Effect of medicinal plants, Heavy metals and antibiotics against pathogenic bacteria isolated from raw, Boiled and pasteurized milk

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Abstract: Present study has been undertaken to isolate and identify the bacterial flora in raw, boiled and pasteurized milk. Agar disc diffusion method was used to determine their sensitivity using medicinal plants, antibiotics and heavy metals. Methylene blue reduction test was used to test the quality of milk samples. Total 10 pathogenic strains were isolated, five strains were isolated from raw milk, three from boiled milk and 2 two from pasteurized milk. To determine optimum conditions for growth, these pathogenic microorganisms were incubated at various temperatures and pH. Gram's staining and biochemical tests revealed that these pathogenic bacteria include *Lactobacillus* sp., *E. coli*, *Salmonella* sp., *Pseudomonas* sp., *Streptococcus* sp. and *Staphylococcus.* Ribotyping revealed S2 as *Pseudomonas fluorescens*, S5 as *Lactococcus lactis* and S9 as *Lactobacillus acidophilus*. Prevalence of pathogenic organisms provided the evidence that contamination of milk arises during milking, transportation and storage of milk. Raw milk is more contaminated than other two types of milk because it contains highest percentage of pathogenic organisms and pasteurized milk was found to be of best quality among three types. So it is recommended to drink milk after proper boiling or pasteurization. Proper pasteurization and hygienic packing of milk is essential to minimize contamination in milk which can save human beings from many milk borne diseases. Our study suggests that antimicrobial use in animal husbandry should be minimized to reduce the hazard of antibiotic resistance. Plant extracts are better alternative against pathogenic bacteria in milk.

Keywords: Milk borne pathogens, antibogram analysis, resistogram analysis, medicinal plants, antibacterial activity, molecular identification of microbes.

INTRODUCTION

Milk is a main constituent in human food in the whole world besides it acts as an excellent home for growth of countless microorganisms particularly pathogenic bacteria. Therefore, the quality of milk is very important for the health of a community. Milk can be taken normally as raw milk, boiled milk and pasteurized milk.

Milk and milk products serve as prime habitat to enormous variety of microorganisms and their presence in milk leads to the large scale variations in attributes of milk like taste, odor and quality of milk are affected the most. Adulteration in milk and milk products by presence of microorganisms arises from inside of udder, outside of udder, by milker man and equipment which is used for storage to transport to long distances. Milk is a sterile substance in the udder of a mammal but as soon as milk passes out of the teat of cow, it is inoculated by normal flora of the animal (Talaro, 2005). The origin of pathogenic microorganisms present in milk may be from a cow, or it may be human, and in some cases it may be transmitted by both (Seguin *et al.,* 1999 and Khan *et al.,* 2000).

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Pathogenic bacteria in raw milk are involved in spoilage of milk and these pathogens are predominantly resulting of poor handling and unclean conditions. These pathogenic microorganisms in raw milk are responsible for many milk borne illnesses like tuberculosis, brucellosis and typhoid fever especially in developing countries (Goff and Horst, 1995). Most of the milk borne disorders arise by drinking milk are caused by *Salmonella* sp., *Listeria monocytogenes, Staphylococcus aureus, Campylobacter* sp., *Yersinia* sp., *Escherichia coli* and *Clostridium botulinum.*

Boiling of milk in developing countries including Pakistan is a common practice and it increases the shelf-life of milk as well as also enhances the taste of the milk. Metwally *et al.* (2011) has reported that there is a noticeable decrease in total bacterial load when raw milk was exposed to boiling of 15 seconds. Lipids and proteins in colloidal forms in milk that have a shielding role in micro-organisms against heat treatments given to kill microorganisms, consequently some micro-organisms are expected to survive in milks even after boiling **(**Pathak *et al.,* 2012).

Pasteurization is a thermal process used to reduce health risks from pathogenic microorganisms present in milk and also to lengthen shelf life of product. Spoilage of

processed milk is predominantly due to activity of bacteria that results in loss of physical quality (Boor and Murphy, 2002 and Hayes and Boor, 2001). Among microorganisms found in milk, psychotropic bacteria are considerably the most important cause that is altering the quality of pasteurized milk (Sorhaug and Stepaniak, 1997).

Various antibiotics are used to treat different cases of mastitis (Morin *et al.,* 1998) Antibiotics are usually used to cure diseases of cattle and besides they are being used as preservatives for milk (Devriese *et al.,* 1997). Antibiotic treatment has become ineffective because the excessive use of these antibiotics has managed to develop multiple antibiotic resistances among microorganisms causing diseases (Johnston *et al.,* 1983). Low amount of antibiotics can affect some susceptible bacteria while resistant bacteria grow as such in presence of antibiotics. These resistant bacteria are responsible for spreading their resistance characteristics to bacteria that were formerly non-resistant so resistant bacteria have broad range effects (Lacy *et al.,* 1998).

MATERIALS AND METHODS

Sources, collection and transportation

A total number of 5 raw milk samples (10 ml each) were collected from different dairy owners from various areas of Lahore, Pakistan and its surrounding districts. The milk samples were collected directly during milking procedure in sterilized capped bottles. After collection samples were brought to the laboratory in an ice box and were tested within 24 hours of collection for microbiological examination. A total number of 5 boiled milk samples (10 ml each) in triplicate were studied. Each sample was heated to three holding times i.e. ≥ 5 min, 10 min and 15 min, respectively. After boiling samples were saved in an ice-box and brought to the laboratory for microbiological examination.

A total number of 5 pasteurized milk samples (10 ml each) were collected from milk vendors in Lahore, Pakistan and transported to laboratory for microbiological study.

