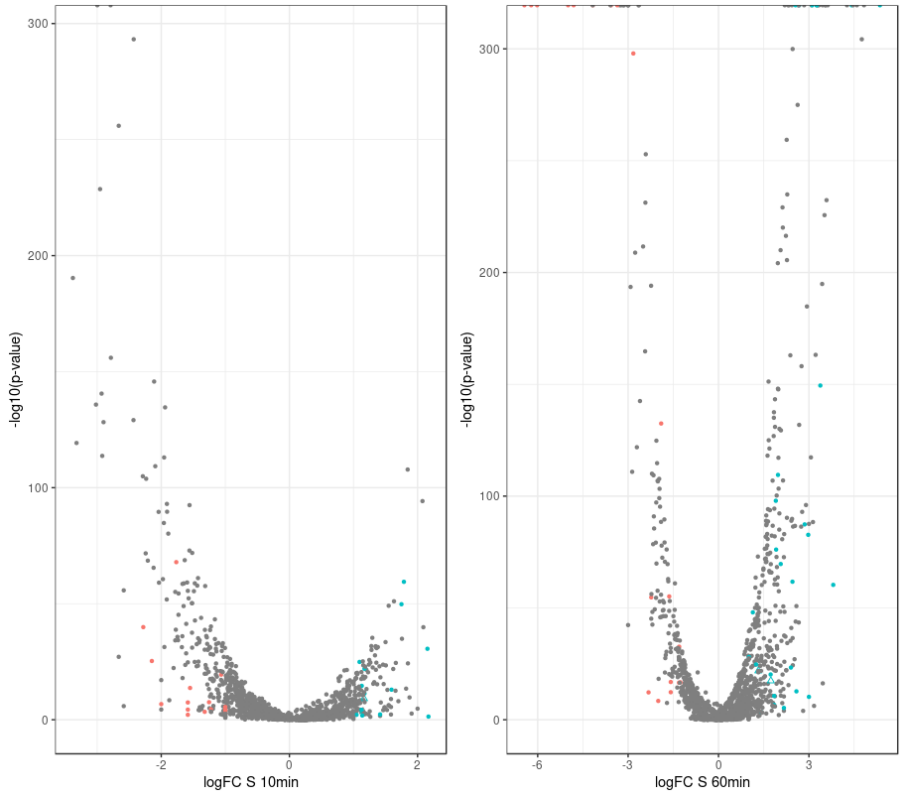


Figure 2: MDS plot to display differences in the azithromycin-induced gene expression profile at 10 and 60 min. Dissimilarity in the expression profiles between the replicates, strains, and conditions were calculated and plotted.

377 **A**



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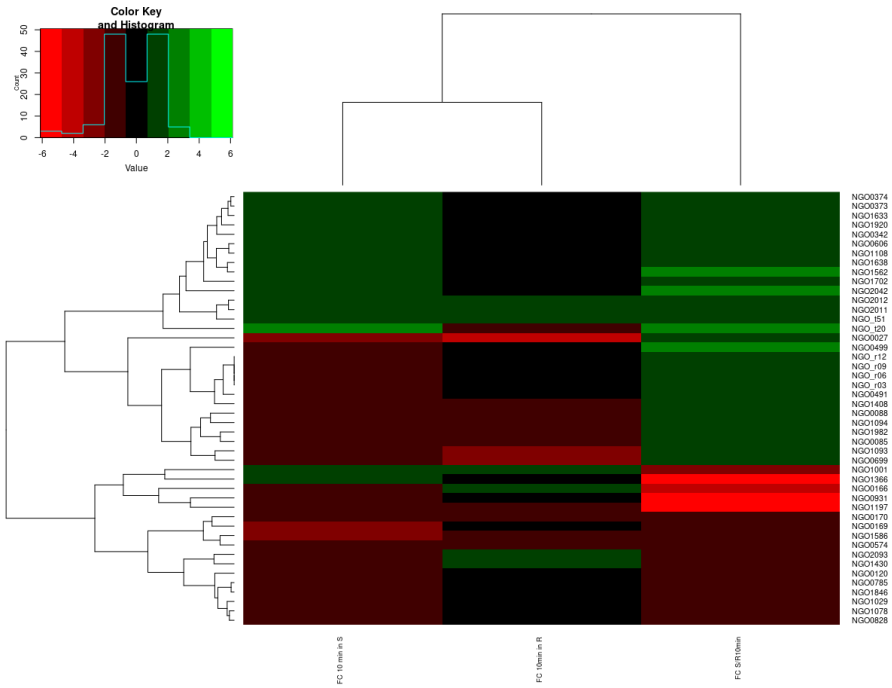
379 **B**



