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RESEARCH ARTICLE OPEN ACCESS

Isolation and Identification of Pathogenic Bacteria Showing Resistance against Disinfectants

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Abstract

Successive use and misuse of disinfectant against certain microbes may trigger resistance, causing the problem of ineffectiveness of disinfectant due to development of resistance strains. Ultimately, it is speculated to raise public health issues worldwide. Keeping in view the said problem an experiment was designed to test the efficacy of different disinfectants against some pathogenic bacteria isolated from selected restaurants and cafeterias kitchen. A total of 45 isolates were purified and identified. Amongst them, three isolates; *Klebsiella pneumoniae*, *Macrococcus caseolyticus*, *Staphylococcus sciuri* were selected for their pathogenicity while *Terribacillus halophilus* were selected for significant tolerance to high temperature and salt regimes. Different parameters including Minimum Inhibitory Concentrations (MIC) and Minimum bactericidal concentration (MBC) were determined for disinfectant; sodium hypochlorite, benzalkonium chloride and chloroxylenol. MIC values was: 2500µg/ml to 5000µg/ml for sodium hypochlorite; 0.54µg/ml to 17.3µg/ml for Benzalkonium chloride; 18.7µg/ml to 150µg/ml for chloroxylenol. MBC values was: 5000µg/ml to 33320µg/ml for sodium hypochlorite; 8.6µg/ml to 138µg/ml for Benzalkonium chloride; 75µg/ml to 300µg/ml for chloroxylenol. Conclusively, *K.pneumoniae* showed high resistance to sodium hypochlorite and benzalkonium chloride compared to other bacteria used in this study. Among the studied, *Staphylococcus sciuri* were the most tolerant and resistance to chloroxylenol.

Keywords: Disinfectant, Resistance, Pathogenic bacteria.

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INTRODUCTION

Killing or eliminating bacteria through Disinfectants and Antiseptics particularly in microbiological laboratory, hospitals, food industry, humans and animal care centers are generally exercised for the purpose[1,2]. Selection of disinfectant and antiseptic counter to pathogenic microorganisms is become a problem due to extensive use to check and control the growth of microbes in living and non-living entities. The extensive application of disinfectant and antiseptic products has encouraged the assumption: promotion of resistance in microbes especially in cross resistance to antibiotics[3-6]. Currently, chemicals are available in the market in the form of disinfectants and antiseptics having ingredients like halogen compounds, alcohol, chlorohexidine, sodium hypochlorite, peroxides, phenols and quaternary ammonium compounds (QAC) such as benzalkonium chloride[7,8]. Ethanol, Chloroxylenol, Chlorohexidine are commonly used in microbiology laboratories[9]. Disinfectant in food industry is important to keep hygiene and to protect the product in food processing and production areas[2]. Quaternary ammonium compounds, chlorine-based biocides and peroxides are the most disinfecting agent that are used in food industry[4]. The use of disinfectant is increasing to keep high level of hygienic in food industry environment. The wide use of disinfectant impels selective pressure on bacteria and can lead to emergence of disinfectant-resistance microorganisms[10]. Disinfectant antimicrobial characteristics towards some of the pathogenic bacteria have been documented. However, microorganisms are developing resistance to these disinfectant and antiseptic, continuously[2]. Hence, for suitable selection of chemical it is essential to assess the usefulness of disinfectant or antiseptic against particular pathogen[11,12]. All over the world resistance of pathogenic bacteria against antimicrobial products has been detected and will become a major health threat which lead to treatment failure in human and animal infections[13]. Bacteria have the ability to adapt new environment rapidly even in the presence of antimicrobial molecules[14]. Ultimately, increases resistance in pathogenic bacteria along antimicrobial uses[15]. It is difficult to eradicate these pathogens with antibiotics due to new resistant strains development and new virulent pathogens appearance. New formulations with some non-antibiotic agents have been introduced which are mainly focusing breaking the chain of infections in home, industries and hospitals[16].

In this study, we investigated the presence of pathogenic and disinfectant resistant bacteria in some food facility providers (Restaurants, cafeterias) located in south Makkah city. This study will Determine the efficacy and deficiencies in hygiene practices of antimicrobial product in food facilities, the presence of pathogenic and disinfectant resistance bacteria at food preparation rooms.

MATERIALS AND METHODS

Samples collection

Samples was collected using cotton swabs from five different location including three restaurants and two cafeterias located in south Makkah from different kitchen accessories including food preparation place, Cutting boards, spoons and cutting tools. A total of 25 swabs were collected from these locations. Further, Swabs was cultured in nutrients agar media at 30°C for 24 hours.

