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1 **Bacterial resistance to microbicides: Development of a predictive protocol**

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14 **Abstract**

15 Regulations dealing with microbicides in Europe and the United States are evolving and now
16 require data on the risk of resistance development in organisms targeted by microbicidal
17 products. There is no standard protocol to assess the risk of resistance development to
18 microbicidal formulations. This study aimed to validate the use of changes in microbicide
19 and antibiotic susceptibility as initial markers for predicting microbicide resistance and
20 cross-resistance to antibiotics. Three industrial isolates (*Pseudomonas aeruginosa,*
21 *Burkholderia cepacia, Klebsiella pneumoniae*) and two *Salmonella enterica* serovar
22 Typhimurium strains (SL1344 and 14028S) were exposed to a shampoo, a mouthwash, eye
23 make-up remover and the microbicides contained within these formulations (chlorhexidine
24 digluconate; CHG and benzalkonium chloride; BZC), under realistic, in-use conditions.
25 Baseline and post- exposure data were compared. No significant increases in minimum
26 inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) were
27 observed in any strain after exposure to the three formulations. Increases in the MIC and
28 MBC of CHG and BZC of up to 100-fold were observed in SL1344 and 14028S but were
29 unstable. Changes in antibiotic susceptibility were not clinically significant.
30 The use of MICs and MBCs combined with antibiotic susceptibility profiling and stability
31 testing generated reproducible data that allowed for an initial prediction of microbicide
32 resistance development. These approaches measure characteristics that are directly relevant
33 to the concern over resistance and cross-resistance development following use of
34 microbicides. These techniques are low cost and high-throughput, allowing manufacturers to
35 provide data to support early assessment of risk of resistance development to regulatory
36 bodies promptly and efficiently.

37

38 Keywords: microbicides, resistance, predictive protocol, regulation

39 **INTRODUCTION**

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40 Microbicides have been extensively used in the control of bacteria for decades, and
41 are commonly incorporated into a variety of products including disinfectants,
42 cosmetics, preservatives, pesticides and antiseptics. Despite this ever-increasing use,
43 bacteria generally remain susceptible to microbicides when they are used correctly.
44 However, the indiscriminate use of microbicides in a wide range of environments
45 has raised concerns about the selection of microbicide and antibiotic-resistant
46 bacteria (1, 2). Despite the establishment of the European Union (EU) biocidal
47 product regulation (BPR) (http://eur-
48 lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:167:0001:0123:EN:PDF
49 accessed 24th November 2014) to regulate the authorisation and use of biocidal
50 products throughout the EU, the total amount of microbicide use in the EU remains
51 unknown (2).
52
53 Of particular concern are formulations that contain microbicides at low
54 concentrations which may increase the risk of selection for resistance amongst target
55 or non-target microorganisms (2). Resistance or reduced susceptibility to
56 microbicides and/or antibiotics as a result of exposure to low microbicide
57 concentrations has been demonstrated extensively in the laboratory setting (3-7).
58 Despite the lack of *in vivo* or *in situ* studies reporting a link between microbicide
59 exposure and antibiotic resistance development, *in vitro* studies have clearly
60 demonstrated the possibility of microbicide and antibiotic resistance development in
61 bacteria. This has lead committees such as the Scientific Committee on Emerging
62 and Newly Identified Health Risks (SCENIHR) to produce reports and opinions on
63 the knowledge gaps and research concerns associated with resistance. In their 2010
64 opinion paper SCENIHR stated that data on microbicide usage are lacking together

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65 with an understanding of the microbicides most at risk for the development of
66 bacterial resistance
67 (http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_028.pdf,
68 accessed 24th November 2014). SCENIHR recommended the standardisation of
69 methodologies used to monitor resistance levels and suggested the development of a
70 standard protocol that could determine the risk of resistance development in a
71 particular microorganism as a result of microbicide exposure.
72
73 In support of the requirement for such a protocol, the new BPR (EU 528/2012) states
74 that it is a requirement of biocidal product manufacturers to provide information on
75 the likelihood of resistance development to their product in target organisms. In
76 particular the following articles state:
77 "(13) Active substances can, on basis of their intrinsic hazardous properties, be
78 designated as candidates for substitution with other active substances, whenever such
79 substances considered as efficient towards the targeted harmful organisms become
80 available in sufficient variety to avoid the development of resistances amongst
81 harmful organisms…"
82 "(25) … The use of low-risk biocidal products should not lead to a high risk of
83 developing resistance in target organisms."
84 "(33) When biocidal products are being authorized, it is necessary to ensure that,
85 when properly used for the purpose intended, they are sufficiently effective and have
86 no unacceptable effect on the target organisms such as resistance...".
87 In addition, the U.S. Food and Drug Administration (FDA) has also issued a
88 proposed rule to require manufacturers of antibacterial hand soaps and body washes
89 to demonstrate that their products are safe for long-term daily use, more effective

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90 than plain soap and water in preventing the spread of certain infections and do not
91 select for resistance (http://www.gpo.gov/fdsys/pkg/FR-2013-12-17/pdf/2013-
92 29814.pdf accessed 24th November 2014). A standard protocol that could determine
93 the risk of resistance development would allow microbicidal product manufacturers
94 to provide this information to the BPR and FDA promptly and efficiently.
95 Our work focuses on the development of such a protocol and has involved the
96 assessment of several laboratory techniques that can be used to measure microbicide
97 resistance (e.g. minimum inhibitory concentration (MIC)/minimum bactericidal
98 concentration (MBC) determination, antibiotic susceptibility testing, and phenotype
99 stability testing) in terms of ease of use, high throughput, cost and reproducibility.
100 Our recommended protocol encompasses MIC, MBC and antibiotic susceptibility
101 determination combined with bacterial phenotype stability testing as initial markers
102 of bacterial microbicide resistance or antibiotic cross-resistance. This study aims to
103 validate the use of these techniques in a *combination* protocol with the testing of
104 three commercially available formulations and the corresponding active microbicides
105 contained therein.
106
107
108
109 **MATERIALS AND METHODS**
110 **Bacterial strains.** A range of Gram-negative bacteria was selected for the testing of
111 three antimicrobial formulations and the corresponding microbicides contained
112 within each formulation. The bacteria included *Salmonella enterica* serovar
113 Typhimurium strains SL1344 and 14028S (obtained from the University of
114 Birmingham, UK), *Burkholderia cepacia* (UL2P; Unilever culture collection, UK),

