Prevalence, Antibiotic Resistance and *In-vitro* Activity of Yogurt Against Some Gram Negative Pathogenic Bacteria isolated from Arar Hospital, KSA

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Abstract: In recent decades, the antimicrobial resistance of bacteria isolated from hospital has increased. Gram negative bacterial strains are the most frequent bacterial strains isolated from infected specimens. This study analysed gram negative infections in Arar Hospital in North Border Province, KSA, in order to estimate their frequency and antimicrobial susceptibilities. Out of 40 cases admitted to hospital, 35%, 25% and 15 strains of *Klebsiella pneumoniae*, *E. coli* and *Pseudomonas aeruginosa* respectively were isolated from different clinical specimens. Antibiotic resistance profiles of these strains to antibiotics were plotted. All the tested strains were multiple antibiotic resistance (MAR), resisting to at least two antibiotics. Higher rates of susceptibility were demonstrated isolates against ciprofloxacin. Also the purpose of this study was to investigate any relation between the proven antibiotic resistance of isolated bacteria and the presence of plasmids. Molecular sizes of the detected plasmids were 1.6 kbp in *Enterobacter cloacae* and 1.8 kbp in *Pseudomonas aeruginosa*. The aim of this study also was to determine the inhibitive effect of 7 different types of yogurt, three of them were homemade and the other was industrial on several pathogen and contaminant bacteria. All of the yogurt types exhibited antimicrobial activity against *Klebsiella* sp. Cattle, goat and sheep homemade were used to investigate there inhibitor activity against some of the pathogenic microbes. Goat homemade yogurt exhibited inhibition on the all tested microbes to produce inhibition zone ranged from 16-25mm at different concentration. The high resistance isolates identified makes it necessary for antibiotic resistance testing to be conducted prior to antibiotics prescription for infection patients in KSA.

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1. Introduction

Gram-negative bacteria are bacteria that do not retain crystal violet dye in the Gram staining protocol. In a Gram stain test, a counter stain (commonly safranin) is added after the crystal violet, coloring all Gram-negative bacteria with a red or pink color. The pathogenic capability of Gram-negative bacteria is often associated with certain components of Gram-negative cell envelope, in particular, the lipopolysaccharide layer (also known as LPS or endotoxin layer). In humans, LPS triggers an innate immune response characterized by cytokine production and immune system activation. Inflammation is a common result of cytokine production, which can also produce host toxicity (Reynolds et al., 2003).

Antibiotic resistance is a form of drug resistance whereby some (or, less commonly, all) sub-populations of a microorganism, usually a bacterial species, are able to survive exposure to one or more antibiotics. Accordingly, pathogenic species which have become resistant cause infections which cannot be treated with the usual, formerly efficacious antibiotic drugs and/or their usual, formerly efficacious, dosages and concentrations. Resistance may be instrinsic or acquired. Some clinically relevant pathogens have developed resistance to multiple antibiotics and are dubbed multidrug resistant (MDR pathogens). More recently, the colloquial term superbug has become widespread in both popular and technical accounts of the phenomenon with which it is synonymous (Reynolds et al., 2003).

Antibiotic resistance is a serious and growing phenomenon in contemporary medicine and has emerged as one of the eminent public health concerns of the 21st century, particularly as it pertains to pathogenic organisms. In the simplest cases, drug-resistant organisms may have acquired resistance to first-line antibiotics, thereby necessitating the use of second-line agents. Typically, the first-line agent is selected on the basis of several advantages including safety, availability and cost; comparatively, the second-line agent is usually broader in spectrum,

possesses a less favourable risk-benefit profile and may be more expensive or, in more dire circumstances, locally unavailable. In the case of some MDR pathogens, resistance to second and even third-line antibiotics is sequentially acquired, a case quintessentially illustrated by *Staphylococcus aureus* in some nosocomial settings. Some pathogens, such as *Pseudomonas aeruginosa*, additionally possess a high level of intrinsic resistance.

It may take the form of a spontaneous or induced genetic mutation, or the acquisition of resistance genes from other bacterial species by horizontal gene transfer via conjugation, transduction, or transformation. Many antibiotic resistance genes reside on transmissible plasmids, facilitating their transfer. Exposure to an antibiotic naturally selects for the survival of the organisms with the genes for resistance. In this way, a gene for antibiotic resistance may readily spread through an ecosystem of bacteria. Antibiotic-resistance plasmids frequently contain genes conferring resistance to several different antibiotics.

