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Full Length Research Paper

Low accuracy of the McFarland method for estimation of bacterial populations

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The McFarland method is designed to estimate bacterial concentrations by means of a turbidity scale (absorbance) which consists of a series of tubes previously calibrated, and with an optical density produced by the precipitation of barium sulphate. This absorbance is compared to bacterial populations. The most used absorbance is the one corresponding to 0.5 on that scale, which assumes a population of 1.5×10^8 cfu/mL (colony forming units per milliliter). In order to verify the accuracy of this scale, 25 different bacterial species were tested, adjusting to 0.5 of the McFarland scale and then an aliquot in plate with agar was cultured in triplicate to account for the population. The results showed very diverse populations, with variations ranging from 30 to 300% of what was expected (0.5×10^8 to 3×10^8 cfu/mL). The most important implications of this are in studies of microbial ecology, in clinical microbiology, in studies on sensitivity to antibiotics and in areas of quality control. It is suggested to take special care when it is required to establish, with more accuracy, the population of a crop.

Key words: McFarland, accuracy, bacterial population.

INTRODUCTION

Many times, especially in clinical services, the rapid identification of a microorganism is required in order to establish an adequate therapy or for simple experimentation. Most of the time, isolation can be made by limiting some of the microorganism's demands, such as feeding, environment, reactions to compounds and/or biochemical characteristics (Joklik et al., 1998). In other cases, it is necessary to express the microbial concentration without specifying the number of cells, for example dry weight, wet weight, turbidimetry or nephelometry (Corral et al., 2012; Cabeza, 2013). However, on very specific occasions, population counting is required, for example, when establishing a specific inoculum or in studies of microbial resistance when it is required to determine the minimum inhibitory concentrations (MIC) or mínimum bactericidal concentration (CMB) or experimental doses 50 (EC_{50}) as indicated by pharmacopoeias, hospitals or research institutes (Pharmacopeia of the United Mexican States (FEUM), 2015; Plant et al., 2016). In the quality control,

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phenotipification and identification of specific strains, it is also important to count the microorganisms. Many laboratories have to guarantee that their results are reliable, which implies starting from well-characterized microbial populations (Gupta et al., 2016; Paez et al., 2008).

The number of viable cells is considered the best measure of cell concentration. However, for many purposes, the turbidity of the crop, measured by photoelectric means, can be linked to the viable population in the form of a standard curve (Brooks et al., 2005). Turbidity is defined as the reduction of the transparency of a liquid caused by the presence of undissolved particles of material other than the liquid itself. Being an indicator of optical appearance, caused by the dispersion and absorption of light energy through the liquid, turbidity can only be measured using optical techniques. It is based on the relationship between the intensity of the incident light and the light dispersed by the medium, by the Lambert-Beer law, which establishes that the turbidity is proportional to the concentration of particles (Patarroyo, 2018; Acebo et al., 2013).

McFarland standards are used as turbidity patterns in the preparation of suspensions of microorganisms. The 0.5 standard of the McFarland scale has a special application in the preparation of bacterial inocula for antimicrobial susceptibility testing and is perhaps the most used procedure when determining a microbial population. It is always believed that this way of counting populations is accurate, due to its widespread use. The objective of the present work was to compare the populations of diverse bacterial species through the McFarland scale and the immediate culture in plates. The results suggest that in some occasions the McFarland scale is a bad, or very bad option to account for populations.

MATERIALS AND METHODS

Reagents

The experimental reagents used included 0.48 M $BaCl_2$ and 0.18 M H_2SO_4 solutions, respectively, along with Muller Hinton agar and Muller Hinton broth.

Crops

Twenty five (25) bacteria provided by the culture collection of the School of Chemical Sciences (Juarez University of Durango State) were tested: *Escherichia coli, Enterococcus* spp*., Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Streptococcus pyogenes, Yersinia enterocolitica, Aeromonas hydrophila, Shigella flexneri, Bordetella bronchiseptic, Proteus mirabilis, Pseudomonas* spp*., Acinetobacter* spp*., Stenotrophomonas maltophilia, Salmonella choleraesuis, Klebsiella* spp*., Serratia marcescens, Alcalientes* spp*., Salmonella paratyphi, Shigella Sonnei, Aeromonas caviae, Hafnia alvei, Providencia rettgeri, Morganella morganii* and *Vibrio cholerae.*

Standard McFarland preparation

0.5 mL of a 0.048 M $BaCl_2$ solution was added to 99.5 mL of a 0.18M H_2SO_4 solution and vortexed for 2 min. The solution was read in a spectrophotometer at a wavelength of 625 nm and it was found that the turbidity (absorbance) was 0.08 to 0.1, corresponding to 0.5 on the McFarland scale.

Each strain was plated on Muller Hinton agar seeded in three fields by cross streak and incubated at 37°C for 24 h. Colonies were then taken, one by one, to be suspended in a 12×75 tube with Muller Hinton broth and vortexed for 2 min, until adjusted to the 0.5 McFarland scale. From each tube, a series of 1:10, 1: 100, 1: 1000, 1:10 000, 1: 100 000, 1: 1,000,000 dilutions were made, each of which was seeded on a plate with Muller Hinton agar, incubated at 37°C for 24 h and then quantified the population expressed as cfu/mL. The assay for each strain was done in triplicate.

Statistics

The reproducibility when adjusting each experiment to the 0.5 scale of McFarland was determined by the percentage of the coefficient of variation of the absorbance of each strain (C.V. = standard deviation/average). The precision was determined by the percentage of the relative standard deviation (RSD) of the population counted in plate vs the expected population according to the McFarland scale, expressed as cfu/mL:

RSD = (1×10^8 cfu/mL - Average population of cfu/mL) / 1×10^8 cfu/mL

The test was considered acceptable when C.V ≤ 15% and RSD ± 15%. Additionally, ANOVA was applied to determine differences between the populations of the strains studied.

RESULTS

The average absorbance for all the trials was 0.09 ± 0.007, with a C.V. equal to 7.7%; so it is inferred that all crops were adjusted with great precision to 0.5 McFarland. Nevertheless, the populations counted on plate showed great variability with Table 1 showing the results.

Only three of the strains presented the expected population: *Acinetobacter* spp, *Alcaligenes* spp and *Hafnia alvei*, the rest showed populations lower or higher than expected population. Five strains showed higher than expected population, almost double, and *A. hydrophila* showed a population six times higher than expected. The rest of the strains presented a population between 50 to 70% lower than expected.

Figure 1 shows the differentiated growth between three different species in their three repetitions (R1, R2 and R3). All inocula were adjusted to 0.5 McFarland; however, the growth of *Pseudomonas* (A) was considerably higher than that presented by *S. aureus* (B). In addition, note that even *S. aureus* shows a greater growth than *Acinetobacter*.

The ANOVA test indicates that there is no significant difference ($p < 0.05$) when adjusting the absorbance of all cultures. However, there is a significant difference ($p > 0.05$) between the counted populations.

Table 1. Percentage of relative deviations of counted populations.

Microorganism	X ± SD * (cfu × 10^6/ mL)***	RSD (%)**
Escherichia coli	57 ± 11	62
Enterocuccus	10 ± 2	93
Staphylococcus aureus	43 ± 15	71
Klebsiella pneumoniae	80 ± 26	47
Acinetobacter pneumoniae	30 ± 10	80
Streptococcus pyogenes	50 ± 14	67
Yersenia enterocolitica	280 ± 34	-87
Aeromonas hydrophila	600 ± 120	-300
Shigella flexnerii	80 ± 20	47
Bordetella bronchiseptica	36 ± 20	76
Proteus mirabilis	20 ± 6	87
Pseudomonas ssp.	60 ± 10	60
Acinetobacter spp.	146 ± 55	2
Stenotrophomonas maltaphilia	40 ± 10	73
Salmonella choleraesuis	16 ± 5	89
Klensiella spp.	56 ± 5	62
Serratia marcencens	13 ± 5	91
Alcalígenes spp.	163 ± 25	-9
Salmonella paratyphi	66 ± 7	56
Shigella sonneii	210 ± 56	40
Aeromonas caviae	56 ± 15	62
Hafnia alvei	123 ± 35	18
Providencia rettgeri	350 ± 25	-133
Morganella morganii	366 ± 30	-144
Vibrio cholerae	43 ± 12	71

* Average ± Standard deviation; ** Relative standard deviation (based on the expected population 150×10^6 fcu / mL); *** Cfu = colony forming units.

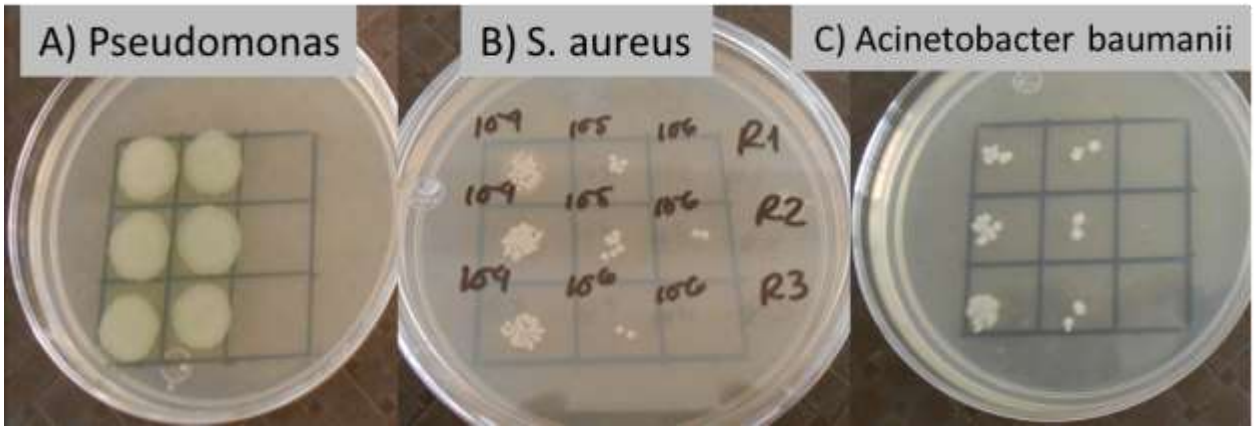

Figure 1. Comparison between the growth of three bacteria A) *Pseudomonas*, B) *S. aureus* and C) *Acinetobacter*, in their respective dilutions 10^{-4}, 10^{-5} and 10^{-6} in three replications (R1, R2 and R3).