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115 *Klebsiella pneumoniae* (UL13; Unilever culture collection, UK) and *Pseudomonas*
116 *aeruginosa* (UL-7P; Unilever culture collection, UK). The 3 Unilever strains were
117 selected as challenge organisms due to their routine use, propagation and handling in
118 Unilever laboratories.
119
120 **Culture and storage of bacteria.** Liquid cultures of all strains were grown in
121 tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) at 37°C (± 1 °C). Strains were
122 stored on protect beads (Fisher Scientific, Loughborough, UK) at -80 °C (± 1 °C)
123 and restricted to a maximum of 2 subcultures from the original freezer stock prior to
124 exposure to a given microbicide. Test inocula were prepared from harvesting an
125 overnight TSB culture centrifuged at 5000 g for 10 min and re-suspended in
126 deionised water (diH$_2$0).
127
128 **Formulations, actives and neutraliser**. A mouthwash (2 mg/mL chlorhexidine
129 digluconate; CHG), eye make-up remover (1 mg/mL CHG) and a shampoo (5
130 mg/mL benzalkonium chloride; BZC) were tested. Selection of these products was
131 based on the fact that they are commonly used home and personal care products. The
132 microbicides CHG and BZC (Sigma-Aldrich, Dorset, UK), the only microbicides
133 contained within the three formulations, were also tested. The neutraliser used was
134 composed of Tween 80 (30 g/L) and Asolectin (3 g/L) (both Sigma-Aldrich, Dorset,
135 UK). Neutraliser efficacy for mouthwash, shampoo and eye make-up remover, and
136 toxicity towards all strains was determined as described previously (3).
137
138 **Antimicrobial susceptibility testing**

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139 ***Suspension testing:*** Test strains were exposed to each formulation and each
140 microbicide at a concentration resulting in a 1-3 log_{10} reduction in CFU/mL, leaving
141 sufficient survivors for further antimicrobial susceptibility testing. Suspension tests
142 were carried out following the British Standard EN 1276 2009 protocol (8). Briefly,
143 bacterial suspensions in deionised water (diH20) produced from overnight cultures
144 were standardised to 1 x 10^8 CFU/mL. Suspensions were used within 15 minutes of
145 preparation. One mL of standardised suspension was added to 9 mL of the desired
146 formulation or active (diluted in diH20) at 1.25 times the required concentration.
147 Concentrations tested were as follows: 0.000125 mg/mL mouthwash/CHG, 0.00015
148 mg/mL shampoo/BZC and 1 mg/mL eye make-up remover/CHG. After exposure for
149 1 min (the estimated length of time spent using each formulation by the consumer), 1
150 mL of this suspension was removed and added to 9 mL of neutraliser. After
151 neutralisation, suspensions were centrifuged at 5000 g for 10 min and the
152 supernatant discarded. The remaining cells were then used in further antimicrobial
153 susceptibility testing experiments. *S. enterica* strains SL1344 and 14028S were also
154 exposed to low BZC and CHG concentrations ranging from 0.0001– 0.004 mg/mL
155 for 5 min.
156
157 ***Determination of the minimum inhibitory concentration (MIC).*** The MIC of each
158 formulation/microbicide was determined for all strains before and after suspension
159 test exposure to a given formulation/active, following the BS EN ISO: 20776-1 (9)
160 protocol. Briefly, a 96 well microtitre plate (Sterilin Ltd, Newport, UK) containing
161 doubling dilutions of a given formulation/active in TSB was set up. Concentration
162 ranges were as follows: Mouthwash/CHG 2 – 0.001 mg/mL, shampoo/BZC 1.25–
163 0.001 mg/mL, eye make-up remover/CHG 0.5 – 0.00048 mg/mL, CHG/BZC

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164 (*Salmonella* strains only) 40 – 0.019 mg/mL. An overnight broth culture of each
165 strain was standardised to 1 x 10^8 CFU/mL and 50 µL volumes of this were added to
166 the microtitre plate. The plate was incubated for 24 h at 37°C. The MIC was defined
167 as the lowest concentration of a formulation/microbicide at which no bacterial
168 growth was observed visually on the microtitre plate. (Approximate cost to test one
169 microbicide and one bacterium in triplicate: < 1€).
170
171 ***Determination of the minimum bactericidal concentration (MBC).*** The MBC of
172 each formulation/microbicide was also determined before and after suspension test
173 exposure of each strain to a given formulation/active. Twenty µL of suspension was
174 removed from each well of the MIC microtitre plate where no bacterial growth was
175 observed and the two lowest formulation/active concentrations at which growth was
176 observed, and added to 180 µL of neutraliser. Twenty-five µl of this suspension was
177 then spotted on to tryptone soya agar (TSA) and incubated at 37°C for 24 h. The
178 minimum bactericidal concentration was defined as the lowest formulation/active
179 concentration where no bacterial growth was observed on the agar plate.
180 (Approximate cost to test one microbicide and one bacterium in triplicate: < 1 €).
181
182
183 ***Antibiotic susceptibility testing.*** The susceptibility of each strain to one or more of
184 the following antibiotics was determined before and after suspension test exposure to
185 a given formulation/microbicide following the British Society for Antimicrobial
186 Chemotherapy (BSAC) disk diffusion protocol (10): chloramphenicol (50 µg),
187 ampicillin (10 µg), ciprofloxacin (1 µg), ceftriaxone (30 µg), piperacillin (30 µg),
188 ceftazidime (30 µg), imipenem (10 µg), meropenem (15 µg), tobramycin (10 µg),