Methylene Blue reduction test (Decolorization assay)

Methylene blue reduction test (MBRT) is employed to check the quality of milk samples. In MBRT, 1 ml of methylene blue was added to 10 ml of milk sample in autoclaved test tube. Then tube was airtight with help of rubber stopper and was inverted slowly for three times to mix thoroughly. Tube was then placed in a water bath at 35ºC and was observed at different time intervals for 10 hours (Pathak *et al.,* 2012).

Isolation of bacteria from raw, boiled and pasteurized milk sample

One ml of each raw milk sample was dissolved in 10 ml of distilled autoclaved water to make dilution. One ml of the diluted sample was taken and 100 µl was poured by micro pipette and spread on nutrient agar plate. Plates were inverted and incubated in an incubator at 37°C for overnight. Various isolated colonies were obtained after spreading and streaking and pure single colony was isolated. For screening of pathogenic bacterial isolates blood agar test was performed. Alpha hemolysis and beta hemolysis confirmed the presence of pathogenic bacteria.

Characterization of bacterial isolate

The obtained bacterial colonies were characterized by employing different tests. For the characterization of obtained bacterial isolates both morphological and biochemical tests were performed. Bacterial isolates were macroscopically observed for colony morphology like shape, color, and texture and bacterial isolates were characterized further biochemically by indole, methyl red, Voges Proskauer, citrate utilization test and carbohydrate fermentation test, gelatin hydrolysis, starch hydrolysis, and urease test etc

Antibiogram analysis

Agar disc diffusion (Bauer-Kirby) susceptibility test was used to assess the sensitivity of antibiotics according to Bauer *et al.* (1966). The disc diffusion test was done on nutrient agar plates. Isolated bacterial colony was spread uniformly by help of sterile spread over the entire surface of the Petri dishes. Before applying discs, the plates were permitted to dry and discs of particular potency were placed with the help of forceps in lamina flow air cabinet after 15min when plates have become dry. Five antibiotics were used *viz*., ampicillin (Amp) 10µg, azithromycin (Azm) 15µg, cefixime (cfm) 5µg, Tetracycline (Ti) 75µg, oxacillin (ox) 5 µg. The plates in inverted position were incubated in an incubator at 37°C for 24 hrs. After 24 hrs plates were observed for the measurement of zone of inhibition around specific discs. The results of the sensitivity tests were expressed as (0mm) for no sensitivity, (below 10mm) for low sensitivity, (11-20mm) for moderate sensitivity and (21-35mm) for high sensitivity.

Determination of antibacterial activity of medicinal plant extract

Aloe Vera extracts

Fresh Aloe Vera leaves were collected, rinsed with running tap water to remove dust or sand and placed in oven at 80°C to dry for 48 hrs. When leaves got dried, they were powdered. Ten g of crushed leaves was soaked in 100 ml of organic solvent i.e. methanol. Methanolic extract was cited in shaking incubator for 48 hrs at 250 rpm. Then filtered extract was centrifuged for 20 min at 8000 g. A sterile flask was used to collect supernatant. Then this extract was stored at 4°C.

Cinnamomum verum extract

One hundred grams of *Cinnamomum verum (*cinnamon) were soaked in 400ml of methanol in sterilized conical

flask (8 with continuous shaking at 130rpm at 20°C overnight in a temperature controlled bio -shaker. The methanol fraction was separated with help of muslin cloth and after that clean Whatman filter paper (No. 2) was used for filtration. Prepared extract was concentrated in a rotary vacuum evaporator at 40°C and the condensed extracts were diluted to 10mg/ml with the solvent methanol and stored at 4°C.

Eucalyptus sp. extract

Leaves of *Eucalyptus* sp. were collected, dried and grounded to form powder and then dipped in 90% ethanol for seven days. After seven days methanol evaporated and extract was filtered and dried in an oven. To get desired concentration methanol was added to extract (Rosina *et al.,* 2009).

Acacia nilotica extract

Leaves of *A. nilotica* were collected and taxonomically identified at department of botany at GC University. Leaves of *A. nilotica* were dried and then grounded to form powder and then dipped in 90% methanol for seven days. Methanol evaporated and extract was filtered and dried in an oven. Ethanol evaporated in seven days and remaining extract was filtered by masculine cloth. Finally the extracts were oven dried at 60^{o}C. To get required concentration ethanol was added to extract (Rosina *et al.,* 2009).

Nigella sativa (Kalwonji) extract

Fresh plants were collected and washed with distilled water and kept for drying. The dried plant material was grinded in electric grinder. 50 g of powdered plant material was soaked in 500 ml methanol. This mixture was kept at room temperature for two weeks at 25°C in bottle with infrequent shaking. Large amount of solvent was separated after evaporation of three weeks. Extracts were filtered and stored at 4°C in refrigerator (Javaid *et al.,* 2013).

Agar disc diffusion assay

Sterilized petri dishes were poured with nutrient agar medium. Discs of eight mm in diameter were dipped in 50 µl of two concentrations of each of five extracts i.e. 50% and 100% (50mg/ml, 100mg/ml) extract. Inoculums were prepared in nutrient broth medium and was compared to McFarland standard (10^8 CFU/mL) and then diluted to 1:50 for microdilution. Plates were streaked with inoculum by injecting 100µl of inoculum by micropipette. After the discs were dried they placed on agar plates by using sterilized forceps and pressed tightly on nutrient agar medium. On each agar plate disc of 50 % and 100 % extract were placed for each of five extract. Agar plates were incubated at 37^{o}C and the minimum inhibitory concentration (MIC) was recorded after 24 hours. After 24 hrs, the inhibited zones were formed on the media and these zones were measured. The antimicrobial activity of each extract was determined by taking the mean diameter of the inhibition zone (mm) formed by plant extracts. The results of the sensitivity tests were expressed as (0) for no sensitivity, (below 10) for low sensitivity, (11-20) for moderate sensitivity and (21-35) for high sensitivity. The MIC is the lowest concentration of the compound (extract) at which the microbe tested does not show evident growth.