DISCUSSION

The results obtained in the present work suggest that McFarland, despite being a fast and widely used method, fails to demonstrate accuracy and precision. Some work had already made similar observations; for example, Procop et al. (2017) suggested that if a McFarland standard is used to prepare an inoculum, it is necessary

to verify the amount of microorganisms in the inoculum periodically, inoculating serial dilutions of the suspension in agar plates. Sutton (2011) reports that this scale has been designed to estimate concentrations of gram-negative bacteria such as *E. coli*, but it must be borne in mind that this estimate becomes uncertain with organisms outside normal use, such as different species of bacteria that differ in size and mass, as well as yeast and mold, and adds that the use of this method requires calibration and validation, which is not normally done. It is common to find real populations counted in plaques lower than what is expected, which is consistent with our results, since 70% of our strains showed lower than expected populations.

In recent works, Zapata and Ramirez (2015) reported similar results when comparing the adjustment of microbial populations by McFarland turbidity and by a Densimat densimeter. When analyzing six different microbial species, they found a statistical difference between both methods, indicating that the adjustment by Densimat resulted in greater precision. Goughenour et al. (2015) also reports a growth of 8.4×10^{6} cfu on the McFarland scale 1.0 when developing a rapid method to identifying fungi and yeasts. According to the McFarland scale, there should be 3×10^{8} cfu. The authors point out the need to standardize and verify the procedures used, even those that supports a proven accuracy as CLSI.

In contrast, García (2007) reports having counted similar to expected populations for G + bacteria like *S. aureus* and G- bacteria like *E. coli*, *Pseudomonas aeruginosa* and *Salmonella* spp. despite their morphological differences (cocci and bacilli). On the other hand, *Bacillus cereus* was shown to have a bacterial concentration 40 times lower despite having been adjusted to the same McFarland scale. Navarro et al. (2010) makes a similar observation when scaling 0.6 McFarland with a range of absorbances that was between 0.152 - 0.157 and a wavelength of 620 nm.

All these differences in counting populations may be due to the great biological diversity of the micro-organisms. The forms, functions and size in them is highly variable (Brooks et al., 2005) so it is expected that a quantification method cannot be applicable in its entirety without presenting restrictions. The method to quantify bacteria by the McFarland scale has the advantage of being fast; however, due to the great variability in the size and shape of the microorganisms, it may result in erroneous readings, since it can have high turbidity produced by a large population of small microorganisms or a small population of large microorganisms.

Based on the populations recorded in our results, it is suggested to rethink the use of the McFarland scale. In cases where a larger population was obtained than expected, it is due to the fact that the bacteria are smaller and a greater number of them are required to equalize the turbidity. This could be solved if for smaller bacteria, smaller scales are introduced to 0.5 of McFarland, such as 0.25 or 0.1. Bacteria such as *E. coli*, Enterococcus and Acinetobacter, among others, should be counted in this way. In contrast, lower than expected populations are due to the fact that the bacteria are smaller, so the use of scale 1 or 2 instead of 0.5 is required. Bacteria such as *Aeromanas* and *Shigella* should be counted in this way.

Conclusions

When accounting for a microbial population, special care must be taken when adjusting for the McFarland scale, especially in research where the results are directly dependent on an exact count.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Efficacy of plasma micro broth dilution assay for antifungal susceptibility testing of *Candida albicans*

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Fungal infection and antifungal drug resistance especially in the treatment of immuno suppressive syndromes, has created an increased demand for reliable and affordable methods of *in vitro* testing of antifungal agents that can assist in their diagnosis. This study aimed to investigate antifungal susceptibility testing method that is reliable, flexible, affordable, accurate, cheap and less time consuming in order to reduce the prevalence of antifungal drug resistance using the plasma micro broth dilution assay. Results show differences in the performances of the agar dilution, plasma micro-broth dilution assay and disc diffusion test methods of ketoconazole susceptibility testing using *Candida albicans* with respect to time, cost and reliability. Therefore, development of an *in vitro* antifungal susceptibility testing method that will allow the clinical laboratory to perform with some confidence is imminent.

Key words: Plasma micro broth, antifungal, *Candidas albican, in vitro*.

INTRODUCTION

The rising prevalence of serious fungal infection and antifungal drug resistance especially in the treatment of immuno suppressive syndromes has created an increased demand for reliable and affordable methods of *in vitro* testing of antifungal agents that can assist in their diagnosis. These methods usually performed in the laboratory for susceptibility testing can be grouped into three categories; diffusion, dilution and diffusion-dilution (Alexander et al., 2009). Resistance to antifungal agents has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs, it is not a simple undertaking and there is little doubts that emergent antifungal resistance is a serious global problem.

The phenomenon of switching of some antifungal agents from only prescription to over-the counter (OTC) has provided conducive environment to the misuse of the antifungal agents. The misuse has been implicated in the rise of antifungal resistant strains as the result of selective pressure. Nevertheless, the trends of opportunistic fungal infections have been observed to

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increase in recent decades from 18% to above 60%, which has resulted in the increase use of antifungal agents (Mushi et al., 2017).

Candida albicans is the commonest cause of candidiasis (moniliasis). The yeast is the common commensal of the gastrointestinal tract. Most *Candida* infections are opportunistic, occurring in debilitated persons. Candidiasis is also associated with prolonged broad spectrum antibiotic therapy.

Many different clinical forms of candidiasis are known, involving primarily the mucosal surfaces (thrush), gastrointestinal or urogenital tract, and deep-seated infection such as cadidaemia or meningitis (Imran and Alshammry, 2016a).

Diffusion method such as the disc diffusion method first described by Bawer et al. (1966) require little specialized equipment and are easily customizable. Briefly, a test organism suspension is plated on a culture plate; paper discs containing the drug are deposited on the inoculated plate and incubated (Alexander et al., 2009). Plates are subsequently inspected for growth and the zones of growth inhibition surrounding the paper discs are measured.

Dilution methods allow for high sample throughput and quantitative inference regarding the minimal inhibitory concentration (MIC) needed to inhibit growth *in vitro*, but are associated with higher cost (Jorgensen et al., 1993). Wells containing dilution of the test drug are inoculated with the organism and incubated. The MIC that inhibits fungal growth *in vitro* is determined. Test drugs are most commonly diluted in twofold dilution series potentially leading to broad ranges of concentration being summarized by one MIC value (Turnidge and Paterson, 2007).

Agar dilution involves the incorporation of varying concentrations of antimicrobial agents into an agar medium usually using serial two-fold dilutions, followed by the application of defined organism inoculums to the agar surface of the plate (Alexander et al., 2009).

The rising prevalence of serious fungal infection and antifungal drug resistance has created an increased demand for reliable and affordable methods of *in vitro* testing of antifungal agents that can assist in their diagnosis. The techniques employed are often taken for granted.

Early investigators recognized these techniques, which are the disc diffusion, the agar dilution and the micro-broth dilution tests, and observed that there were many variables affecting the result of these tests. Consequently, there was recognition (as early as the late 1990s) that standardization of these techniques was required. This has led to many organizations producing standardized antifungal susceptibility testing methodologies that can help in antifungal susceptibility testing. With all this, there is need for the establishment of antifungal susceptibility testing method that is reliable, flexible, affordable, accurate, cheaper and less time consuming in order to reduce the prevalence of antifungal drug resistance.

MATERIALS AND METHODS

An experimental study was carried out on antifungal susceptibility testing using three isolates of *C. albicans* designated A, B and C; all isolated from cases of vaginal candidiasis from Science for Life Foundation Laboratory, Bemenda, North West region of Cameroon. Disc diffusion, agar dilution and plasma micro-broth dilution tests for antifungal susceptibility assays were employed.

Preparation of *C. albicans* suspension

Pure cultures of *Candida albicans* were obtained from the Science for Life Foundation Laboratory. The isolates were inoculated onto Sabouraud dextrose agar (SDA) plates and incubated at room temperature for 48 h after which the colonies on the SDA were further cultured onto SDA slant in a culture tube. It was then sub-cultured onto brain heart infusion agar (BHIA) for 48 h to test for purity. With the aid of a sterile Pasture pipette, the colonies on the surface of the cultures were gently washed with sterile distilled water and vortexed for 15 s, and the cell density was adjusted with a spectrophotometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard at a 530-nm wavelength. The final inoculum suspension was made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with brain heart infusion broth medium, which resulted in 0.5×10^3 to 2.5×10^3 cells/ml. In each case, the inoculum size was verified by enumeration of CFU obtained by subculture on Sabouraud dextrose agar.

Susceptibility testing

Disc diffusion method

Paper discs of 6 mm in diameter were made from Whatman No. 1 filter paper using a paper perforator. The discs were placed in a Petri dish and sterilized in a hot air oven at a temperature of 160°C for 60 min.

Ketoconazole 200 mg (Teva Pharmaceuticals USA, Inc.) was obtained in tablet form and was dissolved in 10 ml of sterile distilled water in a sterile bottle. Two sets of 25 µg discs of ketoconazole were prepared. One set was dried at a temperature of 25°C while the other was dried at 40°C in an enclosed incubator, to rule out the effect of temperature on disc potency.

Prepared and well dried SDA plates were seeded with the already prepared inoculums of *C. albicans* and were kept for 15 min at room temperature for complete absorption. The antifungal discs prepared were carefully placed on the seeded plates, incubated at 37°C, and examined for zones of inhibition after 24 and 48 h. Commercial fluconazole disc was set alongside as positive control while a blank Whatman No. 1 filter paper disc without antifungal agent was placed as a negative control.

Agar dilution method

The antifungal agent (ketoconazole 200 mg) was dissolved in 10 ml of sterile distilled water. Various dilutions were made in sterile molten SDA in test tubes to obtain 50, 25, 12.5, and 6.25 µg/ml concentrations, which were kept in a slanting position at room temperature for solidification. The slopes obtained were inoculated with 50 µl of a uniform suspension of the broth culture of *C. albicans* and all slopes were incubated at 37°C and were checked on 24 and 48 h for growth. Controls were set alongside. For positive control, griseofulvin incorporated into SDA slants at concentration of 500 µg/ml was use, while for negative control, plain SDA slope was used. As such, absence of growth was considered sensitive while growth was considered resistant. After 48 h of incubation, a

Table 1. Antifungal activity of discs loaded with Ketoconazole which dried at 40°C.

Isolates of *Candida albicans*	Zone of inhibition (mm)	Interpretation
A	00	Resistant
B	00	Resistant
C	00	Resistant

Table 2. Antifungal activity of discs loaded with Ketoconazole which dried at 25°C.

Isolates of *Candida albicans*	Zone of inhibition (mm)	Interpretation
A	20	Sensitive
B	22	Sensitive
C	00	Resistant

Table 3. Antifungal activity of agar dilution method (Read after 24 h).

