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1 **"How inkjet printing technology can defeat multidrug-resistant pathogens"**

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15 **Keywords:** antimicrobial susceptibility testing, broth microdilution, multidrug-

16 resistance, antibiotic resistance, digital dispensing technology, inkjet printing,

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18 **Body of Article**

19 When a patient presents with a serious infection, clinicians will start empiric
20 antimicrobial therapy, an informed prediction as to what will be a successful treatment. We
21 know that when empiric therapy does not match the organism's antimicrobial susceptibility
22 profile, there is suboptimal clinical outcome [1, 2]. The longer the time on inappropriate
23 therapy, the worse the patient does. Unfortunately, with the emergence of multidrug resistant
24 (MDR) pathogens, our empiric therapy predictions are increasingly wrong. This new reality is
25 dramatically illustrated in patients with carbapenem-resistant *Enterobacteriaceae* bloodstream
26 infections where delay in institution of appropriate therapy is associated with significantly
27 increased mortality [3].

28 Therefore, to identify needed corrections to empiric therapy, patient specimens are
29 cultured by a hospital-based clinical microbiology laboratory to isolate and identify the infecting
30 pathogen, and to determine which antimicrobials are active against it. The gold standard
31 methods for antimicrobial susceptibility testing (AST) are broth and agar dilution, which involve
32 preparing a doubling dilution series of antimicrobials and determining the lowest concentration
33 at which bacteria are inhibited (the minimal inhibitory concentration, or MIC). However, these
34 methods are complex and laborious, precluding their use in hospital-based clinical laboratories.
35 Therefore, AST is typically performed using automated platforms, a process which practically
36 takes one day. We call the time between initiation of empiric therapy and the availability of the
37 antimicrobial susceptibility profile the "antimicrobial susceptibility testing gap" (ATG). The gap
38 may be two to three days, taking into account the time needed for isolation of the organisms and
39 AST.

40 Unfortunately, the ATG may be particularly long for MDR bacteria, the type of
41 organisms where our empiric therapy guesses are most likely to be wrong. Specifically, standard
42 AST methods used by hospital-based laboratories often consist of commercially-produced, pre-
43 fabricated, fixed panels of antimicrobials chosen to match hospital formularies. However, for
44 MDR pathogens, we often find that the organism is either (1) resistant to all agents tested or (2)
45 practically resistant because the patient is allergic to or cannot tolerate side effects from active
46 antimicrobials.

47 We therefore need to test second line agents, leading to further delay and a longer ATG.
48 In fact, we often need to test agents of last resort such as colistin and newly released drugs that
49 are unavailable in either pre-made panels or surrogate methods such as disk diffusion. These
50 drugs can only be tested by technically complex broth and agar dilution reference methods,
51 which are unavailable in hospital-based clinical laboratories. Isolates are therefore commonly
52 sent to a reference laboratory for such testing, extending the ATG up to 7 days, a clearly
53 unacceptable delay for MDR pathogens with unpredictable susceptibility profiles.

54 We are therefore in desperate need for solutions to close the ATG and expand the
55 capabilities of hospital-based clinical microbiology laboratories. Current trends in AST systems
56 favor increased automation at the cost of reduced flexibility. However, we believe that such a
57 tradeoff is unnecessary.

58 More specifically, we validated inkjet printing technology as a way to perform automated
59 AST for any antimicrobial agent at will [4]. It turns out that with some engineering tweaks inkjet
60 printers (i.e., the HP D300) can print out things other than ink - in our case, antimicrobial stock
61 solutions. Instead of ink cartridges, the HP D300 utilizes cassettes that can be loaded with up to
62 8 different antimicrobial stock solutions which can then be printed out as a dilution series in any

63 desired format. Per manufacturer's specification, the D300 can print out droplets ranging in size
64 from 11 picoliters to 10 microliters [5]. The size of the droplet determines the amount of
65 antimicrobial in a microplate well, and a standard two-fold dilution series can thereby be created
66 with a single pipetting step.

67 We recently verified the performance of the inkjet methodology in comparison with gold
68 standard, reference broth microdilution AST using 7 antimicrobials including colistin against a
69 large panel of clinical isolates [4]. The new inkjet technology performed just as accurately and
70 with greater precision. Importantly, the flexibility of inkjet technology contrasts with automated
71 AST methods in current use which are typically "locked down" to include only limited dilutions
72 of specific antimicrobials and may also only provide an extrapolated rather than a true MIC.
73 Moreover, because the inkjet printer is much more spatially precise than a human being, we were
74 able to miniaturize testing to a 384-well plate format. This saving of microplate real estate has
75 important implications as it enables performance of additional testing relevant to MDR
76 pathogens.