Identifying the bacteria isolates using conventional methods

Isolates were tested by using conventional methods. Gram stain was done, which classifies bacteria into gram-positive bacteria and gram-negative bacteria. The ability to grow on different range of temperature (40 °C, 50°C and 60°C) and the ability to grow in high salt concentration by culturing bacteria isolates on halophilic agar (HiMedia) at 35°C.

Molecular studies

Bacteria was identified using DNA extraction assay; bacterial selected isolates were cultured overnight in enriching broth media. DNA was extracted following the protocols for further PCR. PCR amplification of 16S rDNA gene and sequencing. Bacteria was identified using molecular studies. DNA isolated using Thermo Scientific GeneJET Genomic DNA Purification Kit, following the protocol bacteria cell DNA were extracted for further PCR. The universal primers (Forward primer 5' - AGA GTT TGA TCC TGG CTC AG -3' and reverse primer 5' - GGT TAC CTT GTT ACG ACTT-3') were used for the amplification of the 16S

rRNA gene fragment. The PCR cycling conditions were as follows: 94°C for 5 minutes, followed by 30 cycles of 91°C for 1 min, variable annealing temperature 55°C for 1 min and 72°C for 2 min and then final extension for 5 min at 72°C. The amplified DNA fragments were gel-purified using QIAquick™ GelExtraction Kit. DNA fragment were sent to Macrogen Inc (Seoul, Korea). and sequence analyses were determined by the BLAST.

Determination of the Minimum Inhibitory Concentrations and Minimum Bactericidal Concentration

Using commercially available biocides, biocide tolerance was measured to determine the Minimum Inhibitory Concentrations for each of the selected biocides using a macro-dilution broth method following the Clinical and Laboratory Standards Institute guidelines[17]. The Minimum Inhibitory Concentrations is the lowest concentration of an antimicrobial product that inhibits the visible growth of a microorganism after overnight incubation. Minimum bactericidal concentration (MBC) was measured by transferring the bacterial culture of each tube by using 1µl loops to a nutrient agar plate after 24 hours. By using growth method 3 to 5 colonies were picked up and resuspend into tube containing 4-5 ml of muller hinton broth by using loops and incubated at 35°c until it achieves the turbidity of 0.5 McFarland standard by measuring absorbance using a spectrophotometer. The absorbance at 625 nm was from 0.08 to 0.13. Inoculum were diluted in MHB, each tube contained approximately 5 x 10^5 CFU/ml. 1ml of prepaid inoculum were added to each test tube by using test tube, two-fold disinfectant was prepared which was the desired final concentration. 1ml of the disinfectant solution were added to the first tube. The final volume was 1ml in each tube for the test.

RESULTS AND DISCUSSION

Identifying the bacteria isolates using conventional methods

Samples obtained from these locations were cultured in nutrient agar and the growth was observed after 24 hours. After culturing the samples, 45 Individual different colonies were phenotypically selected, isolated and purified. The plates were incubated at 30°C and subsequent subculture was done until pure distinguishable single bacteria colonies were obtained. Bacteria isolates were identified using conventional method. All isolate was microscopically observed for gram staining and cell morphology. 40 isolates

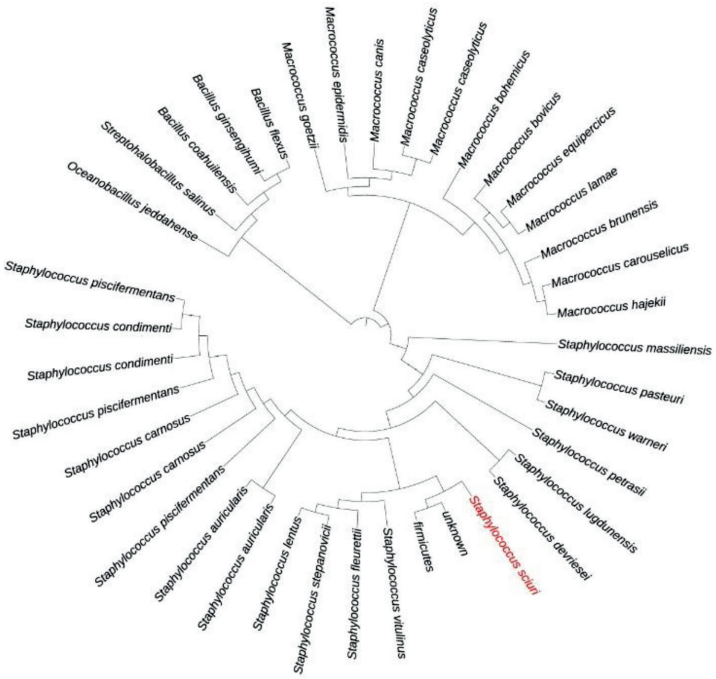

Fig. 1. Phylogenetic analysis of the *Staphylococcus sciuri*, isolates with different S*taphylococcus* strains from GenBank