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189 aztreonam (30 µg) (all from Oxoid, Baskingstoke, UK). These antibiotics were
190 selected due to their use as therapeutic agents in the treatment of infection with the
191 organisms chosen for this study. There are no available BSAC susceptibility
192 breakpoints for *Burkholderia* spp., so breakpoints for *Pseudomonas* spp. were used
193 instead in the case of strain UL2P (*B. cepacia*). (Approximate cost to evaluate
194 susceptibility of 1 strain to 6 antibiotics: < 2 €)
195
196 ***Phenotype stability testing***. The stability of any alterations in antimicrobial
197 susceptibility observed after 5 min exposure of *S. enterica* strains SL1344 and
198 14028S to a range of low CHG and BZC concentrations was investigated via the 24
199 h subculture of surviving organisms through TSB +/- a low concentration of CHG or
200 BZC as described previously (3).
201
202 ***Data reproducibility***. In order to determine the reproducibility of baseline and post-
203 exposure data obtained, the above experiments were performed on 3 separate
204 occasions (each using 3 biological replicates) over a 6 month period, resulting in data
205 values being a mean of 9 results.
206
207 ***Statistical analysis***. A Students t-test was used to compare MIC, MBC and antibiotic
208 zone of inhibition sizes before and after microbicide exposure.
209
210 **RESULTS**
211 Three formulations and their corresponding microbicides were tested on three
212 separate occasions over a 6 month period in order to determine if exposure to a given
213 microbicidal product or microbicide resulted in an alteration in microbicide or

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214 antibiotic susceptibility in test organisms. Data obtained on each occasion were
215 compared in order to determine the reproducibility of the MIC, MBC and antibiotic
216 susceptibility tests, and therefore validate the use of these tests as a high throughput
217 and low cost initial approach in the determination of the risk of resistance
218 development. The mean MIC and MBC for each test organism before and after
219 exposure to mouthwash, eye make-up remover or shampoo and their corresponding
220 microbicides (CHG, CHG, BZC) at the same concentration as that contained within
221 the product are presented in FIG.1. Exposure to one of three formulations or their
222 corresponding microbicides resulted in both increases and decreases in MIC and
223 MBC in individual strains. In the case of shampoo and eye make-up remover an
224 accurate MBC could not be determined as all 5 strains grew in the highest testable
225 concentration of the formulation. The greatest increases in MBC were observed in *S.*
226 *enterica* strain 14028S after exposure to 0.005 mg/mL CHG and mouthwash, and
227 0.015 mg/mL BZC, all of which were found to be significantly different from
228 baseline MBC values. However when considering the post-exposure MBC values
229 observed (0.08, 0.05 and 0.05 mg/mL respectively) it is clear that these values are
230 still below or equal to the concentrations of CHG and BZC present in the relevant
231 formulations when considered as a worst case scenario of product dilution by the
232 consumer. 'Worst case' dilution factors of 1 in 40 (mouthwash) and 1 in 100
233 (shampoo) were estimated based on product use, e.g. rinsing with water. This would
234 result in 0.05 mg/mL CHG in mouthwash and 0.05 mg/mL BZC in shampoo. An
235 MBC of 0.50 mg/mL for BZC is also of less concern as the primary function of BZC
236 in the shampoo is not as an antimicrobial, but as a surfactant. Very few of the
237 remaining observed changes in MIC or MBC were found to be statistically

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238 significant ($p \leq 0.05$), nor did they approach the microbicide concentrations found in
239 the formulations tested after 'worst case' product dilution by the consumer.

240 An important factor in the validation of the use of MIC and MBC determination in
241 an initial assessment of the risk of resistance development was the reproducibility of
242 the data obtained. It is clear from FIG. 1 that both the baseline and post-exposure
243 mean MIC and MBC values were highly reproducible across the 3 separate
244 experiments, as indicated by the small standard deviations observed for each strain
245 and formulation/pure active. Our protocol is based on performing MIC/MBC in two
246 fold dilutions. Standard deviations were calculated based on the MIC or MBC
247 values, which means an increase or decrease in MIC or MBC by one fold dilution
248 will result in a large standard deviation. Error bars (representing SD) on the graphs
249 displayed in FIG. 1 may only indicate an increase or decrease of one doubling
250 dilution.
251
252 There was no clinical change in susceptibility to any of the antibiotics tested after 1
253 min exposure to all 3 formulations and their corresponding microbicides, in the case
254 of all 5 strains (according to BSAC susceptibility breakpoints for
255 Enterobacteriaceae*/Pseudomonas* spp. (10) (data not shown). In the case of some
256 strains and antibiotics, statistically significant changes in the zone of inhibition size
257 were observed. However these differences were often due to an increase in the mean
258 zone of inhibition size and therefore an increase in antibiotic susceptibility [e.g.
259 ciprofloxacin, chloramphenicol, ceftazidime in *K. pneumoniae* after exposure to
260 mouthwash (0.050 mg/mL CHG) or ceftazidime in *P. aeruginosa* after exposure to
261 shampoo (0.015 mg/mL BZC)]. A statistically significant reduction in the mean zone
262 of inhibition size for aztreonam was observed in *P. aeruginosa* after exposure to

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263 0.005 mg/mL CHG, 0.015 mg/mL BZC and 1 mg/mL CHG. However *P. aeruginosa*
264 was already resistant to this antibiotic prior to microbicide exposure and therefore no
265 clinical susceptibility change was observed. It was not possible to clearly determine
266 if clinical changes in susceptibility were observed in *B. cepacia*, as there were no
267 available breakpoints provided in the BSAC protocol, and clinical susceptibility was
268 therefore based on *Pseudomonas* spp.
269 Carrying out this experiment on 3 separate occasions over a 6-month period also
270 allowed for an assessment of the reproducibility of the results obtained. The BSAC
271 method produces consistent and reproducible baseline and post-exposure data (data
272 not shown).
273
274 *S. enterica* strains SL1344 and 14028S were also exposed to a range of low
275 concentrations of CHG and BZC for 5 min before the antimicrobial susceptibility of
276 surviving organisms was determined. Tables one and two show the baseline and post
277 exposure values for SL1344 and 14028S respectively after 5 min exposure to a range
278 of low CHG and BZC concentrations.
279 In the case of both strains post-exposure MIC and MBC values for CHG and BZC
280 were all significantly different from baseline MIC and MBC values ($p \leq 0.05$). For
281 strain SL1344 the greatest increases in MIC and MBC were observed after 5 min
282 exposure to 0.004 mg/mL CHG and 0.004 mg/mL BZC (Table 1). For strain 14028S
283 exposure to 0.001 mg/mL CHG and 0.004 mg/mL BZC resulted in the greatest
284 increases in MIC and MBC in surviving organisms (Table 2). The data appear highly
285 reproducible across all 9 repeats in the case of both strains, as indicated by the low
286 standard deviation values, supporting our recommendation of the use of MIC and
287 MBC determination as an initial indicator of resistance development in bacteria. (As