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381 Figure 3: (A) Volcano plot to show the gene expression profile induced by azithromycin varied
382 significantly at 10 and 60 min between the treated and untreated susceptible strains. Colored
383 points indicate genes that are up or down regulated in the SvC as well as SvR comparisons at

each time point. (B) Heatmap for differentially expressed genes (p-value<0.05 with a FC≥ 1) in SvC and SvR induced by 10min azithromycin exposure.

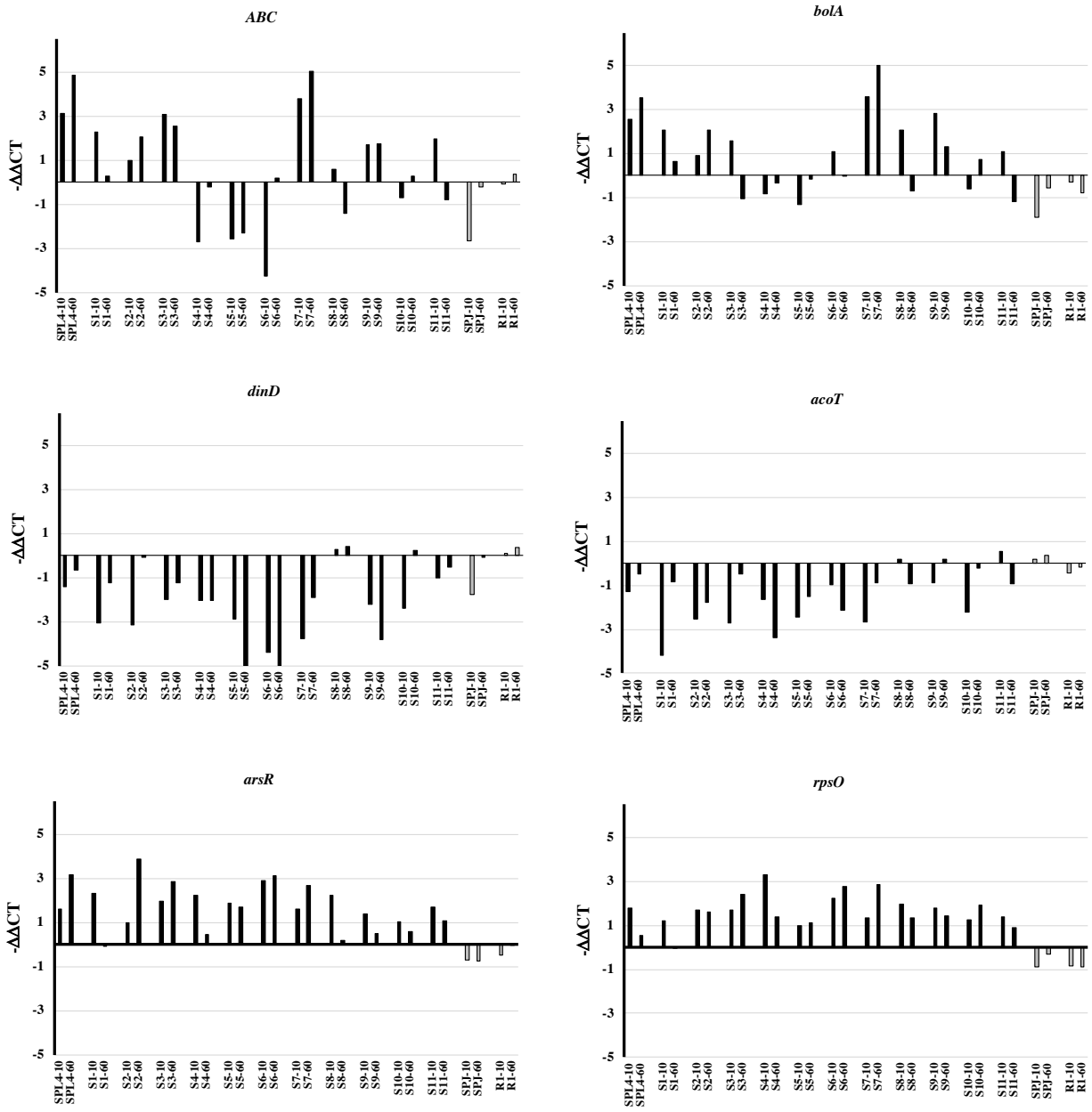

Figure 4. Validation of 6 RNA markers across 14 tested NG isolates using qRT-PCR. Black: susceptible isolates; grey: resistant isolates. *ABC*, *bolA*, *arsR* and *rpsO* upregulated and *dinD* and *acoT* downregulated RNA markers.