(88.8%) were found gram positive and 5 isolates (11.1%) were found negative gram. Growth tolerance of the 45 isolates were also tested for temperature stress (40°C, 50°C and 60°C) and tolerance to NaCl stress using halophilic agar. All 45 isolates were *inoculated on* nutrient agar at 40°C for 24 hours, after 24 hours we found that all the bacteria isolates were able to grow at 40°C. At 50°C Bacteria isolates was inoculated on nutrient agar, we found that 10 isolates (22.2%) was unable to grow at 50°C while 35 isolates (77.7%) grew after 24 hours except for 1 isolate grew after 48 hours. At 60°C we found that 20 isolates (44.4%) was unable to grow, 18 isolates (40%) was able to grow after 24 hours, 3 isolates (6.6%) grew after 48 hours while 4 isolates (8.8%) grew after 72 hours and 16 isolates were selected for further studies.

Molecular studies

About 45 bacteria were isolated from different areas of south Makkah city. These strains were maintained at 35°C for 24 hours. Out of these three of them was identified as pathogenic based on 16s rRNA primer as *Staphylococcus sciuri, Macrococcus caseolyticus* and *Klebsiella pneumoniae as* shown in Fig. 1, 2 and 3. One of them which grow in high salt media was identified as *Terribacillus halophilus* as shown in Fig. 4. The phylogenetic tree of this strain was constructed against the other *pseudomonas* species retrieve from NCBI website. Phylogenetic tree was constructed using online iTol software as shown in (Fig. 1, 2, 3 and 4).

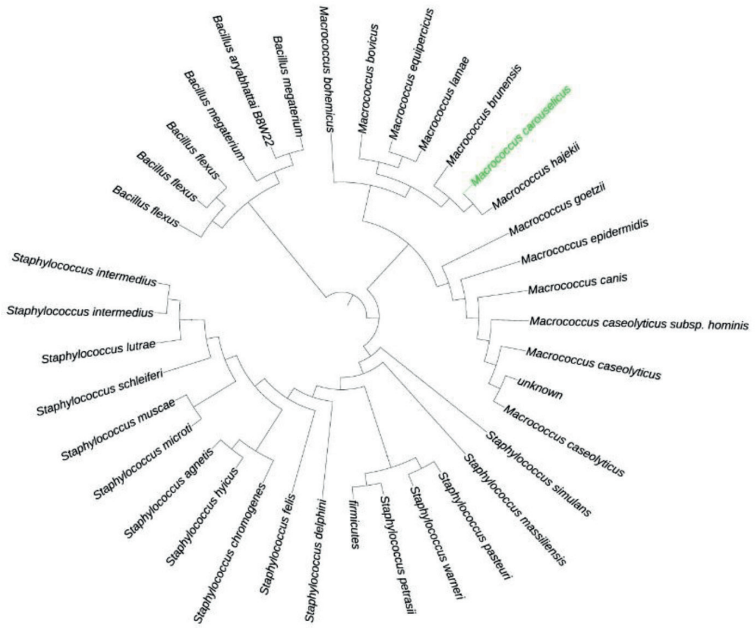

Fig. 2. Phylogenetic analysis of the *Macrococcus caseolyticus*, isolates with different *Macrococcus* strains from GenBank

Table 1. Minimum Inhibitory Concentrations and Minimum Bactericidal Concentration Of Disinfectant Agents For Selected Isolates

Bacteria	*K. pneumoniae*		*T. halophilus*		*S. sciuri*		*M. caseolyticus*	
MIC Chemical Ag.	MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml
Sodium hypochlorite	4165	33320	4165	8330	5000	10000	2500	5000
Benzalkonium chloride	0.54	138	17.3	34.7	1.08	8.6	0.54	34.7
Chloroxylenol	37.5	75	18.7	150	150	300	18.7	150

Determination of the Minimum Inhibitory Concentrations and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) values were investigated. 3 bacterial isolates were selected according to their pathogenicity, *Klebsiella pneumoniae*, *Macrococcus caseolyticus*, *staphylococcus sciuri* and one isolate *Terribacillus halophilus* were selected for exhibiting high tolerance to growth factors at high Temperature