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288 discussed for FIG. 1, occasions where standard deviations appear larger are due to
289 the use of doubling dilutions of a given microbicide/formulation during MIC/MBC
290 testing). Susceptibility to a range of antibiotics was also determined for strains
291 SL1344 and 14028S before and after exposure to low CHG and BZC concentrations.
292 No alterations in antibiotic susceptibility were observed (data not shown).
293
294 The stability of the increases in MBC observed after 5 min exposure of SL1344 and
295 14028S to a range of low CHG and BZC concentrations was investigated via the 24
296 h subculture of surviving organisms through TSB +/- a low concentration of CHG or
297 BZC. Table 3 and 4 show the mean MBC values after 1, 5 and 10 subcultures of
298 surviving organisms through TSB +/- CHG or BZC for SL1344 and 14028S
299 respectively. The high MBC values observed after the initial 5 min exposure to CHG
300 or BZC were lost after 1 subculture in the absence of CHG or BZC. In the presence
301 of a low CHG or BZC concentration, MBC values also returned to baseline levels
302 after 10 subcultures. This was thought to be due to cumulative damage to the cell or
303 the fact that maintaining a high MBC was detrimental to cell survival. The instability
304 of the increased MBC values suggested a low risk of stable resistance development
305 to CHG or BZC in either *S. enterica* strain at the concentrations tested. The values
306 obtained from the phenotype stability tests were reproducible between repeats (as
307 indicated by the low standard deviation values in Tables 3 and 4) and the data
308 therefore supports our recommendation of the use this technique as part of a protocol
309 to predict microbicide resistance development.
310

311 **DISCUSSION**

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312 The principle aim of this work is to design a protocol that can predict bacterial
313 microbicide resistance and antibiotic cross-resistance and give an indication of the
314 risk of resistance development. The purpose of this study was to validate the use of
315 MIC, MBC and antibiotic susceptibility determination before and after microbicide
316 exposure, and phenotype stability testing for use in the initial prediction of bacterial
317 microbicide resistance.
318 The use of existing standard protocols for MIC, MBC and antibiotic susceptibility
319 measurement (i.e. EN 1276, ISO 20776-1, BSAC disk diffusion method) helps to
320 avoid data variability which has been observed previously with MIC values obtained
321 using different methodologies. Schurmaans *et al.* (11) found that MIC values could
322 vary by a factor of up to eight if small alterations were made to the method used.
323 Phenotypic variability was avoided through the use of overnight broth cultures for
324 susceptibility testing, rather than selecting single colonies from an agar plate, which
325 has been demonstrated to result in phenotypic variability in *Burkholderia cepacia*
326 (12), illustrating the importance of consistent inoculum preparation when performing
327 susceptibility tests. In the work carried out here the inoculum was re-suspended in
328 diH20 instead of tryptone sodium chloride (TSC) buffer as TSC has been seen to
329 interfere with log reduction results due to carry over from the inoculum (unpublished
330 data). However the inoculum was used within 15 min of preparation in diH_20 to
331 avoid subjecting bacterial cells to osmotic stress.
332 The MIC, MBC and antibiotic susceptibility values for mouthwash, shampoo, eye
333 make-up remover, CHG and BZC were found to be reproducible between separate
334 experiments at the concentrations tested in all 5 test strains, confirming the
335 appropriateness of using these standard protocols. We concluded that there is a very
336 low risk of resistance development to the formulations and corresponding pure

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337 actives tested, even in the case of the elevated MICs and MBCs observed in strains
338 SL1344 and 14028S as these values were not stable in the absence or presence of
339 CHG or BZC.
340 The use of MIC and MBC in resistance prediction and making a comparison
341 between baseline and post-exposure susceptibility data is supported by our previous
342 work investigating the effect of cationic microbicide exposure on *B. lata* strain 383
343 (3). Our protocol allows the testing of any isolate of interest as data are always
344 compared for the individual isolate rather than general data for the given bacterial
345 species.
346
347 One of the criticisms of *in vitro* techniques used in microbicide resistance
348 measurement is that experimental parameters such as microbicide concentration,
349 exposure time, dilution on application and bioavailability are not reflective of in-use
350 conditions (1, 13). In our work we attempted to accurately reflect product use in
351 terms of exposure time and product concentration (i.e. any dilution of the product as
352 a result of its use). For the purpose of protocol development test concentrations used
353 were considerably lower than those found in the original formulations (i.e.
354 concentrations low enough to obtain surviving organisms), but should be kept
355 realistic when using the techniques recommended here to predict and assess the risk
356 of resistance development. Both formulations and the corresponding active
357 microbicides were tested during protocol development in order to validate the
358 different techniques used, but it must be emphasised that using such a protocol to
359 predict resistance to pure actives alone may be of less relevance than testing the
360 formulation as a whole, as multiple components of a formulation often contribute to
361 the overall microbicidal effect, or could prove antagonistic in the formulation.

