EFFECT OF PAPER QUALITY OF LOCALLY MADE ANTIBIOTIC DISCS ON ANTIBIOTIC SENSITIVITY OF SOME CLINICAL BACTERIAL ISOLATES

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ABSTRACT
The effect of paper quality of four different paper types on antibiotic sensitivity of some clinical bacterial isolates was investigated using the disc diffusion method of antimicrobial susceptibility testing. Antibiotic discs were made from Whatman No.1 filter paper, Copyman printing paper, Conqueror paper and Starfoolscap paper. Their difference in weight, thickness, absorbency and texture were determined and susceptibility testing was carried out with their discs against clinical isolates of Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa, to determine the susceptibility and any effect in zone diameters produced by the discs of the various paper types as indicated by high variance ratio (F-ratios, 79.73).

KEYWORDS
Antibiotic disc, Susceptibility testing, Paper quality and Zone of inhibition.

INTRODUCTION
The early pioneers of microbiology, Pasteur, Koch and Ehrlich made many reference to and observed the actions of biological agents against the growth of microorganisms. William Roberts in 1874 observed that liquid medium in which the mold penicillium glaucum was growing could not easily be contaminated with bacteria1. Two years later, John Tyndall observed that broth supported the growth of either bacteria or moulds but rarely both2. Fleming
also reported the inhibitory effect of penicillin on solid media by observing an area of growth inhibition of staphylococcal colonies adjacent to a penicillin contaminant on an agar plate and this was eventually termed agar diffusion. The concept of attacking invading microorganisms without harming the host was first introduced by Paul Ehrlich when he discovered ‘salvarsan’ which he announced as a magic bullet for the treatment of syphilis. Antibiotic susceptibility testing developed further in 1940s when Heatley introduced the use of absorbent paper for carrying antimicrobial solutions. Filter paper discs incorporated with penicillin were also used by Vincent and his colleague during this period. After the accidental discovery of penicillin in 1928, more and more antibiotics became commercially available. Although these new antibiotics were looked at as wonder drugs initially, resistant bacteria strain soon started emerging and susceptibility test for these drugs became necessary. The reasons for sensitivity testing are for clinical prediction of the likely outcome of treating a patient’s infection with a particular antibiotic agent and for quantitative measurement of susceptibility which can be used to monitor the emergence and prevalence of antimicrobial resistance. Antimicrobial susceptibility tests measures the ability of antibiotic or other antimicrobial agents to inhibit bacterial growth in vitro. It is a test used to determine which antibiotic can kill the organism causing the infection. Antimicrobial susceptibility testing methods may be quantitative, providing an absolute value of the minimum inhibitory concentration (MIC) or minimum Bacteriostatic or Bactericidal Concentration (MBC) of an agent that will inhibit or kill the organism respectively. Examples include Agar dilution and Broth dilution methods and the commercial available E-east MIC strips. It may also be qualitative indicating whether the organism is susceptible or resistant to the antimicrobial. Examples include disc diffusion method and automated system. The testing of sensitivity of pathogenic bacteria to antibiotics has become a necessary procedure in Hospitals and clinical laboratories because it aids the clinician in his choice of therapy. The method of choice is the disc diffusion method and its acceptance has been aided by its simplicity and rapidity. This method was first utilized by Beijerinck in 1889 for studying the effect of different auxins on bacterial growth. It was further developed by Bauer and co-workers in their work to standardize the method. It measures the qualitative action of antimicrobial agent to pathogenic organisms. In disc diffusion method of antimicrobial susceptibility testing, the antimicrobial agent diffuses from a focus or reservoir through a solid medium, inhibiting the growth of an organism to a distance depending on the sensitivity of the organism and many other factors. Bondi and his co-workers initially described the use of filter paper discs as a reservoir in susceptibility testing in 1947 and they are still commonly utilized today in clinical laboratories. The disc diffusion method can be referred to as the Kirby-Bauer method and have been modified by most clinical laboratories due to unavailability of some required materials. For instance Mueller Hinton Agar has been replaced with Nutrient Agar which is now being used by most laboratories and researchers. Disc diffusion test is performed according to standardized methodologies issued by a reference group, such as National Committee for Clinical Laboratory Standards (NCCLS) now known as Clinical and Laboratory Standard Institute (CLSI). Other reference groups include British society for Antimicrobial Chemotherapy (BSAC) and the Swedish Reference Group for Antibiotics (SRGA). These groups promote accurate antimicrobial susceptibility testing, develop interpretative criteria for the results as well as appropriate reporting techniques based on standard reference methods, and also establish quality control parameters for standard test methods. The interpretative criteria for disc diffusion test fall into three categories namely Susceptible (S), Intermediate (I), or Resistant (R). The results are interpreted using the established ‘interpretative criteria’ for each antimicrobial and bacterial species published by the CLSI and recommended by World Health Organization. Many clinical laboratories carry out antimicrobial
susceptibility testing but various constraints result in
the use of inappropriate antibiotic sensitivity discs. Majority of the Hospital laboratories procure
commercial antibiotic discs, while others either prepare their own discs or use both commercial and
self-prepared discs\textsuperscript{16}. The quality of paper used in
the production of sensitivity discs is an important
factor as the specification of these papers varies
somewhat as to weight, thickness, texture, and
absorbability of water, which may affects the result
of the test. In recent times, laboratory workers and
clinicians have questioned whether difference in the
quality of the paper used to produce antibiotic
sensitivity disc can cause any significant changes in
the result of antibiotic susceptibility testing. Paper
quality as stated in this work refers to some
properties of paper such as weight, thickness, texture
and absorbency of water and does not include other
physical, chemical and optical properties of
paper. The aim of this study is to determine if there
are any difference in inhibition zones produced by
antibiotic discs made from papers of different quality
and to determine the susceptibility pattern of some
selected clinical bacteria isolate to antibiotic discs
made from papers with different quality.