Isolates of *Candida albicans*	MIC (µg)	Interpretation
A	25	Sensitive
B	25	Sensitive
C	50	Resistance

Table 4. Results from agar dilution (read after 48 h).

Isolates of *Candida albicans*	MIC (µg)	Interpretation
A	50	Resistant
B	30	Sensitive
C	50	Resistant

Gram stain was done to confirm the presence and absence of growth. The MIC was considered as the lowest concentration that gave no growth.

Plasma micro-broth dilution assay

A serial dilution of antifungal agent (ketoconazole 200 mg) was made in sterile micro titer plate using brain heart infusion broth (BHIB) as diluent in wells of different columns in triplicate to obtain concentrations of 50, 25, 12.5, and 6.25 µg/ml. 50 µl of fresh plasma was added into the wells of the first columns, while a drop of 1% phenol red indicator was added into the wells of the first and second columns. In the third column, only BHIB and ketoconazole was added. In the fourth column, only BHIB was added into the wells with no antifungal agent (positive control). All were done in triplicate to minimize error. One drop (50 µl) of the already prepared inoculums of *C. albicans* (0.25×10^2/50 to 1.25×10^2/50 µl) was added into the different wells using a sterile glass pasture pipette. Chloramphenicol was added into the various wells at a concentration of 10 µg to inhibit the growth of bacterial contamination. It was then incubated at 37°C for 24 and 48 h. Growth was examined macroscopically by colour changes and confirmed microscopically by examining Gram stained preparations and also wet mount from the various wells. Pink coloration was an indicator of growth, that is, resistant, while yellowish coloration indicated absence of growth, that is, sensitive.

RESULTS

According to the results of disc diffusion obtained, all the isolates were resistant to disc that were prepared and dried at 40°C (Table 1). Two Candidal isolates were sensitive to prepared ketoconazole discs which were dried at 25°C with mean zone diameters of 20 and 22 mm, respectively (Table 2). For the agar dilution method, two of the isolates were inhibited after 24 h of incubation with mean MIC of 25 µg (Table 3), and on further incubation for 48 h, only one isolate was sensitive with an MIC of 30 µg (Table 4).

According to antifungal activity using micro-broth dilution assay, macroscopically examination of the micro titer plates read after 24 and 48 h of incubation revealed pink coloration for wells that had growth and yellow coloration for those that did not have growth (Table 5). Results from Gram stained preparations red after 24 h of incubation reveal a MIC of 25 µg. In the column containing the plasma, the first and second dilutions had no yeast cell, while the third and fourth dilutions had 15 and 30 yeast cells/field, respectively. In the column containing broth, indicator and ketoconazole, the first and

Table 5. Macroscopica examination of plasma micro-broth dilution test.

Ketoconazole concentration (µg)	W	X	Y	Z
50	Yellowish	Yellowish	Yellowish	Pink
25	Yellowish	Yellowish	Yellowish	Pink
12.5	Pink	Faint pink	Faint pink	Pink
6.25	Pink	Pink	Pink	Pink

Yellowish: resistant; Pink: sensitive; W: broth with plasma, ketoconazole and indicator; X: broth with ketoconazole and indictor only; Y: broth with ketoconazole only; Z: broth with no antifungal agent.

Table 6. Microscopical view of Micro-broth dilution test (Examined after 24 h).

Ketoconazole concentration (µg)	W (yeast cells/field)	X (yeast cells/field)	Y (yeast cells/field)	Z (yeast cells/field)
50	00	00	00	38
25	00	00	00	41
12.5	15	10	9	39
6.25	30	16	15	38

W: Broth with plasma, ketoconazole and indicator; X: broth with ketoconazole and indictor only; Y: broth with ketoconazole only; Z: broth with no antifungal agent.

Table 7. Microscopically examination of Micro-broth dilution Test (after 48 h).

Ketoconazole concentrations (µg)	W (yeast cells/field)	X (yeast cells/field)	Y (yeast cells/field)	Z (yeast cells/field)
50	00	00	00	37
25	00	00	00	43
12.5	11	12	12	36
6.25	35	17	18	40

W: Broth with plasma, ketoconazole and indicator; X: broth with ketoconazole and indictor only; Y: broth with ketoconazole only; Z: broth with no antifungal agent.

second dilutions showed no yeast cells/field while the third and the fourth dilutions had 10 and 16 yeast cells/field, respectively. In the column containing only broth and ketoconazole, the first and second dilutions had no yeast cell/field while the third and fourth dilutions had 9 and 15 yeast cells/field, respectively. The column containing no ketoconazole had 38, 41, 39, and 38 yeast cells/field in the first, second, third and fourth dilutions, respectively (Table 6). Microscopically, examination of Gram stained preparations for plasma micro-broth dilution assay examined after 48 h, in case of the column containing the plasma, the first and second dilutions had no yeast cell, while the third and fourth dilutions had 11 and 35 yeast cells/field, respectively. In the column containing broth, indicator and ketoconazole, the first and second dilutions had 0 yeast cell/field while the third and fourth dilutions had 12 and 17 yeast cells/field, respectively. In the column containing only broth and ketoconazole, the first and second dilutions had 0 yeast cell/field while the third and fourth dilutions had 12 and 18 yeast cells/field, respectively. The column containing no ketoconazole had 37, 43, 36 and 40 yeast cells/field in the first, second, third and fourth dilutions, respectively (Table 7).

According to results obtained from examination of wet mount from the different wells, all the wells that had growth from the columns that contain plasma were positive for pseudohyphae, while all those that did not contain plasma had no pseudohyphae (Tables 8 and 9).

DISCUSSION

The development of a standardized antifungal susceptibility testing method by the NCCLS has been important, and the *in vitro* results of the MIC have been shown to correlate quite well with clinical outcome (Rex et al., 1997).

The results of this study showed that the agar dilution test detected 2 strains with a MIC of 25 µg/ml, 1 strain resistance with a MIC of 50 µg/ml after 24 h, while the result read after 48 h indicated that all strains were

Table 8. Results from wet mount of Micro-broth dilution test (examined after 24 hours).

Ketoconazole concentrations (µg)	W	X	Y	Z
50	-	-	-	-
25	-	-	-	-
12.5	+	-	-	-
6.25	+	-	-	-

(+): Presence of pseudohyphae; (-): Absence of pseudohyphae.

Table 9. Results from wet mount of Micro-broth dilution test (examined after 48 hours).

Ketoconazole concentrations (µg)	W	X	Y	Z
50	-	-	-	-
25	-	-	-	-
12.5	+			-
6.25	+	-	-	-

(+): Presence of pseudohyphae; (-): Absence of pseudohyphae.

resistant, this correlate with the reference method for agar dilution introduced by the NCCLS (2002).

With the disc diffusion test, the results from the disc prepared and dried at 40°C detected all the yeasts resistant. In addition, the results read at 48 h detected all the yeasts resistant. This results corroborates previous findings (Imran and Al-Karrem, 2016b)

The disc diffusion method gave a zone diameter of 20 and 22 mm for isolates A and B, respectively while isolate C was resistance with a zone diameter of 00 mm when the results were read after 24 h of incubation. When the disc test results read at 48 h were compared with the results obtained by the Cantón et al. (1999) method, it was found that there was correlation in the two methods. In a similar study carried out by Mushi et al. (2017) with 40 *Candida* isolates, MIC was ≥16 µg/ml for 14 strains and 11 (79%) of these were detected by the disc test when results were read after 48 h.

A study by Cantón et al. (1999) with 143 *Candida* isolates from blood cultures evaluated the same commercial disc method that was used. In their study, however, the result of the disc test was read after 24 h and not 48 h. They found that all seven resistant strains and four of seven sensitive strains had zone diameters <22 mm. Of the 129 susceptible strains, 19 strains (15%) had zone diameters <22 mm and were classified as resistant by the disc test. Their results differ from those of the other studies in the sense that, the results were read after 24 h and not 48 h, and it is possible that the shorter incubation time used by Cantón et al. (1999) might explain the differences.

In the present experience, the zone diameters are often difficult to read after 24 h due to poor growth, and this is the reason why 48 h of incubation has been used routinely. The disc test methods used in these different studies vary somewhat, it is necessary that the method should be well suited standardized because resistant strains of *C. albicans* have nearly always been detected.

In a study carried out by Kirkpatrick et al. (1998), most sensitive and resistance strain were also detected. Susceptible strains especially strains for which the MIC was 8 µg/ml was reported as resistant by the disc test. The results obtained by the ketoconazole disc test are quite comparable to the results obtained by the much used oxacillin disc screening test for detection of penicillin-resistant pneumococci (Jette et al., 1999).

The plasma micro-broth dilution test indicated all the 3 strains of *C. albicans* used in this study sensitive to ketoconazole with a MIC of 25 µg when the results were read after 24 and 48 h of incubation. From the plasma broth dilution assay, the column that contained fresh plasma showed excessive growth indicating that the plasma had a positive impact on the growth of *C. albicans.*

The present results from the plasma micro broth dilution assay confirmed that, duration of incubation has been a major factor associated with antifungal susceptibility tests; particularly with *Candida* species. The 48 h yeast incubation time specified in M27-A (NCCLS, 1995), has been a source of controversy ever since it was first chosen (Lozono et al., 1999). Studies on antifungal susceptibility testing, before the NCCLS undertook preparation of a reference method, had already indicated that results read during the exponential phase of yeast growth were consistent and independent of inoculums size, but the principle of exponential-phase and endpoint readings have generally been ignored (Galgiani et al., 1987).

The micro-broth dilution test adopted from the original NCCLS M27-A broth microdilution MIC test is currently

employed in many laboratories. Despite the considerable effort that went into defining and standardizing the M27-A test parameters, many authors have indicated that for tests with *Candida* spp., a 24 h end-point may improve reproducibility and correlate better with responses *in vivo* than the recommended 48 h reading time (Lozano et al., 1999).