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362 Although better representative of microbicide use, long-term (≥ 6 months) studies
363 investigating the effect of exposure to commonly used household microbicides on
364 antimicrobial susceptibility, have failed to demonstrate resistance development in
365 isolated bacteria (14-17). These studies are also costly and do not allow for a prompt
366 response to regulatory bodies. This suggests that in light of new regulatory
367 expectations a compromise may be required, allowing the rapid generation of data
368 and preliminary assessment of risk, using *in vitro* techniques based on existing
369 standard methods whilst controlling parameters such as microbicide formulation,
370 contact time and concentration in order to bring realism to the evaluation. The
371 protocol proposed in this study aims to achieve this.
372
373 A further recommendation of Maillard *et al.* (1) and SCENIHR (2) in the event of
374 the observation of a reproducible change in microbicide susceptibility is the
375 execution of further tests to understand the nature of the change. This could include
376 molecular techniques to investigate changes to the transcriptome and proteome as a
377 result of microbicide exposure. Genotypic alterations as a result of microbicide
378 exposure and their potential as resistance markers have been investigated by
379 numerous groups (18-20), and molecular techniques such as PCR and microarray
380 technology have been successfully used to define microbicide resistance
381 mechanisms. Although useful, molecular techniques can be complex, costly and
382 time consuming and we therefore do not recommend them as a core part of this
383 predictive protocol. Taking this in to account, FIG. 2 shows the proposed protocol
384 steps in the form of a decision tree, as well as potential steps in the event of
385 observed, reproducible resistance. A stable increase in MIC or MBC or change in
386 antibiotic susceptibility could result in risk of resistance development. It must be

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387 emphasised that the exact level of risk can only be determined through further
388 assessment. For example, a stable increase in MBC may not constitute a high level
389 of risk if this new MBC does not approach the concentration of a particular
390 microbicide intended for use (FIG. 2). Some microbicides have a long history of
391 use, and there is a large amount of literature studying their efficacy and any observed
392 bacterial resistance, e.g. chlorhexidine, triclosan, benzalkonium chloride. For these
393 microbicides there may be sufficient evidence available in the literature to support a
394 weight of evidence assessment of the risk of resistance development, before
395 considering the generation of new data on resistance (21, 22).
396

397 Our findings and proposed approach for assessment of risk can be applicable to the
398 wider use of microbicides in various settings where such compounds are applied.
399 This approach is preventative and aimed at being predictive, thereby ensuring that
400 microbicide-containing formulations are safe by design with regards to resistance
401 and cross-resistance risks, either by enabling omission of an ingredient identified by
402 the protocol as undesirable or by using the improved understanding of resistance and
403 cross-resistance mechanisms to design a formulation with an ingredient preventing
404 the expression of a microbicide-relevant resistance mechanism (e.g. efflux pump
405 inhibitors). Such a strategy has already been investigated and documented to
406 decrease bacterial resistance to antibiotics (23).
407

408 With regulatory bodies such as the US FDA and EU BPR requiring information on
409 the propensity of microbicidal products to select for resistant bacteria, it is
410 imperative that relevant, cost-effective, high throughput techniques are available in
411 order for product manufacturers to provide this information. As global harmonisation

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412 of protocols used to measure changes in microbicide susceptibility is now considered
413 a key requirement in moving microbicidal research forward (1,2), we recommend,
414 and here demonstrate, the efficacy of a protocol that allows the prediction of
415 resistance development using simple, low cost and high throughput techniques.
416

417 **Conflict of Interest**

418 This project conducted by Cardiff University was sponsored by Unilever Safety &
419 Environmental Assurance Centre that provided a PhD studentship to L Knapp.

420

421 **REFERENCES**

422 1. **Maillard J-Y, Bloomfield S, Coelho JR, Collier P, Cookson B., Fanning S,**
423 **Hill A, Hartemann P, McBain AJ, Oggioni M, Sattar S, Schweizer HP,**
424 **Threlfall J.** 2013. Does microbicide use in consumer products promote
425 antimicrobial resistance? A critical review and recommendations for a cohesive
426 approach to risk assessment. Microb. Drug Resist. **19**:344-354.

427 2. **SCENIHR** 2010. Scientific Committee on Emerging and Newly Identified Health
428 Risks: Research strategy to address the knowledge gaps on the antimicrobial
429 resistance effects of biocides. Available online at:
430 http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_028.pd
431 f; accessed 24th November 2014

432 3. **Knapp L, Rushton L, Stapleton H, Sass A, Stewart S, Amezquita A, McClure**
433 **P, Mahenthiralingam E, Maillard J-Y**. 2013. The effect of cationic microbicide

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Applied and Environmental Microbiology

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Applied and Environmental Microbiology

434 exposure against *Burkholderia cepacia* complex (Bcc); the use of *Burkholderia*
435 *lata* strain 383 as a model bacterium. J. Appl. Microbiol. **115**:1117-1126.

436 4. **Christensen EG, Gram L, Kastbjerg VG.** 2011. Sublethal triclosan exposure
437 decreases susceptibility to gentamicin and other aminoglycosides in *Listeria*
438 *monocytogenes*. Antimicrob. Agents Chemother. **55**:4064-4071.

439 5. **Escalada MG, Harwood JL, Maillard J-Y, Ochs D.** 2005. Triclosan inhibition
440 of fatty acid synthesis and its effect on growth of *Escherichia coli* and
441 *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **55**:879-882.

442 6. **Mavri A, Smole MS.** 2013. Development of antimicrobial resistance in
443 *Campylobacter jejuni* and *Campylobacter coli* adapted to biocides. Int. J. Food
444 Microbiol. **160**:304-312

445 7. **Thomas L, Maillard J-Y, Lambert RJ, Russell AD.** 2000. Development of
446 resistance to chlorhexidine diacetate in *Pseudomonas aeruginosa* and the effect of
447 a "residual" concentration. J. Hosp. Infect. 46:297-303.

448 8. **British Standards Institute**. BS EN 1276: Chemical disinfectants and antiseptics:
449 Quantitative suspension test for the evaluation of bactericidal activity of chemical
450 disinfectants and antiseptics used in food, industrial, domestic and institutional
451 areas. Test method and requirements (phase 2, step 1). 20-9-2009.

452 9. **BS EN ISO**: 20776-1 (2006) International Organisation for Standardisation.
453 Clinical laboratory testing and in vitro diagnostic test systems - Susceptibility
454 testing of infectious agents and evaluation of performance of antimicrobial
455 susceptibility test devices - Part 1: Reference method for testing the in vitro

Accepted Manuscript Posted Online

Applied and Environmental Microbiology

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Applied and Environmental Microbiology

456 activity of antimicrobial agents against rapidly growing aerobic bacteria involved
457 in infectious disease.