MATERIALS AND METHODS

Microbiological Culture Media

The media used for the study were commercially
obtained and include Nutrient agar, Blood Agar, and
Nutrient broth (Oxoid). They were prepared
according to manufactures instructions\textsuperscript{6}.

Test Organisms

A total of three known recent clinical isolates,
\textit{Escherichia coli}, \textit{Staphylococcus aureus} and
\textit{Pseudomonas aeruginosa} was used in the research
and were obtained from the Medical Microbiology
department of Federal Medical Center Owerri Imo
State Nigeria. They were isolated from patients with
urinary tract infections and were inoculated onto
blood agar plates and transported to the laboratory in
sterile polyethylene bags for microbiological
analysis and biochemical tests.

Identification of Bacteria Isolates\textsuperscript{6}

All bacterial isolates were identified by their cultural
and morphological characteristics on media plates,
Gram reaction and biochemical tests.

Preparation of Paper Disc\textsuperscript{22}

Four types of paper, namely Whatman No.1 filter
paper (P1), Conqueror paper (P2), Copyman printing
paper (P3) and Star-Foolscap paper (P4) were
selected for preparing the discs. Their selection was
based on their frequent use in discs preparation and
their different specifications which include weight,
thickness, texture and absorbency of water (Table1).

Whatman No.1 filter paper Catalog No. 1001 was
commercially obtained from Okey Surgicals stores,
others were obtained from Arinze Stationery store
both in Owerri City, Imo State Nigeria. To facilitate
identification of discs, code names of antibiotics
were printed on the sheets of paper before discs of
6mm were punched out from the four paper types
using an office hole-puncher. Sterilization was done
by autoclave for 15 minutes at 121\textdegree C, and allowed to
cool.

Preparation of Antibiotic Solutions\textsuperscript{6, 22}

Standard antibiotic powders of known concentrations
were commercially obtained for the study. The
antibiotic powders include Ampicillin, Gentamicin,
Penicillin, Ceftriaxone, Ciprofloxacin, Erythromycin, Cefotaxime, Nalixidic acid and
Chloramphenicol. They were all products of
Beecham pharmaceutical Germany. The antibiotic
powders were dissolved in their appropriate solvents
and further diluted in distilled water. The solutions
were prepared to contain the desired disc potency in
0.02ml of the drug solution. The sterile disc was
placed in Petri dishes and 0.02ml of the antibiotic
solution was delivered to the disc using a single
point pipette. Without covering the petri dishes, the
discs were allowed to dry in an incubator at 37\textdegree C for
1 hour. After drying, the discs were placed in a
sterile clean, air-tight container and stored in the
refrigerator at 8\textdegree C. They were tested using known
standard organisms before they were used. The
container was removed from the refrigerator 1 hour
before use in order to adjust the container to room
temperature.

Preparation of Inoculum\textsuperscript{23}

Standard saline solution was used to prepare an
inoculum with density equivalent to a 0.5 McFarland Opacity Standard. In the preparation of the inoculum, 8 colonies of the test organisms were suspended in 5ml of saline and thoroughly mixed and adjusted to required density. To obtain a uniform growth, the bacteria suspensions were agitated a little before inoculation.