Resistogram analysis

To check the capacity of bacteria to withstand heavy metals, MIC of mercury, chromium, copper and cadmium were determined through dilution method. First of all stock solutions of these metals were prepared and filtered. Different concentrations of mercury, chromium, lead; copper and cadmium i.e. 100, 200, 300, 400 and 500µg/ml were added in 20 ml of sterilized broth against bacterial strains and kept in an incubator at 37˚C. Then optical density of each bacterial strain was assessed by using spectrophotometer at wavelength of 590 nm.

Determination of Optimum pH

To determine optimum pH for each bacterial strain, pH of medium was set up to 2, 4, 6, 8, and 10. After setting pH, medium was autoclaved. 100µl of fresh culture of each isolated strain was inoculated in medium and medium was incubated in shaking incubator at 37ºC. After 20 hours, optical density was taken by spectrophotometer at 590 nm (OD_{590}). Each pH was taken in triplicates for each strain. Finally the optimum pH of each strain was determined by plotting a graph between pH on X-axis and Optical density along Y-axis. Optimum pH for each bacterial isolate was found by graph.

Determination of Optimum Temperature

To determine optimum temperature for each isolated strain, medium was incubated at 25ºC, refrigerator temperature, 37ºC, 45ºC and 60ºC. Optical density was taken by spectrophotometer after 20 hours at 590 nm (OD_{590}). Lastly the optimum temperature of each strain was determined by plotting a graph between temperature on X-axis and optical density along Y-axis. Optimum temperature for each bacterial isolate was ascertained by graph.

Genomic DNA isolation

DNA was isolated by phenol chloroform method. Agarose gel electrophoresis (1%) was done to assure that the samples contain isolated genomic DNA. After running gel, DNA bands in the gel were visualized using short wave ultraviolet light provided by a trans illuminator and photographed was taken through Stratagene Eagle Eye still video system.

Ribotyping

Ribotyping is aimed at molecular characterization of pathogenic milk isolates, so their 16SrDNA was partially

Table 1: Decolourization time and grading of milk samples

Samples	Raw milk		Boiled milk		Pasteurized milk	
	Decolourization Time (Hrs)	Quality	Decolourization Time (Hrs)	Quality	Decolourization Time(Hrs)	Quality
S-1	2	Poor	6	Good	8	Excellent
S-2	2	Poor	1.5	Poor	5	Good
S-3	1.5	Poor	7	Good	8	Excellent
S-4	2.5	Poor	2	Poor	7.5	Good
S-5	4	Good	2.5	Poor	10	Excellent

S-1 to S-5: number of milk samples; Hrs: Hours

Table 2: Biochemical characterization of bacterial strains isolated from various milk samples

Test	Bacterial isolates									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Blood agar	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE
Methyl Red	+VE	-VE	-VE	+VE	-VE	+VE	+VE	+VE	-VE	+VE
Indole	+VE	-VE	-VE	+VE	+VE	-VE	+VE	+VE	-VE	+VE
VP	-VE	-VE	+VE	-VE	-VE	-VE	+VE	-VE	+VE	-VE
Catalase	+VE	+VE	+VE	+VE	-VE	+VE	+VE	+VE	-VE	+VE
Citrate	-VE	+VE	+VE	-VE	+VE	+VE	+VE	+VE	+VE	+VE
Urease test	-VE	-VE	+VE	-VE	-VE	+VE	-VE	+VE	-VE	+VE
Glucose fermentation	+VE	-VE	+VE	+VE	-VE	+VE	+VE	-VE	+VE	-VE
Sucrose Fermentation	+VE	-VE	+VE	+VE	+VE	+VE	+VE	-VE	+VE	+VE
Lactose fermentation	+VE	-VE	+VE	+VE	+VE	+VE	-VE	+VE	+VE	+VE

S1-S2 indicates bacterial strains isolated from milk samples; +VE indicates positive; -VE indicates negative

Table 3: Effect of different temperatures on growth of pathogenic bacteria isolated from milk

Bacterial isolates	Temperature (M±SD)				
	15°C	25°C	37°C	45°C	60°C
Control	0.00	0.00	0.00	0.00	0.00
S1	0.165±0.02	0.404±0.01	0.621±0.01	0.441±0.05	0.301±0.001
S2	0.231±0.03	0.426±0.02	0.671±0.005	0.306±0.04	0.210±0.007
S3	0.123±0.01	0.307±0.01	0.540±0.006	0.326±0.06	0.117±0.006
S4	0.31±0.02	0.427±0.05	0.689±0.004	0.376±0.02	0.234±0.004
S5	0.22±0.03	0.488±0.06	0.645±0.003	0.454±-0.08	0.345±0.003
S6	0.214±0.02	0.356±0.07	0.578±0.002	0.052±0.006	0.047±0.02
S7	034.± 0.01	0.447±0.01	0.596±0.002	0.333±0.004	0.190±0.01
S8	0.210±0.01	0.312±0.01	0.593±0.01	0.083±0.003	0.068±0.001
S9	0.315±0.05	0.412±0.02	0.685±0.02	0.583±0.002	0.317±0.001
S10	0.213±0.02	0.393±0.1	0.608±0.05	0.288±0.001	0.189±0.02

S1-S2 indicates bacterial strains isolated from milk samples; (M±SD): Mean ± standard deviation

amplified through polymerase chain reaction. Polymerase chain reaction (PCR) was performed in a thermocycler for 35 reaction cycles. Total reaction mixture (50 µl) was taken. Initial denaturation was done at 94ºC for 5 min, annealing was done at 52°C and elongation at 72°C for 30 sec, 40 sec and 30 sec, respectively. Final extension was given at 72°C for 10 min.