A plasmid is a small DNA molecule that is physically separate from, and can replicate independently of, chromosomal DNA within a cell. Most commonly found as small circular, double-stranded DNA molecules in bacteria, plasmids are sometimes present in archaea and eukaryotic organisms. In nature, plasmids carry genes that may benefit survival of the organism (e.g. antibiotic resistance), and can frequently be transmitted from one bacterium to another (even of another species) via horizontal gene transfer. Artificial plasmids are widely used as vectors in molecular cloning, serving to drive the replication of recombinant DNA sequences within host organisms.

Plasmid sizes vary from 1 to over 1,000 kbp. The number of identical plasmids in a single cell can range anywhere from one to thousands under some circumstances. Plasmids can be considered part of the mobilome because they are often associated with conjugation, a mechanism of horizontal gene transfer.

The term plasmid was first introduced by the American molecular biologist JoshuLederberg in 1952. Plasmids are considered replicons, capable of replicating autonomously within a suitable host. Plasmids can be found in all three major domains: Archaea, Bacteria, and Eukarya. Similar to viruses, plasmids are not considered by some to be a form of life. Unlike viruses, plasmids are naked DNA and do not encode genes necessary to encase the genetic material for transfer to a new host, though some classes of plasmids encode the sex pilus necessary for their own transfer. Plasmid host-to-host transfer requires direct mechanical transfer by conjugation, or changes in incipient host gene expression allowing the intentional uptake of the genetic element by transformation. Microbial transformation with plasmid DNA is neither parasitic nor symbiotic in nature, because each implies the presence of an independent species living in a commensal or detrimental state with the host organism. Rather, plasmids provide a mechanism for horizontal gene transfer within a population of microbes and typically provide a selective advantage under a given environmental state. Plasmids may carry genes that provide resistance to naturally occurring antibiotics in a competitive environmental niche, or the proteins produced may act as toxins under similar circumstances. Plasmids can also provide bacteria with the ability to fix nitrogen or to degrade recalcitrant organic compounds that provide an advantage when nutrients are scarce (Poirel et al., 2005).

In this study investigated the prevalence and antimicrobial resistance of Gram negative bacteria isolates from Arar Hospital. KSA. Also, the purpose of this study was to investigate any relation between the proven antibiotic-resistance of bacteria isolated from patients and the presence of plasmids. Also, the present study is therefore aimed at understanding the protective effect of yogurt from cattle, goat, sheep fresh milk, and four commercial yogurts and their ability to inhibit some of the pathogenic microbes.

2. Material and Methods

Patients and strains

Gram Negative strains were isolated from Forty cases were selected from Clinical samples (Urine, pus, blood, Sputum) carried out at the Arar Hopsital, KSA. All specimens were cultured on MacConky agar plates. All plates were incubated face down and the bacteria were allowed to grow at 37°C for 24-48 hours prior to enumeration and further identification. Pure well-isolated colonies were preceded for their biochemical tests: The isolated colonies were identified by the API 20E system (Bio Merieux, mary, I'E Toil, France).

Antibiotic Susceptibility Studies.

All experiments were conducted using the original stock cultures to avoid the spontaneous loss of antibiotic resistance sometimes associated with frequent subculturing. Antibiotic resistance was assayed using a modified Kirby-Bauer disc diffusion method. Bacterial isolates were inoculated into 5mL of sterile broth and incubated at 37^{o}C for 18 hours. A loopful was then diluted in 5mL sterile phosphate buffered saline (PBS) and seeded onto Müller-Hinton agar plates. After the inoculum has dried, antibiotic discs were applied to the inoculated medium with sterile forceps and pressed down gently to ensure even contact. Antibiotic standard discs (Oxoid) were

used namely, chloramphenicol, tetracycline, erythromycin, gentamicin, and ciprofloxacin. Diameter in mm of clear zones surrounding the antibiotic discs indicating bacterial growth inhibition was measured after 24 hours incubation at 37°C (NCCLS, 2000; Selim et al., 2013).