458 10. **Andrews JM.** 2009. BSAC standardized disc susceptibility testing method
459 (version 8). J. Antimicrob. Chemother. **64**:454-489.

460 11. **Schuurmans JM, Nuri Hayali AS, Koenders BB, ter Kuile BH.** 2009.
461 Variations in MIC value caused by differences in experimental protocol. J.
462 Microbiol. Methods **79**:44-47.

463 12. **Larsen GY, Stull TL, Burns JL.** 1993. Marked phenotypic variability in
464 *Pseudomonas cepacia* isolated from a patient with cystic fibrosis. J. Clin.
465 Microbiol. **31**:788-792.

466 13. **Harbarth S, Tuan SS, Horner C, Wilcox, MH.** 2014. Is reduced susceptibility
467 to disinfectants and antiseptics a risk in healthcare settings? A point/counterpoint
468 review. J. Hosp. Infect..**87:** 194-202.

469 14. **Aiello AE, Marshall B, Levy SB, Della-Latta P, Larson E**. 2004. Relationship
470 between triclosan and susceptibilities of bacteria isolated from hands in the
471 community. Antimicrob. Agents Chemother. **48**:2973-2979.

472 15. **Aiello AE, Marshall B, Levy SB., Della-Latta P, Lin, SX, Larson E.** 2005.
473 Antibacterial cleaning products and drug resistance. Emerg. Infect. Dis. **11**:1565-
474 1570.

475 16. **Cole EC, Addison RM, Rubino JR, Leese KE, Dulaney PD, Newell MS,**
476 **Wilkins J, Gaber DJ, Wineinger T, Criger DA.** 2003. Investigation of
477 antibiotic and antibacterial agent cross-resistance in target bacteria from homes of
478 antibacterial product users and nonusers. J. Appl. Microbiol. **95**:664-676.17.

Accepted Manuscript Posted Online
Applied and Environmental Microbiology
AEM
Applied and Environmental Microbiology

479 17. **Cole EC, Addison RM, Dulaney PD, Leese KE, Madanat HM, Guffey AM.**
480 2011. Investigation of antibiotic and antibacterial susceptibility and resistance in
481 *Staphylococcus* from the skin of users and non-users of antibacterial wash
482 products in home environments. Int. J. Microbiol. Res. **3**: 90-96

483 18. **Bailey AM, Constantinidou C, Ivens A, Garvey MI, Webber MA, Coldham**
484 **N, Hobman JL, Wain J, Woodward MJ, Piddock LJ**. 2009. Exposure of
485 *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan
486 induces a species-specific response, including drug detoxification. J. Antimicrob.
487 Chemother. **64**:973-985.

488 19. **Furi L, Ciusa ML, Knight D, Di Lorenzo V, Tocci N, Cirasola D, Aragones**
489 **L, Coelho JR, Freitas AT, Marchi E, Moce L, Visa P, Northwood JB, Viti C,**
490 **Borghi E, Orefici G, Morrissey I, Oggioni MR**. 2013. Evaluation of reduced
491 susceptibility to quaternary ammonium compounds and bisbiguanides in clinical
492 isolates and laboratory-generated mutants of *Staphylococcus aureus*. Antimicrob.
493 Agents Chemother. **57**:3488-3497.

494 20. **Yu BJ, Kim JA, Ju HM, Choi SK, Hwang SJ, Park S, Kim E, Pan JG.** 2012.
495 Genome-wide enrichment screening reveals multiple targets and resistance genes
496 for triclosan in *Escherichia coli*. J. Microbiol. **50**:785-791

497 21. **SCENIHR** 2012. Scientific Committee on Emerging and Newly Identified
498 Health Risks: Memorandum on the use of scientific literature for human health
499 risk assessment purposes - weighing of evidence and expression of uncertainty.
500 Available online at:
501 http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_s_001.pd
502 f; accessed 24th November 2014

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Applied and Environmental Microbiology
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503 22. **SCENIHR** 2014. Scientific Committee for Emerging and Newly Identified
504 Health Risks: Nanosilver: safety, health and environmental effects and role in
505 antimicrobial resistance. Available online at:
506 http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_039.pd
507 f; accessed 24th November 2014

508 23. **Poole K.** 2001. Overcoming antimicrobial resistance by targeting resistance
509 mechanisms. J. Pharm. Pharmacol. **53**: 283-294.

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510 TABLE 1: Mean baseline and post-exposure MIC and MBC values for strain SL1344 after 5 min exposure to a range of low CHG and BZC concentrations. N=9

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		Biocide concentration (mg/mL) ± SD						
MIC/MBC (mg/mL)	**Baseline**	**0.004 CHG**	**0.001 CHG**	**0.0005 CHG**	**0.0001 CHG**	**0.004 BZC**	**0.001 BZC**	**0.0001 BZC**
CHG MIC	0.03 ± 0.03	0.80 ± 0.00	0.80 ± 0.00	0.40 ± 0.00	0.80 ± 0.00	0.50 ± 2.00	0.40 ± 0.00	0.80 ± 0.00
CHG MBC	0.10 ± 0.06	2.00 ± 0.90	2.00 ± 0.00	0.40 ± 0.00	1.00 ± 0.40	3.00 ± 0.00	2.00 ± 0.00	2.00 ± 1.00
BZC MIC	0.03 ± 0.00	2.00 ± 0.00	0.30 ± 0.20	0.10 ± 0.00	0.70 ± 1.00	3.00 ± 1.00	0.80 ± 0.00	0.70 ± 1.00
BZC MBC	0.03 ± 0.03	2.00 ± 0.00	0.50 ± 0.20	2.00 ± 2.00	1.30 ± 2.00	8.00 ± 0.00	2.00 ± 0.00	3.00 ± 2.00

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522 TABLE 2: Mean baseline and post-exposure MIC and MBC values for strain 14028S after 5 min exposure to a range of low CHG and BZC concentrations. N=9