Amplified DNA extraction and Gel electrophoresis

To confirm the amplification, PCR products were loaded in 1 % agarose gel. Gel was run following same procedure for half hrs at 80 volts and the bands of amplified DNA were visualized under the UV light by using the trans-illuminator. After amplification anticipated band were eluted/cut and kept in eppendrofs. Then using GF-1 DNA recovery kit by Vivantis, gene was cleaned.

Sequencing

The amplified sequence of 16S rDNA gene was sent to Molecular Biological Products, Korea for sequence analysis. The nucleotide sequences were Basic Local Alignment Search Tool (BLAST) searched for blastn and or/ blastx algorithms in NCBI.

Table 4: Effect of different pH on growth of pathogenic bacteria isolated from milk

Bacterial isolates	pH				
	2	4	6	8	10
Control	0.00	0.00	0.00	0.00	0.00
S1	0.041±0.001	0.058±0.005	0.065±0.005	0.522±0.001	0.499±0.001
S2	0.556±0.001	0.456±0.007	0.609±0.01	0.342±0.005	0.593±0.006
S3	0.097±0.006	0.279±0.007	0.682±0.002	0.526±0.003	0.326±0.006
S4	0.081±0.001	0.432±0.02	0.567±0.03	0.321±0.006	0.298±0.006
S5	0.689±0.001	0.628±0.003	0.728±0.02	0.567±0.04	0.367±0.002
S6	0.095±0.001	0.503±0.005	0.509±0.004	0.298±0.006	0.149±0.003
S7	0.194±0.001	0.357±0.002	0.535±0.007	0.338±0.005	0.023±0.001
S8	0.130±0.01	0.248±0.001	0.461±0.001	0.354±0.01	0.232±0.005
S9	0.070±0.002	0.438±0.004	0.648±0.003	0.640±0.001	0.415±0.01
S10	0.122±0.001	0.320±0.001	0.446±0.01	0.346±0.003	0.291±0.005

S1-S2 indicates bacterial strains isolated from milk samples; (M±SD): Mean ± standard deviation

Table 5: Molecular characterization of bacteria

Bacterial Isolate	Description	Max score	Total score	Query cover	E Value	Max indent	Source
S2 (697 bps)	*Pseudomonas fluorescens*	1288	1288	100%	0.0	100	Raw milk
S5 (670 bps)	*Lactococcus lactis*	1238	1238	100%	0.0	100	Raw milk
S9 (687 bps)	*Lactobacillus acidophilus*	1269	1269	100%	0.0	100	Boiled milk

RESULTS

Decolourization assay

Results for decolarization assay asre shown in table 1. It was observed that all collected pasteurized milk were decolourized after 5-10 hours using the methylene blue reduction test and was classified as a class 1 and class 2 milk (excellent and good quality milk). On the other hand S-3 sample of raw milk were decolourized within 1.5 hrs followed by S-1, S-2, S-4 and S-5 samples. Similarly S-2 sample of boiled milk was decolourized within 1.5 hrs followed by S-4, S-5, S-1 and S-3 (table 1). These results indicated that pasteurized milk is of good quality and raw milk to be of poor quality having most of bacterial contamination.

Isolation and identification of bacterial pathogens

Results of bacterial isolates characterization are shown in table 2. All bacterial isolates showed hemolytic activity on blood agar plates. Morphological and biochemical characterization showed that common pathogens in milk were S1 (*Staphylococcus* sp.), S2 (*Pseudomonas fluorescens*), S3 (*Klebsiella* sp.), S4 (*E. coli*), S5 (*Lactococcus lactis*), S6 *(Listeria* sp.), S7 (*Salmonella* sp.), S8 (*Corynebacterium*sp.), S9 (*Lactobacillus acidophilus*), S10 (*Proteus* sp.).

Effect of different temperatures and pH

It was observed that the Optimum temperature for all bacterial strains was 37°C (fig. 1). The optimum pH for S1 and S2 was 8, for S3 was 6, for S4-S8 was 4-8, and for S9 and S10 was 4-10 (fig. 2). The optical density (OD_{590}) was also recorded through spectrophotometer and shown in table 3 and 4.

Resistogram analysis

MIC of heavy metals such as mercury, copper, lead, cadmium against pathogenic strains was determined. Minimum inhibitory concentration was smallest heavy metal concentration at which bacteria show minimum growth or no growth. Different concentrations of heavy metals were checked for minimal inhibitory concentration of heavy metals. S1 (*Staphylococcus* sp.) showed no growth at 500 µg/ml for lead, 400 µg/ml for mercury, 500 µg/ml for cadmium showing its sensitivity but for chromium S1 showed growth of up to 500 µg/ml showing S1 is resistant to chromium. S2 (*Pseudomonas fluorescens*) showed minimal inhibitory concentration at 500 µg/ml for mercury but it showed resistance to chromium, cadmium and for lead showing growth up to 500 µg/ml. S3 (*Klebsiella* sp.) showed minimal inhibitory concentration at 500 µg/ml for cadmium and showing resistance for lead, chromium and cadmium. S4 (*E. coil*) showed minimal inhibitory concentration at 500 µg/ml for mercury and showing resistance for lead, chromium and cadmium.