Isolation of Plasmid DNA

For purifying plasmid DNA from Escherichia coli cells, the Qiagen Spin Miniprep Kit produces quite reliable results. For Qiagen buffer compositions, please see the Qiagen Buffers page. Note: Qiagen buffer also works for Epoch Life Science's spin columnswhich are sold in bulk at a much lower price. Yield and purity are quite close. This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 37. Please read "Important Notes" on pages 19–21 before starting. Note: All protocol steps should be carried out at room temperature. Resuspend pelleted bacterial cells in 250 µl Buffer P1 (kept at 4 °C) and transfer to a microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet. Add 250 µl Buffer P2 and gently invert the tube 4–6 times to mix. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. Add 350 µl Buffer N3 and invert the tube immediately but gently 4–6 times. To avoid localized precipitation, mix the solution gently but thoroughly, immediately after addition of Buffer N3. The solution should become cloudy. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting. Centrifuge for 30–60 s. Discard the flow-through. Spinning for 60 seconds produces good results. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step. Although they call this step optional, it does not really hurt your yield and you may think you are working with an endA- strain when in reality you are not. Again for this step, spinning for 60 seconds produces good results. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s. Spinning for 60 seconds produces good results. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer. IMPORTANT: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions. They are right about this. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min. If you are concerned about the concentration of the DNA, you can alternatively add 30 µL water to the center of the column, incubate at room temperature on the bench for 5 mins and then centrifuge for 1 min. This will increase the concentration of DNA in your final sample which can be useful in some cases. See notes below for why you should elute in water rather than the Buffer EB they recommend if you plan to sequence your sample. Even if you are not sequencing, it may be beneficial to elute in water. For instance, if you elute in buffer EB and you are using this DNA in a restriction digest, then the additional salts in your sample can affect the salt content of your digest. This may matter with some finicky enzymes (Diab et al., 2004).

Agarose Gel Electrophoresis of the Plasmid DNA

Agarose gel electrophoresis was carried out using the tris-borate EDTA buffer (TBE). Gels were prepared by adding 1% agarose and 5µL ethidium bromide (stock solution of 10 mg/mL) to the TBE buffer as described by Hammad and Dora (1993).

Yogurt Samples Collection

Homemade yogurt will be made in our house and code A-C. Commercial yogurt will be purchased from local market in Sakaka, Al-Jouf. All manufacturers' names have been replaced with code names in order to avoid any commercial consequences, the samples were coded D-G.

Determination of Antimicrobial Activity

The antimicrobial activity of yogurt was determined using the following techniques: A total of 7 yogurt were sampled (3 of them were homemade and the other were brought from the market). Each sample was centrifuged to get the yogurt supernatant that contain the nutrient. Supernatants of each yogurt samples were checked for antibacterial activity against pathogenic bacteria in inoculated Mueller-Hinton agar plates. Yogurt supernatants were inoculated in the wells in pre-seeded agar plates. Reference standard of different antibiotics was inoculated in the same Petri plates as a positive

control. The plates were kept undisturbed for 2 to 3 h and then incubated for 24 h at 37°C aerobically. Finally, the plates were examined for the presence of inhibition zones by the help of Digital caliper in mm (Selim 2011, Selim et al., 2012) .

3. Results and Discussion

Most antibiotic- resistant microbes emerge as a result of genetic change and subsequent selection processes by antibiotics. The resistance factor may be chromosomal, that developed as a result of spontaneous mutations and extrachromosomal resistance (plasmid resistance). R factors are a class of plasmids that carry genes for resistance to one and often several antibiotics and heavy metals. Genetic material and plasmids in particular, can be transferred by transduction, transformation, conjugation and transposition mechanisms (Brooks et al., 1995: Diab et al. 2002: Selim and Hagagy 2013).

Forty isolates were recovered from clinical specimens over the course of this period. *Klebsiella pneumoniae* (14 strains) accounted for 35% of total isolates (Table 1 and Fig. 1). This followed by *E. coli* (10 strains, 25 %) and other organisms (40 %). As can be seen in table 2 and Fig. 2, higher rates of susceptibility were demonstrated isolates against ciprofloxacin.

Table 1. Frequency of bacterial isolates in different studied clinical specimens from Arar Hopsital, KSA.