		Biocide concentration (mg/mL) ± SD			
MIC/MBC (mg/mL ± SD)	**Baseline**	**0.005 CHG**	**0.001 CHG**	**0.015 BZC**	**0.004 BZC**
CHG MIC	0.030 ± 0.03	0.10 ± 0.00	1.00 ± 0.00	0.40 ± 0.00	0.80 ± 0.00
CHG MBC	0.06 ± 0.03	1.00 ± 0.90	20.00 ± 0.00	50.00 ± 0.00	3.00 ± 0.00
BZC MIC	0.04 ± 0.03	0.80 ± 0.00	0.10 ± 0.00	0.80 ± 0.00	2.00 ± 0.00
BZC MBC	0.08 ± 0.02	1.00 ± 0.00	2.00 ± 0.60	1.00 ± 0.00	20.00 ± 0.90

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523 TABLE 3: Mean baseline and post-exposure MBC values for strain SL1344 after 1, 5 and 10 subcultures in TSB +/- 0.004 mg/mL CHG or BZC.

524

525 SC = subculture * = significantly different from baseline ($p \leq 0.05$)

	Baseline MBC (mg/mL)	**5 min CHG 0.004**	**1 SC**	**5 SC**	**10 SC**	**1 SC (CHG)**	**5 SC (CHG)**	**10 SC (CHG)**
CHG MBC (mg/mL ± SD)	0.10 ± 0.90	5.00 ± 0.00*	0.08 ± 0.00	0.09 ± 0.00	0.06 ± 0.00	0.15 ± 0.40	0.10 ± 0.40	0.10 ± 0.00
BZC MBC (mg/mL ± SD)	0.03 ± 0.00	1.50 ± 0.00*	0.04 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.19 ± 0.00*	0.50 ± 0.20*	0.06 ± 0.00
	Baseline MBC (mg/mL)	**5 min BZC 0.004**	**1 SC**	**5 SC**	**10 SC**	**1 SC (BZC)**	**5 SC (BZC)**	**10 SC (BZC)**
CHG MBC (mg/mL ± SD)	0.10 ± 0.90	5.00 ± 0.00*	0.20 ± 0.30	0.10 ± 0.00	0.10 ± 0.00	0.80 ± 0.40*	0.80 ± 0.40*	0.10 ± 0.00
BZC MBC (mg/mL ± SD)	0.03 ± 0.00	3.00* ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.78 ± 0.00*	0.60 ± 0.20*	0.03 ± 0.00

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531 TABLE 4: Mean baseline and post-exposure MBC values for strain 14028S after 1, 5 and 10 subcultures in TSB +/- 0.004 mg/mL CHG or BZC.

532 SC = subculture * = significantly different from baseline ($p \leq 0.05$)

533

	Baseline MBC (mg/mL)	5 min CHG 0.001	1 SC	5 SC	10 SC	1 SC (CHG)	5 SC (CHG)	10 SC (CHG)
CHG MBC (mg/mL ± SD)	0.06 ± 0.03	5.00 ± 0.00*	0.01 ± 0.00	0.06 ± 0.00	0.09 ± 0.00	0.80 ± 0.40*	0.80 ± 0.40*	0.06 ± 0.00
BZC MBC (mg/mL ± SD)	0.08 ± 0.02	3.00 ± 0.00*	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.19 ± 0.00*	0.20 ± 0.00*	0.06 ± 0.00
	Baseline MBC (mg/mL)	**5 min BZC 0.004**	**1 SC**	**5 SC**	**10 SC**	**1 SC (BZC)**	**5 SC (BZC)**	**10 SC (BZC)**
CHG MBC (mg/mL ± SD)	0.06 ± 0.03	5.00 ± 0.00*	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.40 ± 0.20*	0.70 ± 0.70*	0.06 ± 0.00
BZC MBC (mg/mL ± SD)	0.08 ± 0.02	3.00 ± 0.00*	0.07 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	0.19 ± 0.00*	0.20 ± 0.00*	0.06 ± 0.00

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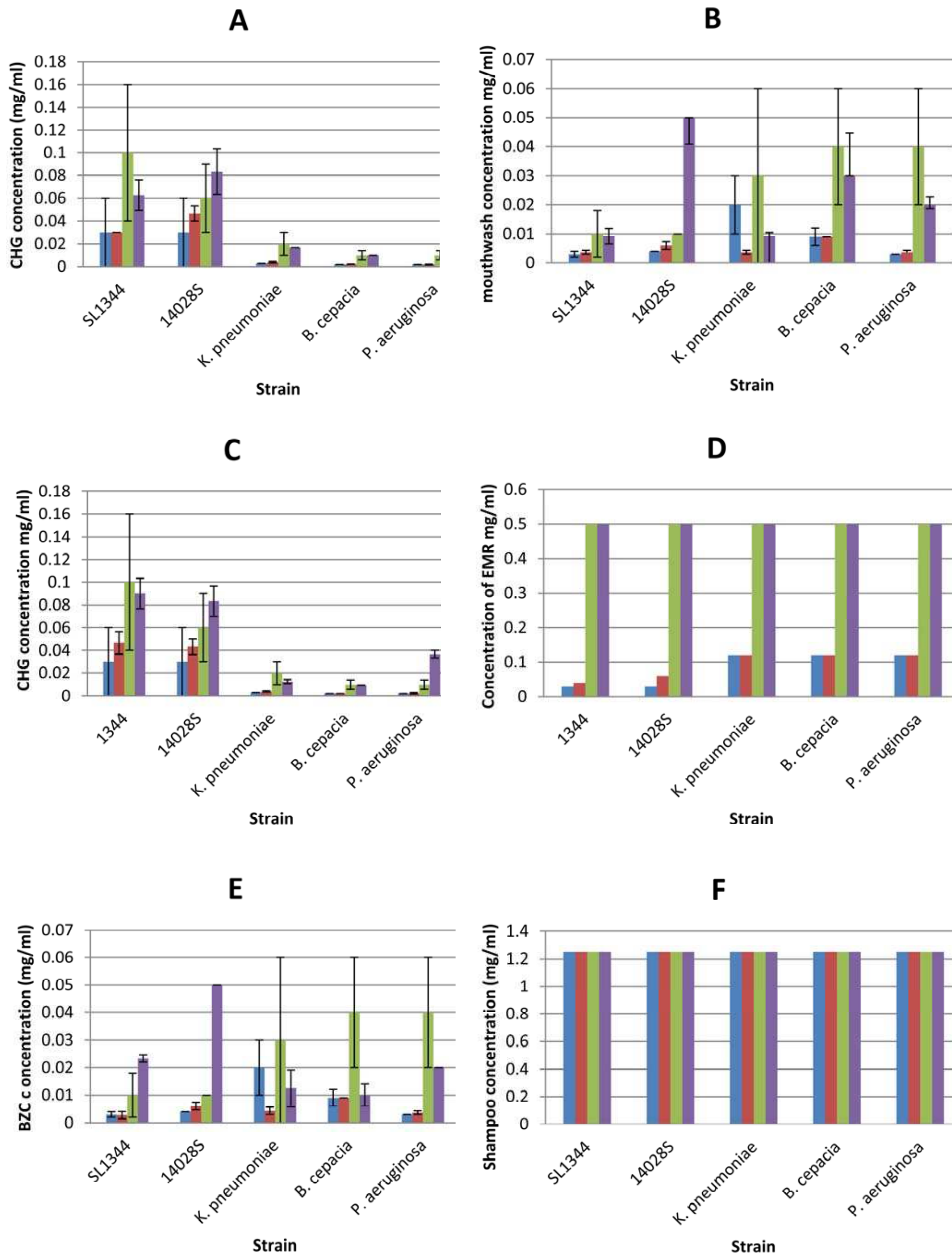