S5 (*Lactococcus lactis*) showed minimal inhibitory concentration at 500µg/ml for mercury and showing resistance for lead, chromium and cadmium. S6 (*Listeria* sp.) showed minimal inhibitory concentration at 500 µg/ml for mercury and chromium and showing resistance for lead and cadmium. S7 (*Salmonella* sp.) showed minimal inhibitory concentration at 500µg/ml for chromium and cadmium and showing resistance mercury and lead. S8 (*Corynebacterium* sp.) showed minimal inhibitory concentration at 500 µg/ml for cadmium and showing resistance mercury, chromium and lead. S9

(*Lactobacillus acidophilus*) showed minimal inhibitory concentration at 500µg/ml for cadmium and showing resistance mercury, chromium and lead. S10 (*Proteus* sp.) showed minimal inhibitory concentration at 500µg/ml for cadmium and showing resistance mercury, chromium and lead.

The sensitivity test showed that among four tested heavy metals for pathogenic bacteria, maximum sensitivity was revealed to mercury followed by cadmium and minimum sensitivity was revealed to lead allowing the increase in growth pathogenic strains up to 500 µg/ml. The number of microorganisms declined with increasing concentration of heavy metals tested on pathogenic microorganisms representing the lethal effect of these heavy metals on the increase of microorganisms.

Antibiogram analysis

Antibiotics were used against pathogenic bacteria to check the antibiotic susceptibility of pathogenic bacteria in milk. Resistance of pathogenic bacteria of isolated pathogenic strains was determined by disc diffusion method. According to the results, S1 (*Staphylococcos* sp.) showed largest zone of inhibition against oxacillin of 17.3±0.3 mm and showed minimum zone of inhibition against tetracycline was 3.0±0.16 mm. S2(*Pseudomonas*) showed largest zone of inhibition against oxacillin of 15.0±0.15 mm and showed minimum zone of inhibition against teteracycline was 5.0±0.5 mm showing maximum resistance for cefixime. S3 (*Klebsiella* sp.) showed largest zone of inhibition against azithromycin of 3.0±0.32 mm and showed no zone of inhibition against test ampicillin, tetracycline and oxacillin. S4 (*E. coli*) showed largest zone of inhibition against oxacillin of 20.0±0.02 mm and showed minimum zone of inhibition against tetracycline was 1.5±0.05mm. S5 (*Streptococcus*) showed largest zone of inhibition against azithromycin of 6.6±0.16 mm and showed no zone of inhibition against ampicillin and cefixime. S6 (*Listeria* sp.) showed largest zone of inhibition against azithromycin of 6.66±0.07 mm and showed minimum zone of inhibition against both oxacillin were of 1.5±0.01mm. S7 (*Salmonella* sp.) showed largest zone of inhibition against oxacillin of 17.0±0.1 mm and showed minimum zone of inhibition against tetracycline was 2.33±0.15mm. S8 (*Corynebacterium* sp.) showed largest zone of inhibition against oxacillin of 15.5±0.3 mm and showed minimum zone of inhibition against was tetracycline of 3.5±0.01 mm, showing no zone for azithromycin and cefixime indicating its strong resistance for azithromycin and cefixime. S9 (*Lactobacillus* sp.) showed largest zone of inhibition against oxacillin of 19.0±0.15mm and showed minimum zone of inhibition against azithromycin was 3.3±0.1mm. Ampicillin and cefixime were completely resistant for S9 as both of them gave no zone of inhibition. S10 (*Proteus* sp.) showed largest zone of inhibition against both oxacillin of 15.44±0.02 mm and showed minimum zone of inhibition against tetracycline of 3.5±0.1mm but gave no zone of inhibition against azithromycin and cefixime showing its strong resistance for azithromycin and cefixime.

Multiple resistances of isolated pathogenic strains isolated from raw, boiled and pasteurized milk were found against antibiotics employed. Ampicillin showed highest resistance among all antibiotics used followed by cefixime. Ampicillin proved to be completely insensitive for all the strains except S6, S7 and S10. Oxacillin was proved to have best antimicrobial effect against nearly all the pathogenic strains isolated from milk followed by azithromycin and tetracycline.

Antibacterial activity of medicinal plants

Antibiotics are leading source for cure of microbial infections. Overuse of these antibiotics has led to development of multi drug resistance against pathogenic strains. Prevalence of resistance pathogenic strains has raised the need of new microbial agents against pathogenic strains. Plants or herbal extracts have replaced antibiotics to some extent. For this purpose plant extracts were prepared and employed to check antimicrobial activity of these pathogenic strains. Five different plant extracts were tested against all strains through agar disc diffusion method. According to the results, plant extract sensitivity of S1*Staphylococcus* sp.) was determined. S1 showed largest zone of inhibition against *A. nilotica* of 5.0±0.12 mm and showed minimum zone of inhibition against Aloe vera was 0.32±0.01 mm. S2(*Pseudomonas* sp.) showed largest zone of inhibition against *N. sativa* of 3.0±0.16mm and showed minimum zone of inhibition (0.32±0.14mm). S3 (*Klebsiella* sp.) showed largest zone of inhibition against *A. nilotica* of 7.5±0.01mm and showed minimum zone of inhibition against cinnamon was 0.4±0.01mm. S4 (*E. coli*) showed largest zone of inhibition against *A. nilotica* of 6mm ± 0.8 and showed minimum zone of inhibition against cinnamon was 0.35 ±0.1mm. S5 (*Streptococcus* sp.) showed largest zone of inhibition against *A. nilotica* of 6.0±0.01mm and showed minimum zone of inhibition against cinnamon was 0.7±0.12mm. S6 (*Listeria* sp.) showed largest zone of inhibition against *A. nilotica* of 7.0±0.16mm and showed minimum zone of inhibition against *Aloe vera* was 0.2±0.04mm.