Species	Blood	Pus	Urine	Sputum	percentage
E. coli	3	6	1	0	25
Enterobacter cloacae	4	0	0	1	12
Pseudomonas aeruginosa	0	5	0	1	15
Proteus mirabilis	0	0	0	5	12
Klebsiella pneumoniae	11	3	0	0	35

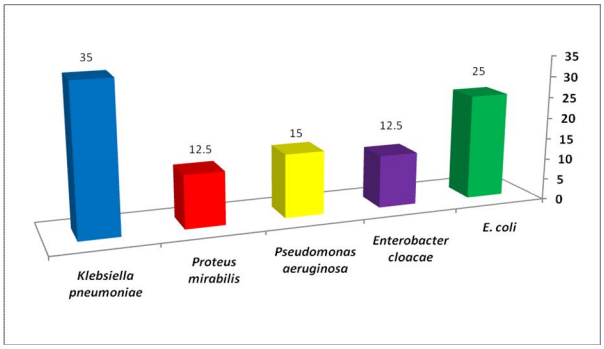


Figure 1. Showed the percentage of isolated bacteria.

Plasmid profiles of the gram-negative bacterial isolates under study were determined. Only 5 out of 7 isolates were found to contain plasmids (Figure 5). No plasmids could be detected for the other two isolates (Fig. 5 and table 3). Molecular sizes of the detected plasmids were 1.6 kbp in *Enterobacter cloacae* and 1.8 kbp in *Pseudomonas aeruginosa* (Fig. 5).

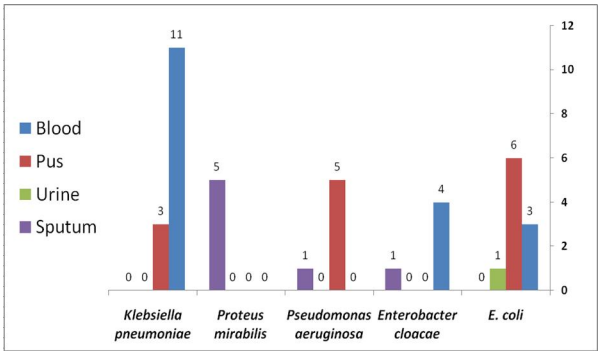


Figure 2. Prevalence of isolated bacteria in different clinical specimens

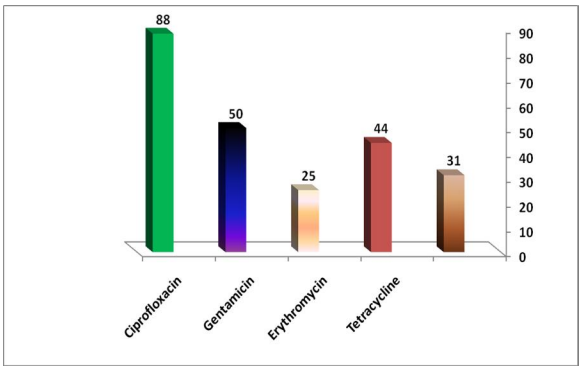


Figure 3. Percentage of sensitivity of isolated gram negative bacteria in against studied antimicrobial agents

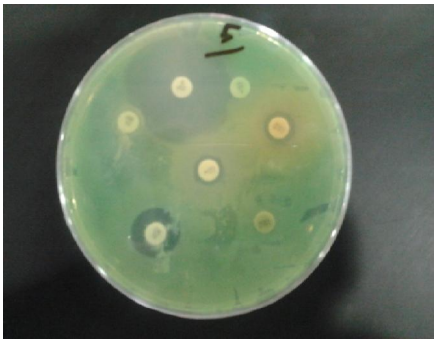


Figure 4. Showed the inhibition zone of studied antimicrobial agents on *Pseudomonas aeruginosa*.

Table 3. Molecular size of isolated plasmids size.

Species (Source)	Total Size(Kbp)
Enterobacter cloacae **(Blood)**	1.6
Escherichia coli **(Blood)**	1.6
Escherichia coli **(Pus)**	1.75
Pseudomonas aeruginosa **(urine)**	1.8
Proteus mirabilis **(Sputum)**	1.8

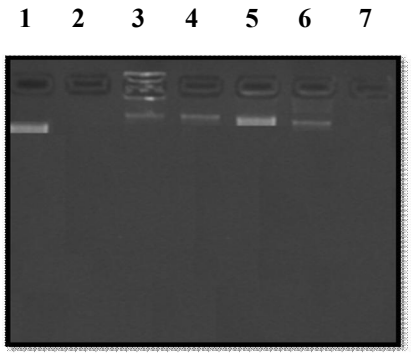


Figure 5. A brief description of the plasmids detected in studied bacterial. Where:

Lane1: *Proteus mirabilis* (Sputum) *
Lane2: *Pseudomonas aeruginosa*
Lane3: *Escherichia coli* (Blood)*
Lane4: *Escherichia coli* (Pus) *
Lane 5 : *Pseudomonas aeruginosa* (urine) *
Lane6: *Enterobacter cloacae* (Blood) *
Lane7: *Klebsiella pneumoniae* (Pus)

*Plasmid bearing isolates.