Conclusion

This study is at an exciting stage in the development of an *in vitro* antifungal susceptibility testing method that will allow the clinical laboratory to perform with some confidence. It was realized that there were many differences in the performances of the agar dilution, plasma micro-broth dilution assay and disc diffusion test method of ketoconazole susceptibility testing using *C. albicans* with respect to time, cost and reliability. However, the plasma micro-broth dilution assay for antifungal susceptibility testing of *C. albicans* is the cheapest, most reliable, and less time consuming to be performed in the clinical diagnostic laboratory of less developed countries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antimicrobial susceptibility pattern of Gram negative bacteria isolated from intensive care units in Al-Ahsa, Kingdom of Saudi Arabia

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Antimicrobial resistance by bacteria isolates continues to receive attention globally. This investigation looks into the antibiotic susceptibility pattern of Gram negative bacteria isolated from intensive care unit patients in Al-Ahsa, KSA. Bacteria samples were classified based on the CDC criteria for the definition of ICU infections. Gram negative bacteria had been isolated on MacConkey agar using basic bacteriological technique. Identification and antimicrobial susceptibility was carried out using the GN cards of the Vitek 2 compact system. The results showed non-ESBL producing *Klebsiella pneumoniae* to be the most frequently encountered, isolated from 21% (n=23) of the patients. Other isolates were ESBL producing *Escherichia coli* (9.47%) and *K. pneumoniae* (3.77%), *E. coli* (15.09%), *Pseudomonas aeruginosa* (10.38%), *Proteus mirabilis* (9.43%), *Acinetobacter baumannii* (8.5%), and Carbapenem resistant *K. pneumoniae* amongst others. Resistance to five antibiotic groups was seen in *A. baumannii, Enterobacter*, *E. coli*, *K. pneumoniae* ESBL *K. pneumoniae*, non ESBL *K. pneumoniae* and *P. aeruginosa*. The association between bacteria resistance to antibiotic groups was statistically significant with a p-value of 0.00001. The encountered isolates showed both multi-drug resistance as well as extensive drug resistance against the tested drug. This information is being provided for Al-Ahsa and would be important for regional surveillance.

Key words: Intensive care unit, Gram negative, patients, multi-drug resistance.

INTRODUCTION

Hospital intensive care units are said to account for less than 10% of total hospital beds in most hospitals. However more than 20% of nosocomial infections are said to be gotten from ICUs (Dror and Keith, 2016). World Health Organisation (Ducel et al., 2013) report defines nosocomial infections as “those infections occurring in hospitalised patients or those in healthcare settings in whom the infection was neither present nor incubating at the time of admission”. With the rise in morbidity and mortality resulting from ICU infections all over the world, attention is being drawn by researchers (Iwuafor et al., 2016; Sugata et al., 2015; Molay et al., 2014) to the

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reporting of pathogens associated with these infections. Also, besides the effect of ICU infections on morbidity and mortality, there is the effect on the cost of treatment to be considered (Blot, 2008). A wide range of organisms inclusive of Gram negative and Gram positive bacterial isolates have been associated with these infections (Maazuddin et al*.,* 2014). Encountered pathogens have included both coagulase positive and negative Staphylococci, *Proteus mirabilis* and *Klebsiella pneumoniae* (Iwuafor et al*.,* 2016). Other reports listed ICU infectious agents to include *Acinetobacter baumannii, Pseudomonas aeruginosa*, species of candida amongst other organisms (Kayaaslan et al*.,* 2016; Zaman et al*.,* 2015).

The antimicrobial susceptibility patterns of encountered ICU isolates have also been receiving much attention, particularly in this era of multidrug resistant bacterial superbugs (Theuretzbacher, 2013). According to Maazuddin et al*.* (2014), nosocomial infections are frequently caused by multidrug resistant (MDR) strains of bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *S. aureus* (VRSA) and vancomycin resistant enterococci (VRE). This view was also supported by Zaman et al*.* (2015) who reported an increase in resistance by Gram negative bacteria (GNB) to antimicrobials used for their treatment. Also, it is stipulated (Brusselaers et al*.,* 2011) that pathogens associated with ICU infections exhibited higher resistant rates to commonly used antimicrobials when compared to those in community or hospital wards. Antibiotics are generally used in the treatment of bacterial infections. However, the advantages of such usage is being hindered by an increase in the emergence of MDR bacteria strains worldwide (Yezli et al., 2014). In Saudi Arabia, there is a recorded increase in the prevalence of bacteria pathogen that are highly resistant to antimicrobial of choice (Yezli et al*.,* 2014; Memish et al*.,* 2012; Alsultan et al*.,* 2014). High levels of antibiotic resistance by GNB such as *A. baumannii* and *P. aeruginosa* isolated from intensive care units have been known to lead to a significant morbidity and mortality (Al-Ahmadey et al*.,* 2013).

There is an urgent need to reduce both morbidity and mortality rates caused by ICU infections. Iwuafor et al. (2016) however postulated that such a reduction would be dependent on the availability of adequate and accurate data for individual local regions. They were of the view that there was an over dependence on available data from different regions which they say is not a reflection of the realities at various local regions. They argued that aetiological resistance pattern differ even in units of the same hospital. Earlier reports (Barai et al*.,* 2010) indicated that the organisms causing infections, along with their susceptibility to antibiotics not only varied from country to country but varied also from one hospital to another and even among ICUs within a hospital. As the world is gripped with thoughts of the possibility of returning to pre-antibiotic era, there is the need for regular surveillance of clinical isolates and their susceptibility to available antibiotics. Regional difference must also be taken into consideration. The present investigation looks into the GNB isolates associated with ICU infections in Al-Ahsa, south-eastern region of Saudi Arabia with a view to providing information of such in this region.

MATERIALS AND METHODS

Description of the study setting

The isolates were collected from the laboratories of five hospitals for a period of six months, from January 2016 to June 2016. Four of the hospitals were in Al-Ahsa and one in Al-Khobar. All of the hospitals are located in the South eastern region of Saudi Arabia.

Ethics approval and consent to participate

Ethical approval was not required as the samples were part of the routine in standard care of patients.

Sample collection and criterial for collection

Selection of ICU samples was based on the criteria by Centre for Disease Control and Prevention (CDC) definition for ICU infections. This stipulates that ICU associated infections are those that occur after 48 h of ICU admission or within 48 h after transfer from an ICU (Deep et al*.,* 2004). For exclusion criteria, samples from patients who had stayed in ICU for less than 48 h were not included in the study.

Laboratory samples included endo tracheal aspirates, surgical abdominal site swabs, catheter tips, sputum, diabetic wounds, urine, type 1 necrotising fasciitis, ear swabs and vagina swabs. A total of 93 samples were collected and used for the investigation.

Processing of samples and antimicrobial susceptibility test

Only Gram negative isolates were used for the investigation. They had been isolated and identified in the respective hospital laboratories using basic microbiological and biochemical identification test. Samples were transported to the College of Medicine, Microbiology Laboratory on ice bed and stored at -80°C until required. At the College of Medicine Laboratory, they were cultured using MacConkey agar following basic microbiological techniques for the preliminary identification of isolate. Confirmation of the isolates was by the Vitek 2 compact system (bioMe´rieux) according to the manufacturer's guidelines using the GN ID and AST Cards.

The antimicrobial susceptibility profile of the isolates was tested against the following antibiotics: Augmentin (AUG), ceftriaxone (CRO), cefotaxime (CTX), ceftazidime (CAZ), Cefepime (FEP), gentamicin (GM), amikacin (AK), Imipenem (IMP), Meropenem (MEM), Ciprofloxacin (CIP), Levofloxacin (LEVO), piperacillin-Tazobactam (TZP), Colistin (CS), Tigecycline (TG), and Sulphamethoxazole/trimethoprim (SXT) using the GN cards of the Vitek 2 compact system.

ESBL producing isolates were detected using the Vitek 2 compact automated system based on the antimicrobial susceptibility pattern. ESBL production confirmation was carried out using the cefepime/cefepime plus clavulanic acid Etest strips (AB

Table 1. Demographic information.

Demographics	N = 93	Frequency (%)
Gender		
Male	65	69.89
Female	28	30.12
Age group		
≤ 20	6	6.45
20 -30	3	3.23
31 - 40	7	7.53
41 - 50	16	17.2
51 - 60	19	20.43
61 - 70	9	9.7
71 - 80	17	18.28
81 - 90+	16	17.2

Biodisk. Solna, Sweden), following manufacturer's guidelines.

Multi-drug resistance (MDR) was assessed based on the method of Zhanel et al. (2008) as resistance to 3 or more of the following antibiotic groups; Aminoglycosides, Cepharlosporins, Carbapenems, Fluoroquinolones and Penicillin. While extreme drug resistance (XDR) was classified as defined by Magiorahos et al. (2012), it was not only being resistant to multi antimicrobials but also exhibiting the likelihood of being resistant to all.

Statistical analysis

Data was analysed with excel Microsoft software and GraphPad Prism. One-way ANOVA test was used to compare treatment of ICU isolates to the different antibiotic groups. Statistical significance was taken at $p<0.05$.

RESULTS

A total of 93 specimens analysed were collected from both male and female patients. Based on gender, 28 (30.12%) were females while 65 (69.89%) were males. The age of patients ranged from 6 months to 98 years old. Table 1 shows the percentage distribution of these ages. Six (6.45%) of the patients were below 20 years, 3 (3.23%) were in the age range of 20 - 30, while 7 (7.53%) were between 31 - 40 years of age. The patient age breakdown continues as follows: 41 - 50 years, 16 (17.20%) patients, 51 - 60 years, 19(20.43%), 61 - 70 years 9 (9.78%), 71 - 80 years had 17 (18.28%) of the patients while those between 81 - 90 years of age were 16(17.2%).

The encountered bacterial isolates and their characteristics

K. pneumoniae were the most frequently ICU bacteria isolates with 23(21%) being non ESBL producing. The ESBL producing *K. pnemoniae* constituted 3.77% of all the ICU isolates while an equal percentage (3.77%) were carbapenem resistant (CRE). ESBL - producing *Escherichia coli* made up 9.43% of the total ICU isolates while non ESBL *E. coli* producers made up 15.09% of the total isolates and the results are presented in Figure 1. Also, encountered ICU bacteria isolates were *P. aeruginosa* (10.38%), *P. mirabilis* (9.43%), *A. baumannii* (8.5%), and *Proteus vulgaris* (6.6%). *Enterobacter* constituted 5.66% of the total isolate, while *Serratia marcescens* and *Stenotrophomonas maltophilia* made up 2.83 and 0.94% of the total isolates respectively (Figure 1).

Characteristics of patient specimens

Of the confirmed infections, 15.5% of the patient isolates were from the sputum. Infections associated with urinary tract were 10.7% of the total ICU samples while bloodstream infections were from 11.9% of the patients. The most common infections were from wound swabs constituting 32.1% of patients with diabetic wounds, bed sores as well as post-surgical abdominal swabs. Tissue specimens were from 7.1% of patients with necrotising fasciitis while endotracheal aspirates made up 4.8% of the total ICU samples. Catheter related infection were 2.4% of total isolates, with infections from ear and throat making up 2.4 and 1.2% respectively of the total isolates. The infection sites were not given for 11.5% of the total patient samples, and the results are presented in Figure 2.