S7 (*Salmonella* sp.) showed largest zone of inhibition against both *A. nilotica* and *N. sativa* of 6.0±0.16 mm and showed minimum zone of inhibition against cinnamon was 0.7±0.06 mm. S8 (*Corynebacterium* sp.) showed largest zone of inhibition against *A. nilotica* of 6.0±0.16 mm and showed minimum zone of inhibition against Aloe vera was 0.2±0.1 mm. S9 (*Lactobacillus* sp.) showed largest zone of inhibition against both *A. nilotica* of 5.5±0.02 mm and showed minimum zone of inhibition against cinnamon was 0.75±0.03 mm. S10 (*Proteus* sp.)

showed largest zone of inhibition against both *Eucalyptus* of 4.0±0.1 mm and showed minimum zone of inhibition against Aloe vera was 0.6±0.12 mm.

Extract of cinnamon was *A. nilotica* was most effective showing the antibacterial activity against all pathogenic strains tested except *Lactobacillus* sp. which gave maximum zone of inhibition against *Eucalyptus.* Least effective extract of all extract were of cinnamon and *Aloe vera.*

Sequencing of PCR-fragment and analysis

The genomic DNA of all biochemically analyzed bacterial isolates were isolated and run at agarose gel for further studies i.e. ribotyping. Specific sequences of bacterial DNA were amplified (fig. 3) and sequenced. The homology of the amplified products were analyzed through NCBI nucleotide data blast system (table 5). These results indicated that S2 strain showed 100% homology with *Pseudomonas flourescens* (), S5 strain showed 100% homology with *Lactococcus lactis* (TTTGTATTCTTTAAAAGAAATCTGCTCAAAAATA GTTTCATTTAAATCAGGGCTTAGTTGATATGTTAT AATTAGGGAATGGAAAACTTTTACAAAGTAGGA ACAATTGTTAATACACAGGGACTTCAAGGTGAAG TTCGAATACTACCATCAACAGATTTTGCTAACGA GCGCTTTTCAAAAGGGGCAGTATTAGCACTTTTT GATGATAAAGATAACTATATTCAAGATTTAAAAG TGAAATCTGGTCGATTACAAAAGAATTTTTATGT GGTCAAATTTGAAGGATTTTATCATATCAATGAT GTCGAAAAATATAAAGGTTATGTTGTTAAAATTG CGCAAGAAAATCAAGAAGAGTTAAATGATGGGG AATTTTACTATCATGAAATTATTGGAAGTGATGT TTATGACAATGACATTTTAATTGGTCAGATCTCT GAAATTCTTCAACCAGGTGCCAATGATGTCTGGG TCGTTAAACGTAAAGGAAAACGTGATTTACTTTT ACCTTATATCCCACCTGTTGTCCTGAAAGTAGAT GTGGCTCAGCATCGAGTTGATGTGGACATCATGG AAGGATTGGATGACTAAGGGCATGAGAATTGAT ATTTTAAGTATTTTTCCTGATATGTTTGGTCCACT TAACCAGTCAATAGTTGGCAAGGCT), and S9 showed 100% homology with *Lactobacillus acidophilus* (ATGCAAAATACAACAAATAATTTTGCTATTAATT AAAAGCTTGATGCCAAGTGCTTCAAGCTTTTTTT ATTACTTAAAAGGAGAAAAAGGTATGCAGTACTT TGATGTTGCACGAATTTTGACTACACATGGATTA CATGGAGAAGTTAAAGTAAATGTGATTACGGATT TTCCAGAAGATAGATTTGCTGAGGGGATGCAACT AGAATTAAAAGATGATATTGATCGAGTTTTAACA ATAAAAAAGAGTAGACCATTTAAGCAATTTTGGC TACTTCAATTTGATGAAATAACTGATATTGATGA AGCTGAAAAATTACGTGGAAAAATTTTAGTAATT AGTGAAAAAGATCGTGGTGAATTACCAGATGGA GTTTACTATTACAAGGATATTTTTGATTGTGGTGT AATAGACAATGAGACTGGTAAAAGATTAGGTAA AATAACTGATATTCAATCACCTGGTGCTAACGAT ATTTGGCTTGTGCATGAAGACAATGGTAAAGAAT ATTGGATTCCTAATATTGCTGATGTTGTAAAGAA AATAGATATTGCTGATAAAAAGGTATATGTTGAA TTGATGGAGGGATTGCGAGATGAAGATTAATATC CTGACCCTTTTTCCTGATATGTTTACGCCATTGCA AGTTTCTATGCTGGGACGTGGTCTTGAAGATGGT AAGTGGG).

DISCUSSION

As a result of microbial characterization *Staphylococcus* sp., *E. coil, Klebsiella sp.,Pseudomonas, Listeria* sp. were found to be present in raw milk samples by help of Bergey's manual key. *Salmonella* sp., *Corynebacterium* sp., *Lactobacillus acidophilus* was isolated from boiled milk samples and *Proteus* sp. was found in pasteurized milk samples. This agrees with result of Torkar and Terger, 2008; Mubarack *et al.,* 2010 and Ibtisam and Own, 2009. Quality of milk tested by methylene blue reductase test revealed that all milk samples were of poor quality of all milk samples tested. These results are similar in accordance with the results of Pant *et al.,* (2013) and Srunjna *et al.,* (2011). In present research work ribotyping was done for antibiotic resistant bacteria i.e. S2, S5 and S9 were molecularly characterized as *Pseudomonas flouresecens*, *Lactococcuslactis* and *Lactocbacillus acidophilus* respectively.