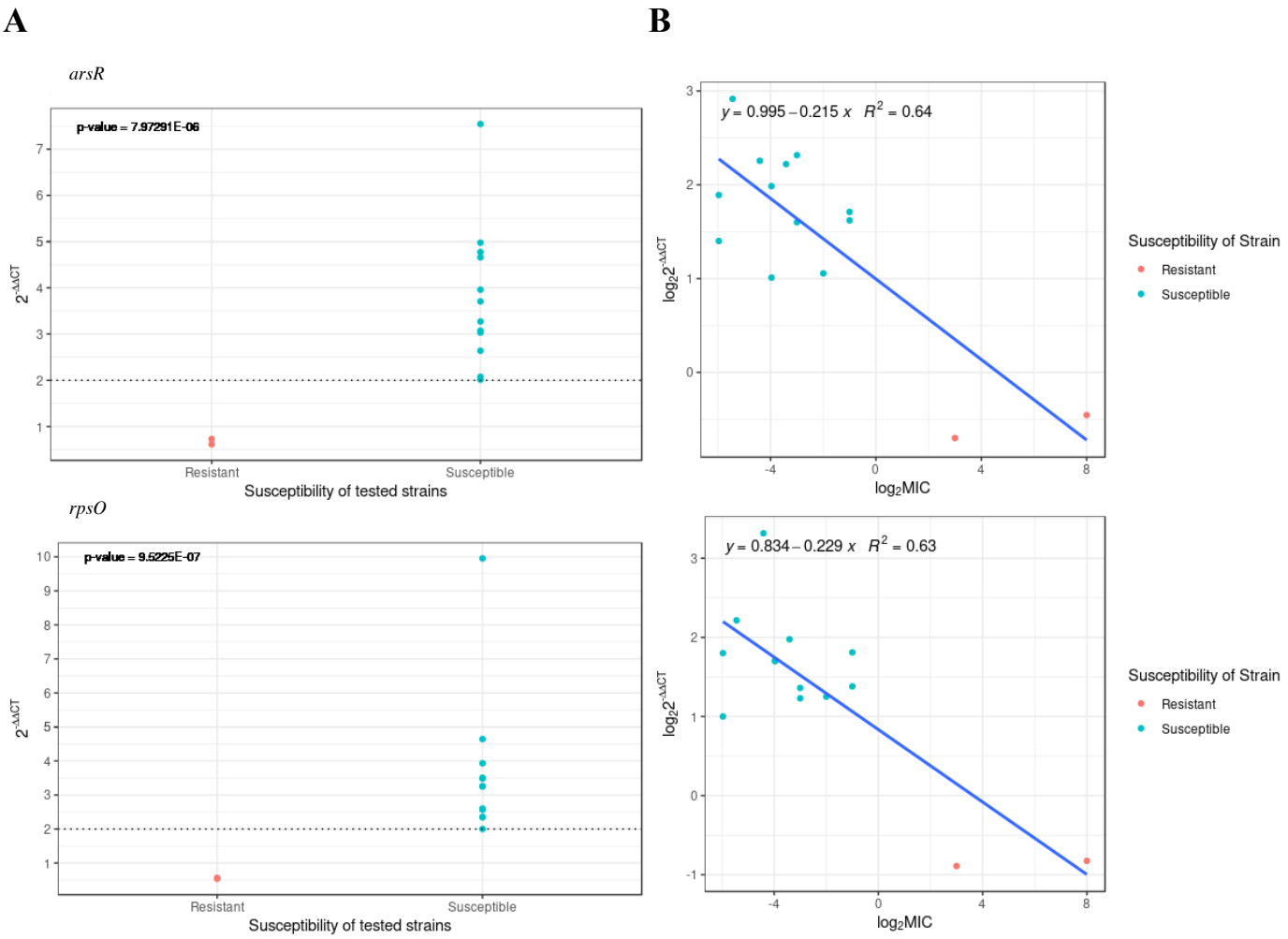

Figure 5. (A) Determination of susceptibility threshold by correlation of MIC and our AST platform using *arsR* and *rpsO* across14 tested NG isolates.(B) Linear fitting for *arsR* and *rpsO* to show how transcriptional response is associated with the MIC across14 tested NG isolates (12 susceptible and 2 resistant).

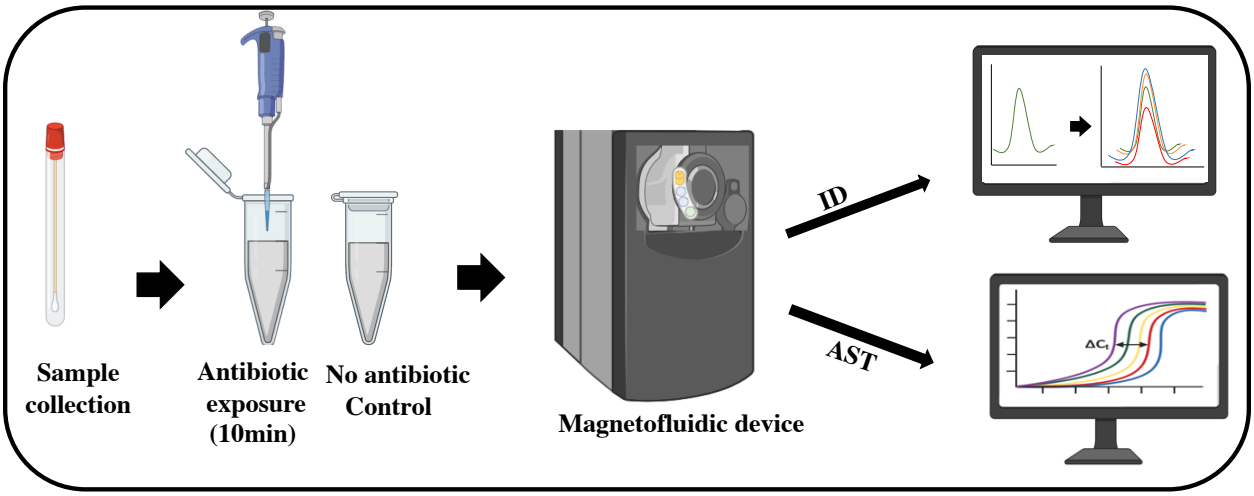

Figure 6. Proposed workflow for combined ID and AST using a novel molecular point-of-care systems such as a magnetofluidic device (*39*). Clinical samples are collected and exposed to antimicrobial for 10min. Control and antimicrobial treated samples are loaded into magnetofluidic device for further ID and AST.
This figure is created with BioRender.com and PPT.

Supplementary data:
Appendix Table1: Susceptibility profile of tested reference and clinical isolates of NG.
Appendix Table2: Candidate markers information including gene description, locus tag and primer sequences used for qPCR validation.
Appendix Table3: List of differentially expressed genes in NG upon 10min and 60min exposure to azithromycin.
Appendix Table 4: List of 568 differentially expressed genes (p-value<0.05) in S/C and S/R induced by 10min azithromycin exposure.
Appendix Table5: Validation of our susceptibility threshold ($2^{-\Delta\Delta CT} = 2$) across 64 clinical isolates from CDC (Antibiotic Resistance Isolate Bank) with a wide range of MIC values.
Appendix Figure1: Heatmap for differentially expressed genes (p-value<0.05) in S/C and S/R induced by 10min azithromycin exposure

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