Antimicrobial susceptibility of the isolates

The results on the antimicrobial susceptibility of the Gram negative ICU isolates are presented in Table 2. For *A. baumannii*, resistance was highest with ceftazidime (77.8%), followed by Cefepime and ciprofloxacin with a 66.7% resistance each. Imipenem, meropenem and piperacillin-tazobactam recorded 55.56% each. One (11%) isolate was resistant to Colistin, Augmentin and Cefuroxime. There was a 33.33% resistance against gentamycin and levofloxacin. While being sensitive to tigecycline, intermediates were seen in all the antibiotics with the exception of the following: Cefuroxime, Cefotaxime, Cefepime, Meropenem and Ciprofloxacin. A high antimicrobial resistance was seen among the *Enterobacte*r. All (100%) were resistant to Cefuroxime and 80% resistant to the following; Ceftriaxone, Cefotaxime, Gentamicin and Ciprofloxacin. There was no resistance to Colistin and Tigecycline, while for other antimicrobials, resistance was as follows: 60% for Ceftazidime, Meropenem and Levofloxacin. Also, there were intermediates to all the tested drugs with the exception of cefuroxime.

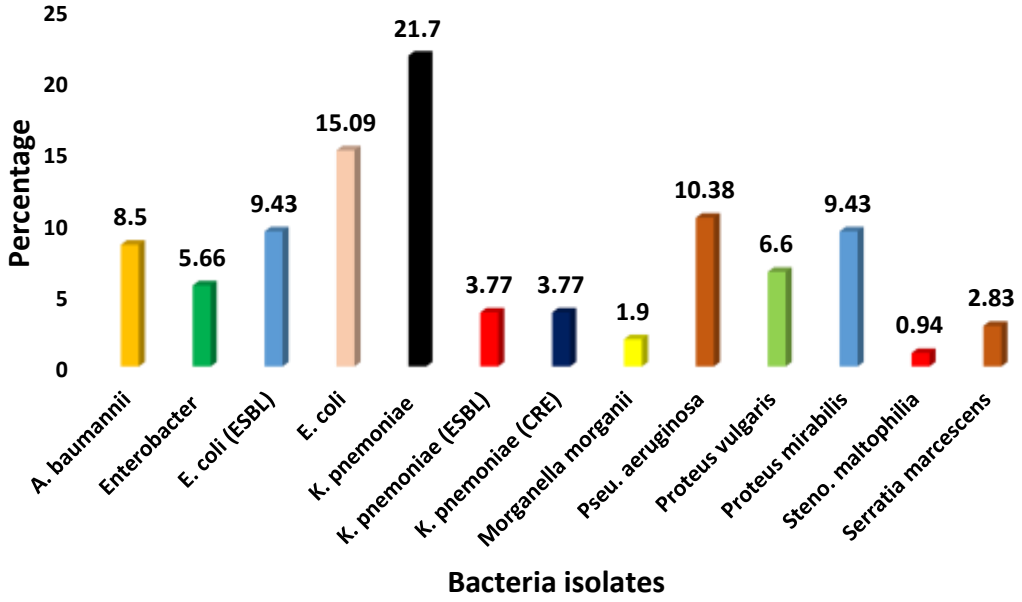

Figure 1. Frequency of bacterial isolated from ICUs.

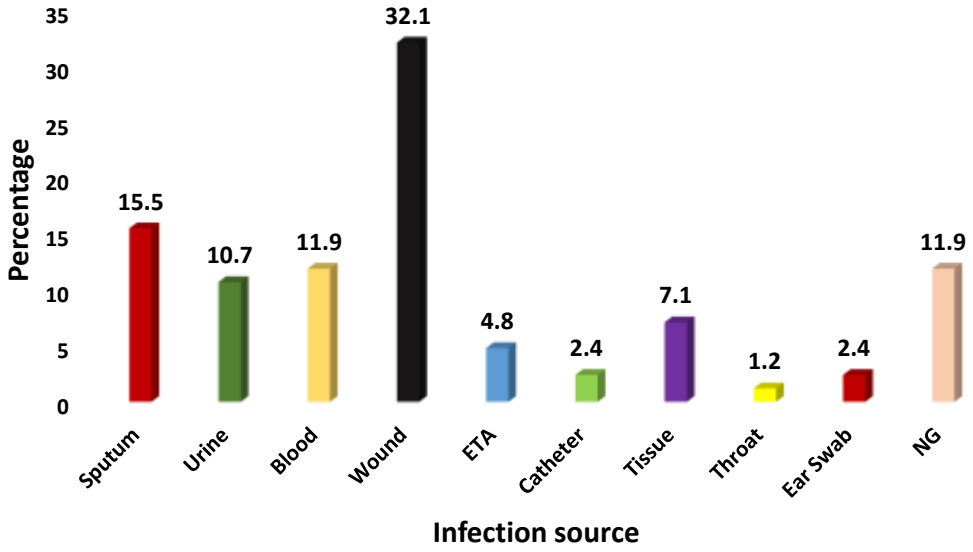

Figure 2. Percentage of each source of sample obtained from the ICU.

Table 3 also shows that carbapenem resistant *K. pneumoniae* exhibited 100% resistance against all the tested antibiotics with the exception of Colistin and Tigecycline. A similar resistant pattern is seen with ESBL producing *K. pneumoniae*. For this isolate, there was 100% resistance against the tested antibiotics with the exception of piperacillin-tazobactam (75%) and sensitive to the carbapenems. For *E. coli,* there was no resistance against Colistin and Tigecycline while ESBL *E. coli* showed resistance against the Carbapenems and Tigecycline.

Fifty percent (50%) of the isolated *P. mirabilis* were resistant to Colistin, with a further 25% being intermediate to this drug. Resistance rates for *P. aeruginosa* were as follows: Augmentin (47.4%), Cefuroxime (52.6%), Ceftriaxone (100%). This isolate was sensitive to Ceftazidime, Cefepime, and Gentamicin amongst others as shown in Table 2. The only *Stenotrophomonas maltophilia* isolate was resistant to all the antimicrobials with the exception of Levofloxacin. The Enterobacter were sensitive to Colistin and Tigecycline with a resistance pattern for other antimicrobials as

Table 2. Percentage of isolated resistant [R] and intermediate resistant [I] to the tested antibiotics.

Organism	Antibiotics [R / I]														
	AUG	CXM	CRO	CTX	CAZ	FEP	GM	AK	IMP	MEP	CIP	LEV	TZP	CS	TG
A. baumannii	11/11	11/0	22/11	22/0	77.8/0	66.7/11	33.3/11	-/-	55.56/11	55.56/0	66.7/0	33.3/33.3	55.6/11	11/0	0/0
Enterobacter.	60/40	100/0	80/20	80/20	60/20	40/20	80/20	40/20	40/20	60/20	80/20	60/20	40/20	0/0	0/0
E. coli (ESBL)	100/0	100/0	100/0	100/0	100/0	100/0	75/0	-/-	0/0	0/0	75/0	75/0	25/12.5	-/-	0/0
E. coli	25/31.3	31.3/0	25/0	6.25/6.25	18.75/12.5	18.75/6.25	25/12.5	12.5/12.5	6.25/0	25/25	37.5/6.25	25/37.5	0/12.5	0/0	0/0
K. pneumoniae	37/21	42.1/5.26	37/5.26	21.05/0	5/0	26.31/0	16/21	21./21	5.3/5.3	10.5/10.5	37/10.52	21/10.52	16/16	0/0	0/0
K. pneumoniae (ESBL)	100/0	100/0	100/0	100/0	100/0	100/0	100/0	-/-	0/0	0/0	100/0	100/0	75/0	-/-	0/0
K. pneumonia (CRE)	100/0	100/0	100/0	100/0	100/0	100/0	0/0	-/-	100/0	100/0	100/0	100/0	100/0	0/0	0/0
M. morganii	67/0	672/0	0/0	0/0	0/0	0/0	0/0	-/-	0/0	0/0	67/0	67/0	0/0	0/0	0/0
P. aeruginosa	47.4/5.3	52.6/0	100/0	5.26/0	0/5.26	0/31.6	0/47.4	-/-	10.53/21	16/10.52	5.3/5.3	10.53/21	0/10.52	0/0	0/0
Proteus Vulgaris	22.2/0	55.6/0	22.2/0	44.4/0	22.2/0	22.2/0	11.1/0	-/-	0/0	0/0	0/11.1	11.1/22	0/0	44/0	11/56
Proteus mirabilis	25/0	75/0	37.5/0	50/0	25/0	37.5/0	50/0	-/-	0/0	0/0	0/0	12.5/37.5	12.5/0	50/25	0/12.5
S. maltophilia	100/0	100/0	100/0	100/0	100/0	100/0	100/0	-/-	100/0	100/0	100/0	0/0	-/-	-/-	-/-
Serratia marcescens	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-/-	0/0	0/0	0/0	0/0	0/0	0/0	0/0

AK = Amikacin, AUG = augmentin, CXM = cefuroxime CRO = ceftriaxone, CTX = cefotaxime, CAZ = ceftazidime, FEP = cefepime, CIP = ciprofloxacin, CS = colistin, GM = gentamicin, IMP = imipenem, LEVO = levofloxacin MEM = meropenem, TZP = piperacillin-tazobactam, TG = tigecycline. -/- = not given; R/I = resistant/intermediate.

Table 3. Multi-drug resistance (MDR) characteristics of ICU isolates to the antibiotic groups.

Organism	Antibiotic grouping {No. (%)}					Total (%)
	Aminoglycosides	Cepharlosporins	Carbapenems	Fluoroquinolones	Penicillins	
A. baumannii	3 (6.7)	18 (40)	10 (22.2)	8 (17.8)	6 (13.3)	45 (100)
Enterobacter	6 (14.6)	18 (43.9)	5 (12.2)	7 (17.1)	5 (12.2)	41 (100)
E. coli (ESBL)	6 (10.71)	40 (71.43)	0.00	0.00	10 (17.86)	56 (100)
E. coli	6 (16.67)	17 (47.22)	2 (5.56)	7 (19.44)	4 (11.11)	36 (100)
K. pnemoniae	7 (12.3)	28 (49.1)	3 (5.3)	9 (15.8)	10 (17.5)	57(100)
K. pnemoniae (ESBL)	4(9.76)	20 (48.78)	8 (19.51)	2 (2.88)	7 (17.07)	41 (100)
K. pnemoniae (CRE)	0.00	20 (45.4)	8 (18.2)	8 (18.2)	8 (18.2)	44 (100)
M. morganii	0.00	2 (25)	0.00	4 (50)	2 (25)	8 (100)
P. aeruginosa	9 (16.3)	28 (50.9)	5 (9.1)	4 (7.3)	9 (16.4)	55 (100)
Proteus vulgaris	0.00	15 (88.24)	0.00	0.00	2 (11.76)	17 (100)
Proteus mirabilis	4 (16.7)	17 (70.8)	0.00	0.00	3 (12.5)	24 (100)

The p-value is < 0.00001. The result is significant at $p < 0.05$, using one-way Analysis of variance for independent measures of samples treated simultaneously.

follows: AUG (60%), CXM (100%), CRO (80%), CTX (80%), GM (80%), CIP (80%) and the results are shown in Table 2.