77 Specifically, with limited options for treating MDR pathogens, novel solutions are
78 needed to rescue the ability of available antimicrobials to serve as useful agents. This rescue may
79 take two forms, both of which rely on highly accurate quantitative measures of antibacterial
80 inhibitory levels. First, for many antimicrobials, therapeutic success is predicated on a balance
81 between *in vivo* drug exposure and pathogen susceptibility (i.e., reflected in *in vitro* MIC
82 measurements). Therefore, there may be room to rescue use of antimicrobials for treatment of
83 relatively resistant organisms through augmented dosing. However, here we run into limitations
84 of traditional MIC testing in its ability to help us reliably negotiate within the therapeutic safety
85 window and avoid harmful side effects.

86 Standard MIC values are determined using a doubling dilution series. These values are
87 used to categorize organisms as susceptible or resistant. However, we know that MIC values
88 determined by standard doubling dilution testing may have significant error (± 1 dilution) and
89 certainly may fall between concentrations tested. In 2015, the Clinical and Laboratory Standards
90 Institute introduced a new MIC interpretive category, susceptible dose-dependent (SDD), for the
91 antibiotic, cefepime [6] . This category lies between susceptible and resistant with the specific
92 goal of giving clinicians a new ability to treat relatively resistant *Enterobacteriaceae* by either
93 increasing the antimicrobial dose (MIC = 4 μg ml^{-1}) or increasing both the dose and frequency of
94 dosing (MIC = 8 μg ml^{-1}). Unfortunately, the spacing of concentrations tested in standard
95 doubling AST become larger at higher antibiotic concentrations with unfortunate consequences
96 for MIC-based dosing.

97 For instance, an organism with a cefepime MIC of 10 potentially could still be treated
98 based on pharmacokinetic principles alone. However, one with an MIC of 30 μg ml^{-1} likely
99 could not be. Yet, both, when tested by the classic doubling dilution scheme in current use, may
100 show an MIC of 16 μg ml^{-1} taking into account the fact that only 8, 16, and 32 μg ml^{-1} are
101 traditionally tested in this upper concentration range. Therefore, traditional practice and methods
102 lack the requisite precision to provide a confident basis for SDD dosing regimens for cefepime
103 and other antimicrobials for which SDD dosing regimens will likely be recommended in the
104 future.

105 To accommodate changing AST standards and demand for increased precision, inkjet
106 printing technology can be used to create a finer dilution series centering on critical clinical
107 cutoffs (such as the SDD range). Using this technology, we can easily test concentrations
108 between two-fold dilutions (e.g. 6, 8, 10, 12, . . . μg ml^{-1}) to know more precisely how a

109 particular dosing strategy may affect an organism, and gain further confidence that our MIC
110 determinations are accurate. The importance of this greater accuracy as a foundation for reliable
111 pharmacodynamics (treatment efficacy) studies is self-evident. The importance of this finer
112 precision in defining the appropriate use of antibacterials with a small safety margin (e.g.,
113 colistin) also seems compelling.

114 The second potential salvage strategy is antimicrobial synergy. Synergy for our
115 discussion means that two agents, considered ineffective when tested individually, fully inhibit
116 growth of a pathogen at clinically relevant concentrations (i.e., within susceptible range) when
117 tested in combination. Unfortunately, synergy testing is even more complicated than reference
118 laboratory dilution testing, because it essentially squares the amount of work involved.
119 Therefore, synergy testing is essentially never performed outside of a research setting. However,
120 by loading two antimicrobials into the HP D300, a synergy grid can easily be created, allowing
121 for rapid determination of synergistic activity between two (or potentially more) antimicrobials
122 within a clinically actionable time frame. We have used this technology to identify potential
123 double and triple synergistic combination therapies for *Legionella pneumophila* and anticipate
124 that this methodology will be applicable to a wide variety of pathogens [7].

125 We predict the conceptually simple automation provided by inkjet printing technology is
126 poised to have significant impact on antimicrobial testing. It will open up new options for
127 treatment by enabling precise MIC-dependent dosing, and spur both research into and use of new
128 synergy-based combinations. Importantly, it will also provide flexibility to immediately
129 incorporate newly approved antimicrobials developed for MDR pathogens into hospital-based
130 laboratory testing, drugs that otherwise might not appear in clinical panels for several years. At
131 present, the HP D300 can be used in so-called "laboratory-developed tests" validated by

132 individual clinical laboratories. We have found in our hands that inkjet printer-based AST meets
133 a verification standard recommended by the FDA and the AST community [4]. We therefore
134 envision that inkjet technology could form the foundation of a future clinical platform that
135 addresses unmet needs in AST diagnostics and significantly shortens the antimicrobial testing
136 gap.

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