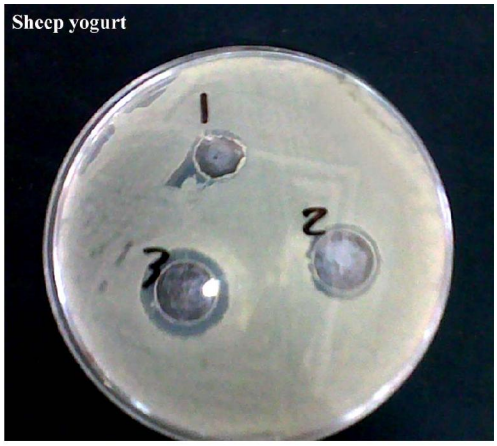
A total of seven different yogurt types were tested for their antimicrobial activity against pathogens bacteria such as (*E. coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Proteus mirabilis*) The antimicrobial activity of yogurt are given in Table 4 and Figure 6. All of the yogurt types exhibited antimicrobial activity against to *Klebsiella pneumoniae*. It has been known that *Lactobacillus bulgaricus* has a preservative effect on the product not only because of the production of lactic acid and hydrogen peroxide, but also by the help of the antimicrobial compounds (example, bacteriocin) it produces. The compound, namely Bulgarican, is inhibitory towards both Gram-positive and Gram-negative bacteria. Some inhibitory compounds against *Staphylococcus* and *Clostridium* species have also been found (Erkus, 2007).

The entire homemade yogurt exhibited obvious inhibition zone when incubated with the pathogens but with different pattern of the appearance clear zone ranged between 12 to 25mm. Significant inhibiting potential has been observed against *Klebsiella pneumoniae* (27 mm) by action of yogurt code (4) at concentration of 250 µL, followed by goat and sheep yogurt that recorded 25 mm at the same concentration (250 µL). These results also are accordance with previous work carried out by Erdourul and Erbulur (2006) but on different strain types, in which supernatants obtained from *Lactobacillus casei* and *L. bulgaricus* exhibited varying degrees of inhibitory activity against strains of *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *Pseudomonas aeroginosa* ATCC 9027, *B. subtilis* ATCC 6633, *Klebsiella pneumonia* ATCC 18833, *Salmonella typhimurium* ATCC 13311, and *Enterobacter cloacae* ATCC 13047. The probiotic potential of these bacteria is also vastly investigated (Diez-Gonzalez, 2007). *Pseudomonas aeruginosa* inhibited only in the presence of sheep yogurt (20 mm, inhibition zone) and by yogurt code (G) that recorded inhibition zone of 17 mm at concentration of 250 µL.

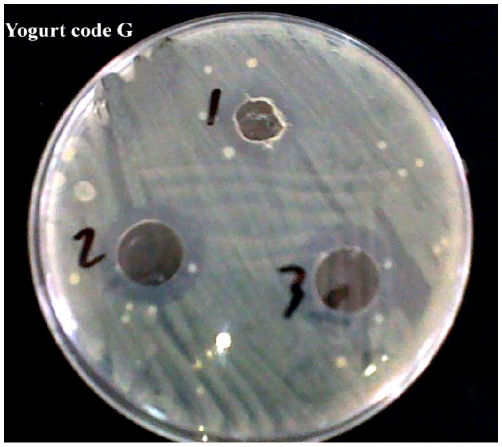
Table (4): Effect of different yogurt types on the test microbes.