From the results presented in Table 2, resistance to five antibiotic groups is seen in the following isolate: *A. baumannii, Enterobacter, E. coli, K. pneumoniae, ESBL K. pneumoniae, and P. aeruginosa.* However, *K. pneumoniae* (CRE) was resistant to four antibiotic groups while *Proteus* species were each resistant to three groups. The result is statistically significant with a p-value of 0.00001 as shown in Table 3.

DISCUSSION

The encountered GNB isolates in the present findings as well as their antimicrobial susceptibility further highlights the healthcare problem facing the world in general and the need for both regional and global surveillance on the susceptibility of these bacteria isolates to commonly used, as well as last line antimicrobials. The monitoring of ICU isolates has become the norm in this era of evolving bacteria that are difficult to treat due to the enormous public health problem caused by these bacteria supper bug. No one is spared for this problem as ICUs are visited by all gender and all ages. In this study, 93 Gram negative isolates were from 28 (30.12%) females and 65 (69.89%) males. This simply implies that there were more males than females in ICUs during the period of this study. Zaman et al*.* (2015), from a study in Jeddah Kingdom of Saudi Arabia (KSA), reported a ratio of 41.14% males to 58.86% females. However, their study comprises both Saudi and non-Saudi nationals while the ICU isolates in the present study were from Saudi nationals only as there were no available data for non-Saudis in this region at the time of the study. Also, similar to the report in the present investigation are those from a previous report (El-Amin and Faidah, 2012) in which there was a higher number of ICU males than females. This therefore indicates that there would be variations in different hospitals, within a country as well as around different regions of the world.

Age-wise, most of the ICU patients in the present study belong to varying age groups with majority of them being between the ages of 51 - 60 years and above. Therefore, age could be a contributing risk factor to ICU infections in this region. Similar findings had earlier been reported in KSA (Zaman et al*.*, 2015; Al-Anazi, 2009). However, there are reports (Kayaaslan et al*.,* 2016) that advanced age was not found to be a risk factor in ICU mortality cases, thus suggesting non-specific pattern in patient age variations. The aetiological agents responsible for the ICU infections as seen in the present investigation appears to represent the general reflection of GNB isolates as had been reported by other researchers such as Kayaaslan et al. (2016) in Turkey, Zaman et al*.* (2015) in Jeddah, KSA as well as in many regions of the world with slight variations. In this study, *K. pneumoniae* was the most encountered bacterial pathogen followed by *E. coli, P. aeruginosa, ESBL E. coli* and *P. mirabillis*. In Kayaaslan et al*.* (2016) report, pneumonia was considered to be the frequent ICU infection followed by urinary tract infections without stating the prevalent causative organism. However, Pradhan et al*.* (2014), while indicating respiratory tract infections to be the commonest encountered in their study, indicated that the most encountered GNB isolate was *A. baumannii.* Moreso, study by Zaman et al*.* (2015) that reported *E. coli* as the most frequently encountered ICU isolate followed by *K. pneumoniae,* shows that there are variations in results. This could be attributed to a number of reasons which might include the time of study, the place, hospital, country as well as the region of the world. Thus, results could reflect what is obtainable differently for individual countries and hospitals.

As there are differences in aetiological GNB responsible for ICU infections, so it would be expected that their response to treatment may differ. The high level of resistance to antimicrobials shown by the isolates in the present report further highlights the difficulty in treating bacteria supper bugs in the 21st century. While all the isolates were MDR, and based on the definition of extreme drug resistance (XDR) by Magiorakos et al. (2012), the following isolates in the present study were considered to be XDR: *K. pneumoniae* (ESBL), *E. coli* (ESBL), *K. pneumoniae* (CRE), *Enterobacter* and *S. maltophilia.* These findings are similar to those of Zaman et al*.* (2015) who reported ESBL strains to be most resistant to commonly used antibiotics. Also from the same region of the present study, earlier reports (Khanfar et al., 2009) showed high resistance to Ciprofloxacin in ESBL producers while other reports (Al-Ahmadey et al., (2013) found much lower rates in ESBL *K. pneumoniae* in different regions of KSA. To this effect, earlier reports (Yezli et al., 2014) pointed to the fact that ESBLs showed wide variations from one country to another and also within the same country. Therefore, all the results either in similarities or in differences help in emphasizing the need for a constant and regular monitoring of antibiotic susceptibility of bacteria pathogen in different regions of the world. Such is needed as the indiscriminate use of antibiotics by patients and healthcare givers continue all over the world. It might also be necessary to look into all types of clinical isolates as Zhanel et al. (2008) indicated from their investigations that there was a high resistance to antimicrobials in ICU patients than in patients in other regions of hospital.

The antimicrobial resistance seen in *P. aeruginosa* in this study is similar to those from previous studies (Zaman et al., 2015; Memish et al., 2012; Al-Ahmadey et al*.,* 2013) in KSA as well as in other regions of the world (Benachinmardi et al*.,* 2014).

A. baumannii is seen to be resistant against the tested antibiotics, with one isolate being resistant to Colistin in

the present study. The MDR of ICU, A. *baumannii,* from KSA has been reported by researchers (Zaman et al., 2015; Memish et al*.,* 2012; Al-Ahmadey et al., 2013). However, contrary to the findings in the present study, the *A. baumannii* in their study were sensitive to Colistin. There is therefore the possibility of an emerging strain of *A. baumannii* that is resistant to Colistin. It is obvious that there is an urgent and continuous need for surveillance of bacteria antimicrobial susceptibility pattern all over the world. To this effect, Ramsamy et al. (2016) were of the view that the "knowledge of inherent flora and their antimicrobial susceptibility pattern are crucial."

The single *S. maltophilia* isolate encountered in the ICU isolates in the present investigation was resistant against all the tested antimicrobials with the exception of levofloxacin. This bacterium might be of the strain as that encountered in an earlier report (Zaman et al., 2015) in Jeddah. This XDR *S. maltophilia* might be the strain circulating in ICU units in KSA. There is therefore need to trace the source of this bacterium in ICUs in KSA for possible control measures.

Conclusion

The present investigation shows that bacterial isolates from ICU continue to show high antimicrobial resistance patterns. This calls for concerted effort at stemming the tide of regional surveillance for MDR bacteria in the Kingdom of Saudi Arabia".

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and identification of *Escherichia coli*, *Salmonella* and *Pasteurella* from holding grounds of live-bird markets at Addis Ababa, Ethiopia

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A cross-sectional study was conducted to isolate and identify of *Escherchia coli, Pastuerella multocida*, *Salmonella gallinarum* and *Salmonella pullorum* from the holding grounds of five purposively selected Addis Ababa live bird markets from November 2016 to May 2017 using bacterial culture, grams staining and biochemical testing. A total of 90 pooled fecal samples were collected as both deep (35) (bottom layer of the feces) and surface (55) (top layer of the feces). The specimens were inoculated directly into blood agar and incubated at 37°C for 24 h. After subsequent subcultures of the colony on the blood agar, to get pure colonies, the isolates were cultured on MacConkey agar and *Salmonella shigella* agar to differentiate bacterial colonies. Gram staining and biochemical tests were carried out on the pure colonies and a loop of the isolates were inoculated into nutrient broth and kept for further investigation of the bacteria. The results of the study revealed that, out of the 90 total samples examined, 32 (35.55%) of the samples were found to be positive for *E. coli*. Based on the sample types the study indicated that 55 (61.10%) surface fecal samples, 32 (58.20%) samples were positive for *E. coli*, and in 35 (38.90%) deep fecal layers, there was no *E. coli* positive samples. There was a statistically significant difference between sample types and *E. coli* isolation rate (p< 0.05). *P. multocida*, *S. gallinarum* and *S. pullorum* were not found during this study. Live bird markets may serve as source of public health and economically important bacteria; however, further microbiological and epidemiological investigation is needed to determine the types of major bacteria predominating in the Addis Ababa live-bird markets and the roles and magnitudes played by these markets in the epidemiology of these pathogens.

Key words: Addis Ababa, live-bird markets, *Escherichia coli, Salmonella, Pastuerella.*

INTRODUCTION

In Ethiopia, poultry rearing is one of the economically important agricultural activities. The national chicken population is estimated to be 56.53 million (CSA, 2017), and poultry in this country is kept in traditional, small scale intensive and in large scale commercial system. Poultry production system in Ethiopia shows a clear

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distinction between traditional low input systems and modern production system using relatively advanced technology (Tadesse, 2015). The traditional backyard poultry production system which accounts for more than 98% of the poultry in the country is reared for two purposes; eggs and meat production (Asresie and Eshetu, 2015). The largest proportion of eggs and poultry meat consumed in the country comes from indigenous birds produced by rural growers. Hence, there is high movement of poultry and poultry products from rural producers to the urban consumers, which are favoring the spread of infectious agents all over the country (Duguma et al., 2017). The current live-bird marketing system represent a significant and potential hazard to both buyers and sellers in addition to causing a pronounced damage to chicken, yet implementation of bio-security and hygienic practices in the system is generally difficult (Heise et al., 2015).

The poultry population in Ethiopia is not growing as demanded; it is often under gradual decline and is highly constrained by many diseases among other factors (Heise et al., 2015). Viral and bacterial pathogens of poultry are therefore a major concern in both local and international scale, because they have represented a burden to human health and economics throughout times (Habte et al., 2017).

Avian colibacillosis which is caused by avian pathogenic *Escherichia coli* (APEC) is one of the most significant and wide spread infectious bacterial diseases occurring in poultry production. It is responsible for large financial losses for the poultry industry each year due to mortality and loses of production (Ronco et al., 2017).

Fowl typhoid and Pullorum diseases cause infectious enteritis leading to heavy mortality mostly in adult and growing chicks, respectively. These diseases are caused by the bacterium *Salmonella enterica* serovar *Gallinarum* biovar *Gallinarum* and *Salmonella enterica* serovar *Pullorum,* respectively. *Salmonella enterica* serovar *Gallinarum* is highly adapted and seldom causes significant problems in hosts other than chickens, turkeys and pheasants (Getinet et al*.,* 2017).