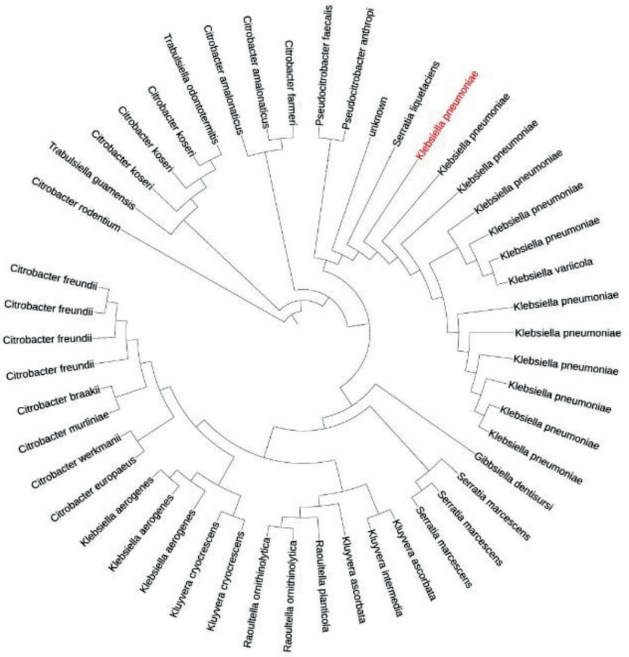

Fig. 3. Phylogenetic analysis of the *Klebsiella pneumoniae*, isolates with different *Klebsiella* strains from GenBank

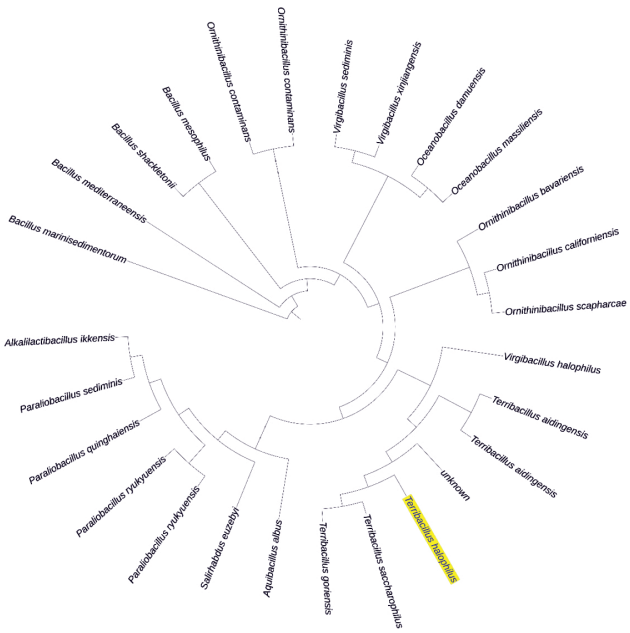

Fig. 4. Phylogenetic analysis of the *Terribacillus halophilus*, isolates with different *Terribacillus* strains from GenBank

and high salt concentrations.

Minimum inhibitory concentration of sodium hypochlorite values was: 5000µg/ml for *S. sciuri*, 2500µg/ml for *M. caseolyticus* and 4165µg/ml for both *K. pneumoniae* and *T. halophilus*. MBC values were: 33320µg/ml for *K. pneumoniae*, 5000µg/ml for *M. caseolyticus*, 8330µg/ml for *T. halophilus* and 10000µg/ml for *S. sciuri*. Benzalkonium chloride MIC values were: 0.54µg/ml for *M. caseolyticus*, and for *K. pneumoniae*, 1.08µg/ml for *S. sciuri* and 17.3µg/ml for *T. halophilus*. MBC values were: 34.7µg/ml for both *M. caseolyticus* and *T. halophilus*, 8.6µg/ml for *S. sciuri*, 138 µg/ml for *K. pneumoniae.* The data is shown in Fig. 5, 6, 7, 8 and table 1. Moreover, Chloroxylenol MIC values were: 18.7µg/ml for both *M. caseolyticus* and *T. halophilus*, 37.5µg/ml for *K. pneumoniae* and 150µg/ml for *S. sciuri*. MBC values were: 75µg/ml for *K. pneumoniae*, 150µg/ml for both *M. caseolyticus* and *T. halophilus*, 300µg/ml for *S. sciuri*.