**Antibiotic Sensitivity Testing**

For the susceptibility test, the disc diffusion method was employed in line with the CLSI guidelines under aseptic condition. For inoculation, a sterile cotton swab was dipped into the inoculum suspension and excess inoculum removed by firmly rotating the swab against the inside wall of the tube above the fluid level. The dry surface of the Nutrient agar plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated twice with the rotation of the plate at an angle of 60° each time. The preparation was allowed to dry for 5 minutes with the lid closed. A pair of sterile forceps was used to place the prepared antibiotic disc evenly and firmly onto the inoculated agar surface. The plates were incubated aerobically at 37°C overnight. After incubation the plates were checked for pure confluent growth and the diameter of the zones of inhibition of growth were measured to the nearest millimeter with a transparent meter ruler.

**RESULTS**

**Paper specification**

The four types of papers used to produce the antibiotic discs used in this study were found to have different physical properties which include weight, thickness, texture, and absorbency. The results were summarized in Table No. 1.

**Susceptibility pattern of tested bacterial isolates**

Antibiotic discs were prepared from each type of paper and placed on agar plates seeded with the test organisms namely *E. coli, S. aureus* and *P. aeruginosa*, so that each plate contains 9 discs, each disc representing a different drug with known concentration. Three of such plates were prepared for each bacterial isolate and for each type of paper and for all the antibiotics tested at a known concentration and the mean zone of inhibition calculated. Tables No. 2-4 summarizes the susceptibility pattern and the average diameters of zones of inhibition produced by the various paper discs against selected bacterial isolates.

**Effect of paper quality**

In all, 324 zones of inhibition were obtained for analysis. The complete data were subjected to statistical analysis of variance so that any significant difference in zones of inhibition due to paper type could be evaluated. Separate analyses of variance were calculated against each organism as shown in Table No. 5. The comparison of the mean zone diameter of the various prepared paper discs is illustrated in Figures No. 1-3. The statistical results of the two-way analysis of variance (ANOVA) shows significant differences in zone diameter produced by the disc of the different paper types for all the organisms and antibiotics tested and were indicated by a high variance ratio (F-ratios, 79.73).

**DISCUSSION**

The manufacture of antibiotic discs and their successful use involve a number of considerations which include the quality of antibiotic, the composition of the discs (paper, tablet or other construction) and the standard of test performance. The aim of the present study was to determine the effect of paper quality of locally made antibiotic discs on some clinical bacterial isolates and their susceptibility pattern. Differences in inhibition zone diameter produced by antibiotic disc made from four different type of paper were compared. The result of this study shows that each type of paper used to produce antibiotics disc affects the diameter of inhibition produced by the disc. This finding is in agreement with the result of the work carried out by Kramer and Kirshbaum in 1961, but disagrees with that of Ostrander and Griffith carried out in 1959, which indicated that unless some other agents, such as certain dyes, were present, the paper used made no difference. Marth and his colleges in 1963 also reported that different grades of paper used for antibiotic discs affect diameter of zones of inhibition produced by the discs. In this present study,
significant variations were seen among the four types of papers locally used to produce antibiotics sensitivity discs. The variation could be due to their different qualities as indicated by their specifications Table No.1. In all the paper types tested, the zones produced by the discs made from Whatman No.1 filter paper (P1) were 3mm larger than zones produced by other paper types. Discs made from Copyman printing paper (P3) gave zones closer to those of Whatman discs (P1) with an average difference of 1.2mm. The disc of Conqueror paper (P2) and Star-foolscap paper (P4) gave smaller zones of inhibition irrespective of the antibiotics and the organism tested, with discs of Conqueror paper producing the lowest zone diameter Tables No.2-4. The analysis of variance showed that there is difference in zone size due to paper type (Table 5). As mentioned earlier, this difference can be said to be due to the different specification or composition that constitute the quality of the papers and this has been indicated by variation in zone size. In view of this, Conqueror paper (P2) has high value for thickness and weight with low absorbency and this may have affected its performance in the test. In addition to this, the weight and thickness of this paper may not have allowed easy diffusion of antibiotics on the agar plates. Whatman No. 1 filter paper (P1) has the highest absorbency and this may have contributed to its good performance in the test. The zones produced by Conqueror paper (P2) and the Star-foolscap paper (P4) were smaller in size and are not within the limit of the published standard for interpretation by the Clinical and Laboratory Standard Institute (CLSI) Table No.6. These variations could cause misinterpretation of result in antimicrobial sensitivity testing as sensitive organisms may be labeled resistant due to the effect of these types of papers. The zones produced by discs made from Whatman No.1 filter paper (P1) were within the limit of the CLSI published standard, unlike the zones produced by Conqueror paper (P2) and Star-foolscap paper (P4).