Pastuerella multocida, the causal agents of fowl cholera and it can cause a zoonotic infection in humans which typically is a result of bites or scratches from domestic pets (Qin et al., 2017). The disease occurs as a fulminating disease with massive bacteremia, and high morbidity and mortality. *P. multocida* is a fairly delicate organism which is easily inactivated by a common disinfectant, sun light, drying; heat and experiment suggest that the organism will survive for a maximum of thirty days in the environment (Saminathan et al., 2016).

The overall poultry trade in Ethiopian is uncontrolled and involves collection of chicken from multiple households having few numbers of marketable ages, mixed together starting from the village markets moving to the big terminal markets of big cities. Daily introduction of new birds into live bird markets (LBMs) provides opportunities for replication and adaptation of different pathogens to a new environment and the infection to persist within the market system for extended periods of time (Haftom et al*.,* 2015).

Most bacteria pathogens of poultry especially *E. coli, P. multocida* and *Staphylococcus gallinarum* can be transmitted from one to others including humans in many ways and the most causal agents are excreted from chickens through excreta feces, nasal and oral secretion, and these pathogens can persist in the environmental litters, water, feed, holding and transporting cages, vehicles, clothing of animal attendants (Emery et al., 2017; Goldstone and Smith, 2016).

Fowl typhoid, pullorum disease, avian colibacillosis and fowl cholera are one of the most devastating poultry bacterial diseases (Nhung et al., 2017). Despite those facts, no any studies were conducted to isolate of etiological agents of these diseases particularly in holding grounds of live-bird markets of Addis Ababa and generally in Ethiopia. The objectives of this study were to isolate and identify *E. coli, P. multocida, S. gallinarum*, and *Salmonella Pullorum* from the feces accumulated on the holding grounds of live bird markets and quantity the role of live-bird market in the spread of avian pathogens.

MATERIALS AND METHODS

Study area

The study was conducted in Addis Ababa which is the capital city of Ethiopia. It covers an area of 530.14 km^2 and it lies at an altitude of 2,300 m above sea level. It is located between 9.03° north and 38.74° east, latitude and longitude, respectively. The city receives variable annual rainfall with lowest and the highest annual average temperature ranging between 9.89 and 24.46°C, respectively. This area of study was chosen because it has big poultry markets (thirteen live-bird markets with average of thirty traders per market) that host chicken originating from different parts of the country.

Study population

Study population were chicken's holding grounds in selected live-bird market of Addis Ababa, namely Akaki, Merkato, Mesalamia, Saris and Shola.

Study design

A cross-sectional study was undertaken from November 2016 to May 2017 with the objectives of isolation and identification of *E. coli, P. multocida, S. gallinarum*, and *Salmonella Pullorum* from the feces accumulated on the holding grounds of live bird markets and also to assess the role of live-bird market in the spread of avian pathogen.

Sampling method

There are about thirteen live-bird markets in Addis Ababa with average of thirty traders per markets from which five markets were purposively selected which are Akaki, Merkato, Mesalamia, Saris and Shola based on number of chicken. A total of 90 pooled fecal

samples out of which 35 were deep and 55 were surface sample type were collected randomly from chicken's holding grounds found in the selected market places (Akaki, Merkato, Mesalamia, Saris and Shola). Fecal samples were chosen to be collected from the chicken's holding grounds to know whether these areas were serving as sources of the selected bacteria for the chickens kept there and sold to different households either for consumption or further rearing.

Study methodology

Sample collection

Samples were collected using sterile cryovials, swabs, sterile water, water-proof markers, gloves, and scissors during the beginning of the two rounds of the biggest festival (Christmas and Easter) in Ethiopia. These time periods were chosen, because many chickens were brought to the live-bird markets of Addis Ababa from different areas of the country during these times (especially from Dire Dawa and Harar in the East, Gojjem and Gondar in the North, Asella, Bale, Borena, Hawas and Wolayta in the South, and Ambo and Jimma in the West). Collection of the fecal samples was done both from the deep and surface layer of accumulated feces in the chicken's holding grounds. The thickness of the feces in the holding cages varies from 2 to 5 cm; therefore fecal samples were designated as 'surface' as samples collected from the top layer and 'deep'as samples collected from the bottom layer of the feces accumulated in the chicken's holding cages. From 5 different sites (corner and center of cage), surface samples were swabbed with wet sterile swab and pooled into one sample, and similarly, the samples from the deep layer of the same cages were collected separately in a pool containing 5 samples. The collected samples were then labeled using markers and kept in an ice box containing ice packs and transported using a car and finally submitted to the National Animal Health Diagnostic and Investigation Center (NAHDIC) on the same date of collection. The samples were then stored in a refrigerator at +/-4°C until laboratory investigation were undertaken.

Isolation and identification of bacteria

Bacteriological culture: The specimens were directly inoculated into blood agar, and the plates were inverted and incubated aerobically at 37°C for 24 h, after which the plates were examined for growth. Pure cultures of bacteria were obtained by aseptically streaking representative colonies of different morphological types on blood agar. The colonies were then examined with naked eye for their morphological properties and any change in the media. The isolates were then cultured on MacConkey agar and salmonella-shigella agar to differentiate colonies of bacteria and a loop of the isolates were inoculated into nutrient broth for further investigation.

Grams staining: This method used to differentiate bacteria into Gram-negative (pink) and Gram-positive bacteria (purple). It was done based on the ability of the bacteria to retain the color of stains used during gram reaction. Gram-negative bacteria were decolorized by alcohol, losing the purple color of the primary stains (crystal violet), but Gram-positive bacteria were not decolorized by alcohol and remain as purple. After decolorization step, a counter stain (carbol fuchsine) was used to impart a pink color to the decolorized Gram-negative bacteria.

Biochemical tests: These are a series of tests that were used to identify bacteria into its generic level based on the types of enzyme produced and metabolism type performed by bacteria with sugars. Different primary and secondary biochemical tests used were Catalase, Oxidase, Motility, Oxidative-Fermentative test (O-F) and the IMViC series that consisted of four definitive tests: indole production, the methyl red test, the Voges-Proskauer test, and the citrate utilization test.

For the isolation of *E. coli* like colonies (colonies with no hemolysis on blood agar and colonies with lactose fermenting on macConkey agar), Catalase (+), Oxidase (-), Motility (+), O-F (F) tests were used to make sure that the isolated colonies were suggestive for *E. coli* after which secondary biochemical tests, IMViC (+/+/-/-) were used to identify the isolated *E. coli* from other coliform bacteria.

The colonies which were suggestive for *P. multocida* (absence of hemolysis on blood agar and no growth on macConkey agar) and *Salmonella Gallinarum* and *Salmonella Pullorum* (absence of hemolysis on blood agar, non lactose fermanter on macConkey agar and ability to grow on Salmonella-shigella agar) were subjected to gram stain and different tests (Catalase, Oxidase, Motility, O-F, and IMViC tests.

Data management and analysis

Data collected from the study area were recorded, and stored in Microsoft® Excel for Windows 2010 and transferred to Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM SPSS, 2011). Data were coded and analysed using descriptive and analytical statistics (chi-square) as appropriate. Analyses were then made to determine if there were significant differences among markets, origins of chicken and between time of sampling and sample types for *E. coli*, *P. multocida*, *S. gallinarum* and *S. pullorum* isolation. For all the analyses, confidence level (CL) is at 95% and $P \leq 0.05$ were set for significance.

RESULTS

The holding grounds of Addis Ababa live bird markets were wooden cages which were not frequently cleaned and because of this the excreta from chicken held in them made layers thicker than 2-5 cm. In the study area, there were mixing of poultry originating from different parts of the country and the major sources of poultry were as far as Dire Dawa and Harar in East; Gojjem and Gondar in the North; Arbaminch, Assella, Bale, Borena, Hawasa and Wolayta in the South and Ambo and Jimma in the West and, most of them located at distance of greater than 300 km. Different live-bird markets sampled in Addis Ababa frequently consist of gathering of native chickens that satisfy local tastes and that are held in cages, baskets or tied-up for sale.

Out of the total 90 pooled fecal samples collected from Addis Ababa live-bird markets holding grounds, 32 (35.55%) of the samples were found to be positive for *E. coli*. The result revealed that, out of 90 fecal samples 55 (61.10%) were surface fecal samples, of which 32 (58.20%) was positive for *E. coli* and 35 (38.90%) were deep fecal samples, of which there was no *E. coli* positive sample (Table 1).

The result of laboratory investigation also indicated that, out of 90 pooled fecal samples, 13 (14.44%) and 5 (5.56%) were revealed the morphological properties of *Pastuerella multocida* (absence of hemolysis on blood agar and no growth on macConkey agar), *Salmonella*

Table 1. Frequency (RF) and isolation rate of *E. coli, P. multocida*, *S. Gallinarum* and *S. Pullorum* from pooled fecal samples from live-bird markets at Addis Ababa.

Variable	Category	No. (%) tested	RF (%) of pooled samples positive for		
			E. coli	S. Gallinarum and S. Pullorum	*P. multocida*
Type of sample	Deep	35 (38.90)	0(0)	0(0)	0 (0)
	Surface	55(61.10)	32 (58.20)	0 (0)	0 (0)
Origin of birds	East	19 (21.12)	6 (31.60)	0 (0)	0 (0)
	North	13 (14.44)	5(38.50)	0 (0)	0 (0)
	South	45 (50)	17 (37.78)	0(0)	0(0)
	West	13 (14.44)	4(30.80)	0 (0)	0 (0)
Market places	Akaki	16 (17.78)	5 (31.25)	0 (0)	0 (0)
	Merkato	21 (23.33)	6 (28.60)	0 (0)	0 (0)
	Mesalamia	14 (15.56)	5 (35.71)	0 (0)	0 (0)
	Saris	23 (25.55)	10 (43.50)	0 (0)	0 (0)
	Shola	16 (17.78)	6 (37.50)	0 (0)	0 (0)
Time of sampling	Christmas	39 (43.33)	15(38.46)	0 (0)	0 (0)
	Easter	51 (56.67)	17 (33.33)	0 (0)	0 (0)

Gallinarum and *Salmonella Pullorum* (absence of hemolysis on blood agar, non-lactose fermanter on MacConkey agar and ability to grow on salmonella-shigella agar), respectively (Figure 1) however, no specific simultaneous laboratory results (gram-negative, catalase (+), oxidase (+), motility (-), O-F (F) and Indole (+) for Pastuerella multocida; and gram-negative, catalase (+), oxidase (-), motility (-), and IMViC (-/+/-+)) for *Salmonella Gallinarum* and *Salmonella Pullorum*) were recovered revealing that there was no positive samples for these organisms (Table 1).