Yogurts Types		*E. coil*	*P. aeruginosa*	*K. pneumoniae*	*P. mirabilis*
A	**100µL**	-	-	19	-
	200µL	23	-	22	16
	250µL	26	-	24	20
B	**100µL**	15	12	18	21
	200µL	19	16	25	23
	250µL	20	20	25	24
C	**100µL**	-	-	16	-
	200µL	16	-	22	-
	250µL	17	-	25	19
D	**100µL**	-	-	21	-
	200µL	17	-	25	23
	250µL	19	-	27	19
E	**100µL**	-	-	20	-
	200µL	-	-	23	-
	250µL	-	-	24	-
F	**100µL**	-	-	17	-
	200µL	-	-	21	-
	250µL	-	-	24	-
G	**100µL**	-	-	15	-
	200µL	16	15	21	-
	250µL	18	17	24	-

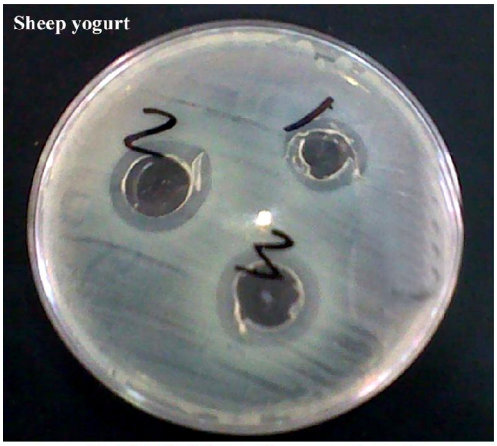
Where: A: cattle yogurt, B: goat yogurt, C: sheep yogurt, D-G codes of the commercial yogurt and -: not detected.



Pseudomonas sp.

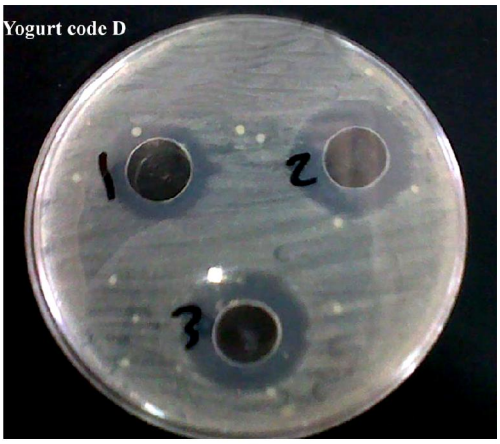


Klebsiella Sp.

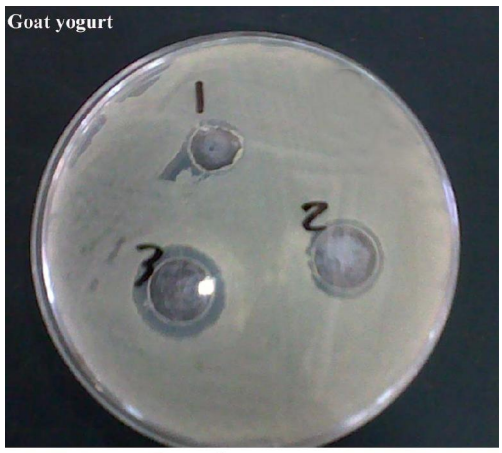


Escherichia coli, wound swab

Klebsiella Sp.



Klebsiella Sp.



Pseudomonas sp.

Figure 6 . Antimicrobial activity of different types of yougrts. Where 1, 2 and 3 differencon concentartion of yogurts.

4. Conclusion

Gram negative bacterial strains are the most frequent bacterial strains isolated from infected

specimens in Arar Hospital in North Border Province, KSA. The high resistance isolates identified makes it necessary for antibiotic resistance testing to be conducted prior to antibiotics prescription for infection patients in KSA.

The plasmid-linked resistance patterns of 7 gram negative isolates were investigated. For five of them plasmids were observed and plasimd-linked resistance. In recent studies plasimd-linked resistance, especially for pathogenic bacterial isolates, are still of critical importance. Detailed characterization of these plasmids is needed in future for more understanding about transferability, gene expression and stability.

This study showed that, homemade yogurts had shown antimicrobial activity against some pathogenic microbes especially *E. coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Proteus mirabilis*. Whereas these isolates had determinate resistance to some commercial antibiotics. Our results showed that certain strains which would be used in the manufacture of homemade yogurts using natural starters have various antimicrobial activity and antibiotic resistances. Therefore, in yogurt manufacturing, we consider it careful in use these strains. Also, there is need for more research on antibiotic resistance profiles of yogurt bacteria. Further research will be focus on varied antibiotic resistance of microbial strains isolated from homemade yogurts.

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