Bacteria exposed to sodium hypochlorite showed high tolerance comparing with other disinfectant used in this study. *K.pneumoniae* were the highest resistance bacteria as MBC result were 33.3 mg/ml. Benzalkonium chloride were highly effective to inhibit bacteria growth according to the result showed on table 1 except for *T. halophilus* which was 17.3µg/ml, however regarding MBC result *K. pneumoniae* were high up to 138µg/ml. Chloroxylenol were less effective than Benzalkonium chloride and *S. sciuri* were the highest tolerance and resistance comparing with other bacteria. The results indicate that the misuse of disinfectant products for disinfection processes may increase bacteria resistance to disinfectant which will lead to disinfection failure and cause threat to public health as previous study by[18] confirmed that three strains of *K.pneumoniae* showed reduced susceptibility against Chlorhexidine, Benzalkonium chloride and Trigene, and another study by[19] about three clinical isolates *K.pneumoniae* that exposed to sub-inhibitory concentration of chlorhexidine showed increase in MIC values from 4-8µg/ml to 128 µg/ml for all three strains. The presence of pathogenic bacteria that can tolerate disinfection process's and contaminate food facilities and the prevalence of antimicrobial resistance (AMR) bacteria considered as a major public threat and could lead to treatment failure.

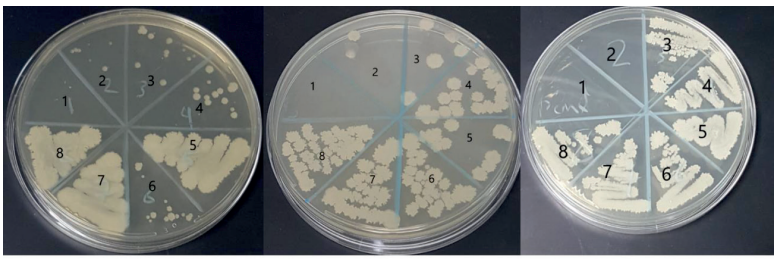

Fig. 5. *K. pneumoniae* MBC (a) Sodium hypochlorite #1 = 33320µg/ml, (b) Benzalkonium chloride #1 = 138 µg/ml and (c) Chloroxylenol #2 = 75µg/ml.

Fig. 6. *S. sciuri* MBC, (a) Sodium hypochlorite #2 = 10000 µg/ml, (b) Benzalkonium chloride #5 = 8.6 µg/ml and (c) Chloroxylenol #1 = 300 µg/ml.

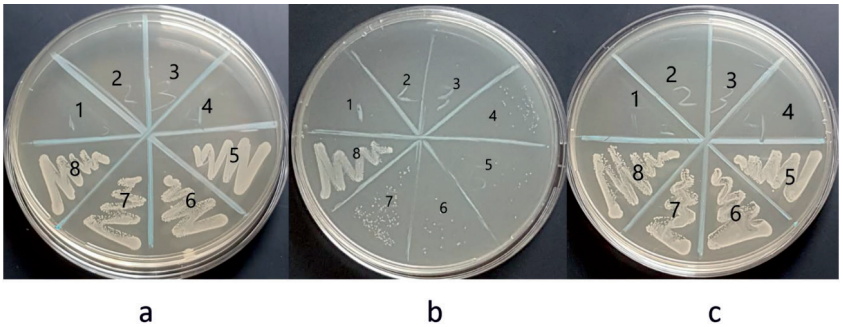

Fig. 7. *T. halophilus* MBC, (a) Sodium hypochlorite #4 = 8330 µg/ml, (b) Benzalkonium chloride #4 = 34.7µg/ml and (c) Chloroxylenol #2 = 150 µg/ml.

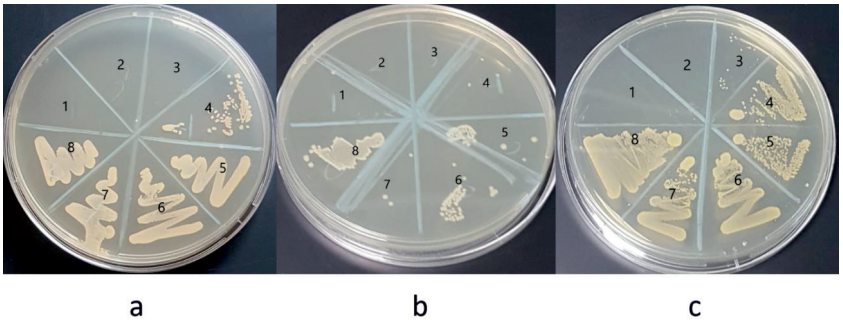

Fig. 8. *M. caseolyticus* MBC, (a) Sodium hypochlorite #3 = 5000 µg/ml, (b) Benzalkonium chloride #3 = 34.7µg/ml and(c) Chloroxylenol #2 = 150 µg/ml.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHORS' CONTRIBUTION

Authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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