In view of the antibiotics tested, Ampicillin (10µg), Chloramphenicol (30µg), Nalixidic acid (30µg), Erythromycin (15µg), and Penicillin (10 units) disc made from the four types of papers gave no significant zone when tested against P. aeruginosa. Cefotaxime (30µg), Ciprofloxacin (30µg), Ceftriaxone (30µg), and Gentamicin (10µg) discs gave larger zones showing high activity against the clinical bacterial isolates Figure No.1-3. This is also in agreement with the report of Ochei and Kolhatkar which stated that pseudomonas species are resistant to most routine antibiotics, but are susceptible to the aminoglycosides and cephalosporin.

### Table No.1: Specification of the papers used for antibiotic discs

<table>
<thead>
<tr>
<th>Paper Discs</th>
<th>Weight per disc (g)</th>
<th>Thickness (Inches)</th>
<th>Absorbency (ml/min)</th>
<th>Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whatman No.1 filter paper (P1)</td>
<td>0.004</td>
<td>0.0045</td>
<td>0.080</td>
<td>Rough</td>
</tr>
<tr>
<td>Conqueror paper (P2)</td>
<td>0.006</td>
<td>0.0051</td>
<td>0.013</td>
<td>Rough</td>
</tr>
<tr>
<td>Copyman printing paper (P3)</td>
<td>0.003</td>
<td>0.0041</td>
<td>0.060</td>
<td>Smooth</td>
</tr>
<tr>
<td>Star Foolscap paper (P4)</td>
<td>0.001</td>
<td>0.0031</td>
<td>0.025</td>
<td>Smooth</td>
</tr>
</tbody>
</table>

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Table No. 2: Susceptibility pattern and the average diameters of zones of inhibition produced by the various paper discs against *E. coli*

<table>
<thead>
<tr>
<th>Antibiotic (Concentration per discs)</th>
<th>Mean zones of various paper discs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1</td>
</tr>
<tr>
<td>Ampicillin (10 µg)</td>
<td>20mm</td>
</tr>
<tr>
<td>Gentamicin (10 µg)</td>
<td>22mm</td>
</tr>
<tr>
<td>Cefotaxime (30 µg)</td>
<td>29mm</td>
</tr>
<tr>
<td>Ciprofloxacin (5 µg)</td>
<td>34mm</td>
</tr>
<tr>
<td>Chloramphenicol (30 µg)</td>
<td>22mm</td>
</tr>
<tr>
<td>Ceftriaxone (30 µg)</td>
<td>30mm</td>
</tr>
<tr>
<td>Nalixidic acid (30 µg)</td>
<td>26mm</td>
</tr>
<tr>
<td>Erythromycin (15 µg)</td>
<td>20mm</td>
</tr>
<tr>
<td>Penicillin (10 units)</td>
<td>23mm</td>
</tr>
</tbody>
</table>

Table No. 3: Susceptibility pattern and the average diameters of zones of inhibition produced by the various paper discs against *S. aureus*

<table>
<thead>
<tr>
<th>Antibiotic (Concentration per discs)</th>
<th>Mean zones of various paper discs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1</td>
</tr>
<tr>
<td>Ampicillin (10 µg)</td>
<td>28mm</td>
</tr>
<tr>
<td>Gentamicin (10 µg)</td>
<td>22mm</td>
</tr>
<tr>
<td>Cefotaxime (30 µg)</td>
<td>28mm</td>
</tr>
<tr>
<td>Ciprofloxacin (5 µg)</td>
<td>22mm</td>
</tr>
<tr>
<td>Chloramphenicol (30 µg)</td>
<td>20mm</td>
</tr>
<tr>
<td>Ceftriaxone (30 µg)</td>
<td>25mm</td>
</tr>
<tr>
<td>Nalixidic acid (30 µg)</td>
<td>16mm</td>
</tr>
<tr>
<td>Erythromycin (15 µg)</td>
<td>25mm</td>
</tr>
<tr>
<td>Penicillin (10 units)</td>
<td>24mm</td>
</tr>
</tbody>
</table>
Table No. 4: Susceptibility pattern and the average diameters of zones of inhibition produced by the various paper discs against *P. aeruginosa*