According to chi-square analyses of the data, there was a statistically significant difference between *E. coli* isolation rate in sample types collected from surface 32 (58.20%) and those collected from deeper layer 0 (0%)($p < 0.05$). On the other hand, there was no significant difference ($p > 0.05$) among the origins of birds (East, North, South and West), market places (Akaki, Merkato, Mesalamia, Saris and Shola) and between time of sample collection (Christmas and Easter) for the fecal *E. coli* isolation rate (Table 2).

DISCUSSIONS

Out of 90 pooled fecal samples, the overall bacterial isolation rate was, 32 (35.55%) for *E. coli* and zero percent isolation rate for *Pastuerella multocida, Salmonella Gallinarum* and *Salmonella Pullorum*. The results of this study indicated that, 32 (58.20%) of 55 pooled fecal samples collected from top surface were positive for *E. coli* as compared to zero percent isolation rate from 35 pooled fecal samples collected from deeper layers. There is statistically significant ($P < 0.05$) association between *E. coli* isolation rate and sample types. This might be due to once the layer of the feces accumulated in the holding cages become deeper and deeper, it might creates anaerobic and acidic condition in which the organisms might not survived. In addition, the presence of a greater number of *E. coli* on the surface layer of the feces might be due to wind action and daily contact with external environment of the chicken.

Previous investigation indicated that some strains of *E. coli,* the one marked as Avian Pathogenic *Escherichia coli,* share a significant genetic similarities with the strains isolated from humans (Kołsut et al., 2017) and that numerous new genetically modified strains of *E. coli* can be found every day with potential pathogenic effect on humans and birds (Hussain et al., 2017). In this investigation although serotyping or genotyping of *E. coli* was not conducted, the abundant occurrence (58.2%) of *E.coli* positive surface fecal samples has both public health and economic implications. This study was the first and original report; it may serve as a basis for further researches.

During the course of this study, *P. multocida, Salmonella Gallinarum* and *Salmonellas Pullorum* were not recovered from all the fecal samples. This might be either due to limitation of the study period and absence of out-breaks at their origins or according to Milton (2015) it might be due to susceptibility of these organisms to external environment (heat, sun light, drying, a common disinfectants). In addition, this could be due to fact that most birds in the markets are of adult age and Antonio et

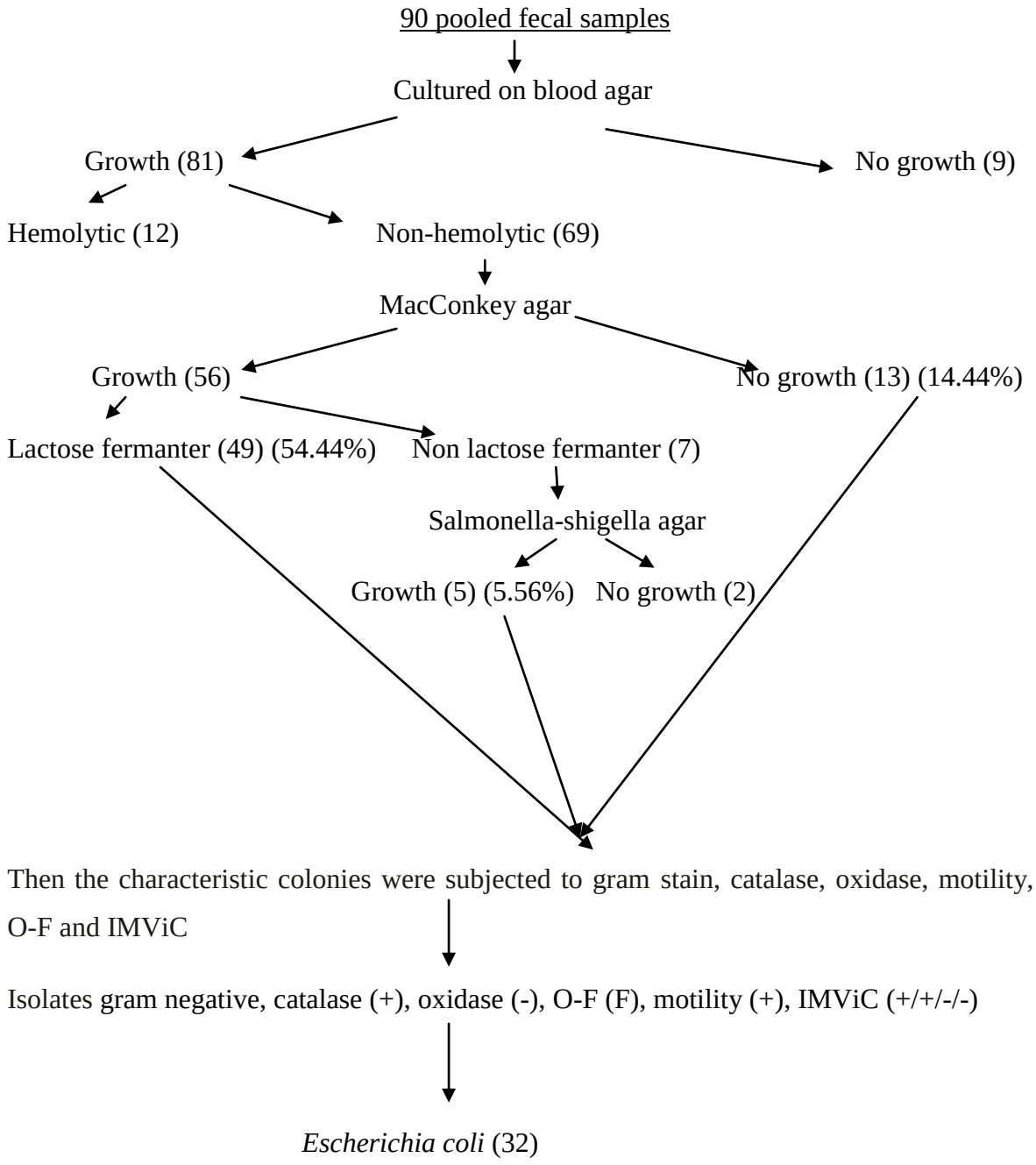

Figure 1. Flow diagram showing the schematic representation of laboratory works.

al. (2017) reported that *Salmonella Pullorum* affect only chicks less than three weeks of age.

In the study area, there were mixing of poultry originating from different parts of the country and the major sources of poultry were as far as Dire Dawa and Harar in the East; Gojjem and Gondar in the North; Arbaminch, Assella, Bale, Borena, Hawasa and Wolayta in South and Ambo and Jimma in the West and, most of them located at distance of greater than 300 km. Haftom et al. (2015) reported that live-bird markets are essential for marketing poultry in developing countries and they are preferred places for many people to purchase poultry for consumption and /or for further rearing throughout the world. Such mixing of chicken from different origins with their own health problems creates fertile grounds for exchanging and dispatching pathogens (Siraju et al., 2016).

Different live-bird markets sampled in Addis Ababa frequently consist of gathering of native chickens that satisfy local tastes and that are held in cages, baskets or

Table 2. Chi-square (X^2) tests for association between different variables (factors) and fecal *E. coli* isolation or positivity.

Variables	Category	No. of sample tested (%)	No. positive (%)	X^2	*P*-value
Origin of birds	East	19 (21.12)	6 (31.60)	0.406	0.939
	North	13 (14.44)	5 (38.50)		
	South	45 (50)	17 (37.78)		
	West	13 (14.44)	4 (30.80)		
Sample type	Deep	35 (38.90)	0(0)	31.599	0.00
	Surface	55 (61.10)	32 (58.20)		
Markets	Akaki	16 (17.78)	5 (31.25)	1.233	0.873
	Merkato	21 (23.33)	6 (28.60)		
	Mesalamia	14 (15.56)	5 (35.71)		
	Saris	23 (25.55)	10 (43.50)		
	Shola	16 (17.78)	6 (37.50)		
Time of sampling	Christmas	39 (43.33)	15 (38.46)	0.254	0.615
	Easter	51 (56.67)	17 (33.33)		

tied-up for sale. It was found that the high customers' demand for local breed chickens that might led to the transportation of birds over very long distances during holiday seasons.

Live-bird markets in Addis Ababa involves collection of chicken from multiple households who own few numbers of marketable ages, then mixed together at various density starting from the village markets to the big terminal markets in Addis Ababa that leads to spread of infection across the long distance. In addition, a high bird density in holding ground creates stressful condition and cross-infection and increased surface contamination. Thus, the isolation rate of *E. coli* from feces of holding grounds of birds from different origins found in the study area can also indicates the role of live bird markets in the biosecurity of avian pathogens of economic and public health importance and spread of pathogens along their long course of distance to market and after redistribution from markets.

CONCLUSION AND RECOMMENDATIONS

The existing customary practices of uncontrolled bird movement from long distance of origin is inhumane, stressor and spread diseases and can bring new infection. More badly mixing densely birds favors cross transmission and ground contamination. Therefore the worst scenario birds move to home or farms in public transport which can risk both farmers and public. In the study area, the holding grounds of live-bird were wooden cages which were not frequently cleaned and because of this the excreta from chicken play a great role as source for spread of avian pathogens. The significantly higher proportion of *E. coli* on surface type fecal samples indicates the lack of biosecurity measures revealing that, there may be potentially pathogenic micro-organisms present in the droppings of Addis Ababa live bird markets, thus the fact cannot be over emphasized as live bird market pose a public health hazard to humans and environment if their droppings accumulated in one place for a long period of time. During this study, *P. multocida, Salmonella Gallinarum* and *Salmonella Pullorum* were not isolated. Based on the above conclusion, the following recommendations are given:

(i) Further microbiological and epidemiological investigation is needed to correctly determine the types of major bacteria predominating in the Addis Ababa live-bird markets and the roles and magnitudes played by these markets in the epidemiology of these pathogens
(ii) Personnel should be educated on the significances of infectious diseases agents and the needs to apply biosecurity practices to prevent dissemination of these agents
(iii) Live bird that leaves the markets should be housed separately from other birds for periods of time to minimize the potential dissemination of infectious agents of poultry
(iv) The surface layer pathogens should be reduced by training merchants to exercise all in- all out put principle with proper cleaning and disinfection
(v) Further study should be done on tracking the spread of out-breaks through live-bird markets.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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