Antibiotics are used as source of treatment of microbial infections. Discovery of these antibiotics and their uses lead to an idea in the medical world that antibiotics will wipe out all infection diseases caused by pathogenic microorganisms (Rosina *et al.,* 2009). Nowadays use of antibiotics in developing countries in common. Antibiotic resistance has developed in bacteria due to their misuse or overuse. These resistant antibiotics are ineffective against pathogenic microorganisms and cause high rate of death (Hart and Kariuki, 1998). Farmers use antibiotics for their treatment of animals. These antibiotics residues remain in milk and provide an opportunity for development of antibiotic resistance bacteria in milk (Booth, 1998). These antibiotics residues are harmful for public health.

Mastitis (inflammation of mammary glands) is due to gram-positive bacteria rarely by certain gram-negative bacteria. Mastitis is usually treated by the employing antibiotics. The transmission of antibiotic resistance genes occur between phylogenetically distant bacterial genera and in this way resistance among different genera of bacteria emerges that is cause of failure of cure of wide spread infections by antibiotics. Emergences of antibiotic resistance among the pathogenic bacteria create a problem of inefficacy of present therapy by antibiotics and their capacity to minimize infectious in both animals and humans.

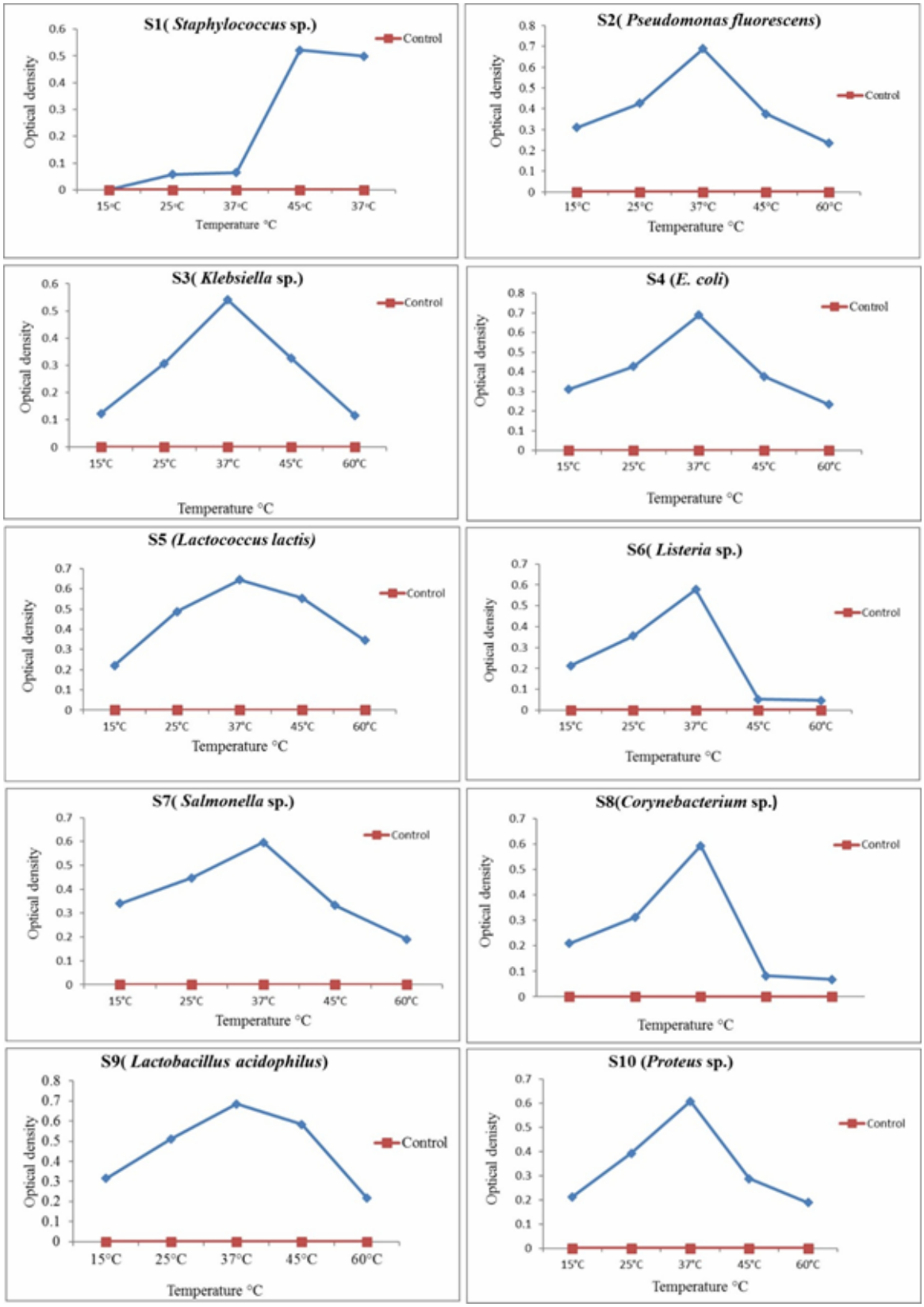

Fig. 1: Optimization of bacterial growth at various temperatures. S1 (*Staphylococcus* sp.), S2 (*Pseudomonas fluorescens*), S3 (*Klebsiella* sp.), S4 (*E. coli*), S5 (*Lactococcus lactis*), S6 *(Listeria* sp.), S7 (*Salmonella* sp.), S8 (*Corynebacterium* sp.), S9 (*Lactobacillus acidophilus*), S10 (*Proteus* sp.)