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540 FIG 1: MIC and MBC values for 5 test organisms re and after exposure to 3 formulations and their corresponding pure actives. N=9. Blue = baseline MIC. Red = post-
541 exposure MIC. Green = baseline MBC. Purple = post-exposure MBC. Error bars correspond to the SD. MIC and MBC were performed in two fold dilution (see text for
542 detailed information). A) 0.005 mg/ml CHG; B) mouthwash (0.005 mg/mL CHG); C) 1 mg/mL CHG; D) Eye-maker remover (neat: 1 mg/mL CHG); E) 0.015 mg/mL
543 BZC; F) Shampoo (0.015

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546 Figure 3: Proposed protocol for use in the prediction of bacterial microbicide resistance. Grey boxes are examples of further work that could be carried out to investigate
547 mechanisms behind changes in antimicrobial susceptibility.

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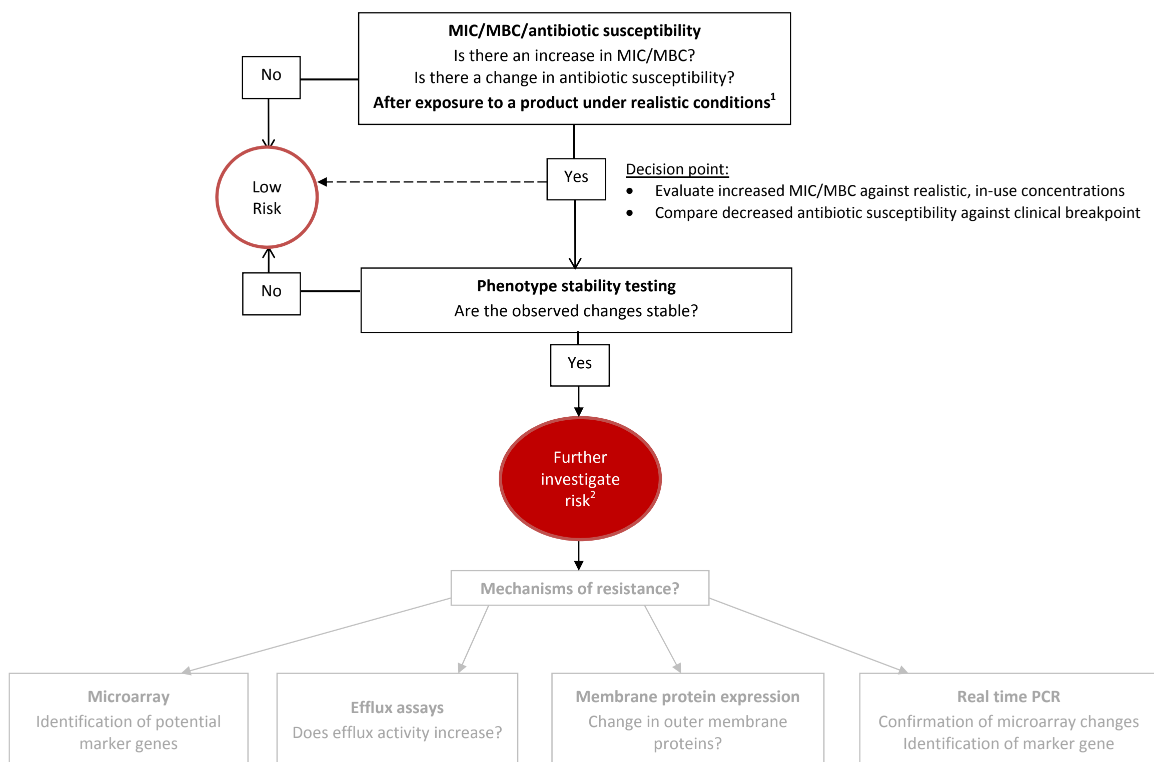

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564 Footnotes for figure 3

565 [1] Realistic conditions refers to those under which the product will be used. Factors such as concentration, contact time and product formulation should be considered in
566 order to represent product use as accurately as possible.

567 [2] If reproducible and phenotypically stable changes in antimicrobial susceptibility are observed after exposure to a particular product under realistic, in-use conditions,
568 further investigation into the risk can be carried out. This may involve the elucidation of possible mechanisms behind susceptibility changes such as the examples shown in
569 the grey boxes in figure 3, leading to better understanding of the level of risk. This investigation could be extended beyond the examples given in figure 3, and could
570 include the exploration of additional resistance markers and the use of additional techniques.

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