<table>
<thead>
<tr>
<th>Antibiotic (Concentration per discs)</th>
<th>Mean zones of various paper discs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1</td>
</tr>
<tr>
<td>Ampicillin (10 µg)</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin (10 µg)</td>
<td>21mm</td>
</tr>
<tr>
<td>Cefotaxime (30 µg)</td>
<td>20mm</td>
</tr>
<tr>
<td>Ciprofloxacin (5 µg)</td>
<td>27mm</td>
</tr>
<tr>
<td>Chloramphenicol (30 µg)</td>
<td>-</td>
</tr>
<tr>
<td>Ceftriaxone (30 µg)</td>
<td>21mm</td>
</tr>
<tr>
<td>Nalixidic acid (30 µg)</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin (15 µg)</td>
<td>-</td>
</tr>
<tr>
<td>Penicillin (10 units)</td>
<td>-</td>
</tr>
</tbody>
</table>

(-): No significant zone

Table No. 5: Analysis of variance for the effect of paper types on zones of inhibition produced by the test organisms

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Source</th>
<th>D.F</th>
<th>S.S</th>
<th>M.S</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Zones (Blocks)</td>
<td>8</td>
<td>816.89</td>
<td>102.11</td>
<td>79.73*</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>3</td>
<td>106.33</td>
<td>35.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>24</td>
<td>10.67</td>
<td>0.4445</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Zones (Blocks)</td>
<td>8</td>
<td>451.39</td>
<td>56.64</td>
<td>61.85</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>3</td>
<td>173.11</td>
<td>57.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>24</td>
<td>22.39</td>
<td>0.9329</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>Zones (Blocks)</td>
<td>3</td>
<td>51.69</td>
<td>17.23</td>
<td>10.99</td>
</tr>
<tr>
<td></td>
<td>Paper</td>
<td>3</td>
<td>60.69</td>
<td>20.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Error</td>
<td>9</td>
<td>16.56</td>
<td>1.84</td>
<td></td>
</tr>
</tbody>
</table>

D.F: degrees of freedom; S.S: sum of squares; M.S: mean squares; F: variance ratio; *: highly significant
### Table No. 6: Published standards for limit of inhibition zones for selected test organisms and antibiotics

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Disc potency</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>10(µg)</td>
<td>16-22mm</td>
<td>27-35mm</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10(µg)</td>
<td>19-26mm</td>
<td>19-27mm</td>
<td>16-21mm</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>30(µg)</td>
<td>29-35mm</td>
<td>25-31mm</td>
<td>18-22mm</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5(µg)</td>
<td>30-40mm</td>
<td>22-30mm</td>
<td>25-33mm</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30(µg)</td>
<td>21-27mm</td>
<td>19-26mm</td>
<td>-</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>30(µg)</td>
<td>23-35mm</td>
<td>22-28mm</td>
<td>17-23mm</td>
</tr>
<tr>
<td>Nalixidic acid</td>
<td>30(µg)</td>
<td>22-29mm</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15(µg)</td>
<td>-</td>
<td>22-30mm</td>
<td>-</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10 units</td>
<td>-</td>
<td>26-37mm</td>
<td>-</td>
</tr>
</tbody>
</table>

(-): Not available

### Figure No. 1: Comparison of the average zones of inhibition produced by the discs from various paper types against *E. coli*

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Figure No.2: Comparison of the average zones of inhibition produced by the discs from various paper types against *S. aureus*

Figure No. 3: Comparison of the average zones of inhibition produced by the discs from various paper types against *P. aeruginosa*
CONCLUSION
The differences in the specifications of some types of papers used locally to produce antibiotic discs clearly outline the need for one specified paper for the preparation of antibiotic disc. More importantly the availability of affordable quality discs is indispensable in antimicrobial susceptibility surveillance of commonly encountered clinical bacterial isolates. The result of this study has shown that in trying to procure antibiotic discs locally, the different paper types used produce a significant effect on the result of susceptibility test. Therefore efforts should be made in the choice of paper, to bring to standard the result of the susceptibility testing which should also be in line with the published standards by the CLSI. The use of good quality papers for discs in susceptibility testing of common organisms encountered will not only improve the result of susceptibility testing but would also guide medical practitioners in their choice of appropriate antimicrobial agents and facilitate appropriate antimicrobial treatment.

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