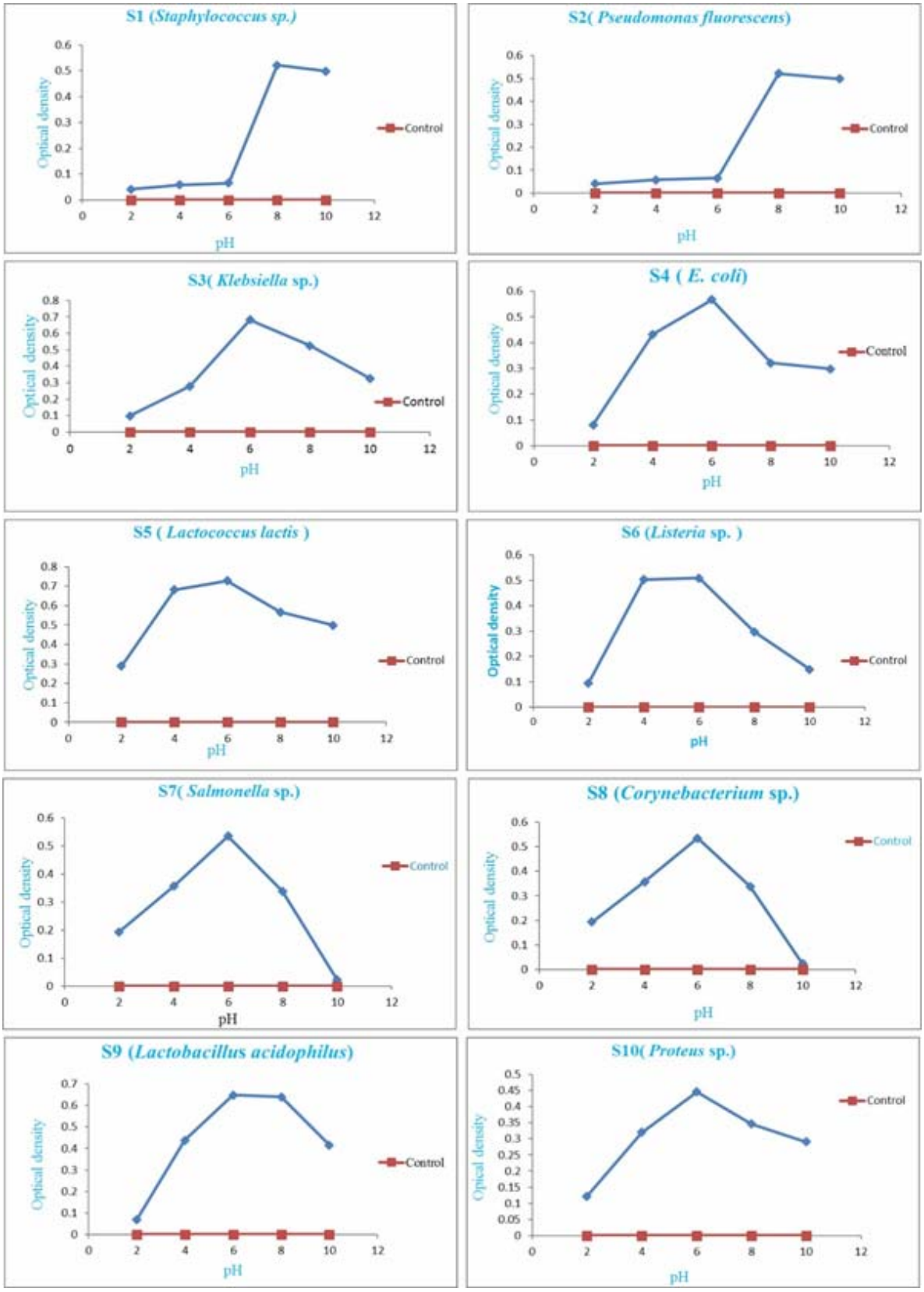

Fig 2: Optimization of bacterial growth at various range of pH. S1 (*Staphylococcus* sp.), S2 (*Pseudomonas fluorescens*), S3 (*Klebsiella* sp.), S4 (*E. coli*), S5 (*Lactococcus lactis*), S6 *(Listeria* sp.), S7 (*Salmonella* sp.), S8 (*Corynebacterium* sp.), S9 (*Lactobacillus acidophilus*), S10 (*Proteus* sp.).

Isolated pathogenic strains were revealed to have multiple resistances against antibiotics used. Ampicillin and cefixime showed the maximum resistance against pathogenic bacteria from milk (table 3). This is a startling outcome because these antibiotics are very frequently used in Pakistan. *Lactococcu slactis* showed highest resistance towards ampicillin and cefixime. These results agree with Ibtisam and Owni, (2009).

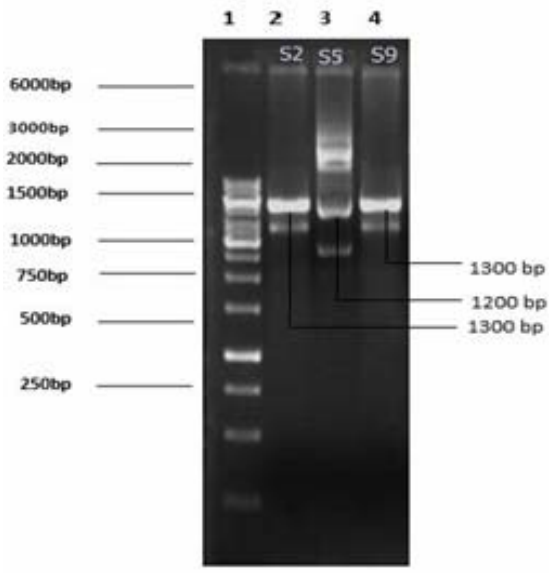

Fig. 3: Amplified PCR products of 16SrDNA

CONCLUSION

From current study it can be concluded that different plant extracts can be used as antibacterial agents against different antibiotic resistant bacterial pathogens from milk. Because of increasing antibiotic resistance of pathogenic bacteria, there is need for alternative natural and cheaper way to control bacterial infections. So these plant extracts can be used as an alternative and effective method in future.

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