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Detection of multi-drug resistant *Streptococcus pyogenes* from raw milk samples in Faisalabad

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Abstract

Milk is a very important source of nutrition for humans as well as animals. It contains carbohydrates, fats, vitamins, and minerals. Due to its nutritious composition, it is an excellent medium for the growth of microorganisms. Milk gets contaminated by different microorganisms including *Streptococcus* species. Considering its importance, the present study was designed to check the microbial contamination of raw milk. 90 samples were collected from different areas of Faisalabad city. Mainly *Streptococcus* species were targeted and isolated. Out of 90, only 23 samples were found positive for *Streptococcus* species. Most species were identified as *Streptococcus pyogenes* by biochemical characteristics. Antibiotic susceptibility testing was performed and isolates were found to be susceptible to Amikacin (30µg), Tazobactam (110µg), Levofloxacin (5µg), Tobramycin (30µg), Ampicilin-sulbactam (20µg), Chloromphenicol (30µg), and Gentamycin (30µg) and were resistant to Cefoxitin (30µg), Tigecycline (15µg), Cefipime (30µ), Ampicilin (10µ), Cefotoxime (10µg), Trimethoprim (25µg), Cefuroxime (30µg), Cefixime (5µg), Tetracycline (10µg). For further confirmation, Minimum Inhibitory Concentration was performed according to CLSI guidelines and results showed that isolates were susceptible to only Ampicillin, Amikacin and Erythromycin. Animals suffering from clinical or sub-clinical mastitis are the source of *Streptococcal* contamination of milk. The current study supports the finding that raw milk can be regarded as a critical source of MDR bacteria. Strict monitoring and the implementation of effective hygienic should be implemented.

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Introduction

Milk is a nutrient-rich,white liquid food produced by the mammary glands of mammals. It is a primary source of nutrition for children and good for bone growth as it is rich in calcium. Its demand in the global market is increasing day by day. It is also used for the production of a variety of dairy products like yogurt, cheese, etc.It containscarbohydrates, fats, vitamins, and minerals(Guetouache *et al.,* 2014).Due to its nutritious composition, it is an excellent medium for the growth of many microbial communities(Soomro *et al.,* 2002). Milk gets contaminated by different microorganisms including *Listeria monocytogenes*, *Streptococcus* species, *Staphylococcus* species, *Compylobacter* species, *Mycobacterium tuberculosis,* etc and these microorganisms adversely affect the quality of milk(Elmoslemany *et al.,* 2009).These pathogenic microorganisms are known to cause serious health hazards to the general population.Milk can serve as a source for transferring these microorganisms from animals to humans (Zoonosis)(Garcell *et al.* 2015). Milk is extremely susceptible to spoilage due to these microorganisms which get into milk from different sources such as Milkman's hands, water, feed, Animal's skin,utensils, environment, etc (Prejit *et al.,* 2007). Microorganisms present in raw milk produce toxins and are responsible for foodborne diseases.Food borne diseases are responsible for >50% cases of mortality to children. These microorganisms are the huge challenge for the dairy industry.

Microorganisms are also present in the milk if the animal suffers from mastitis(Jeykumar *et al.* 2013). Generally, mastitis is divided into Clinical, sub-clinical and chronic*(Haggag et al.* 2018). Mastitis is a multifactorial disease and is very difficult to control. Mastitis can be caused by different bacterial species mainly *Streptococcus* and *Staphylococcus* species. Additionally, different pathogens are typical of different types of mastitis (Clinical, sub-clinical and heifer mastitis). Pathogens involved in bovine mastitis are classified as contagious or environmental pathogen depending upon their epidemiological association with the disease(Azevedo *et al.,* 2015). Contagious pathogens are those which spread from one animal to another while the primary source of pathogen is animal. Environmental pathogens are those which enter the milk during milking whereas the primary source of pathogen is the environment. Due to its economic importance, extensive research is being carried out to determine the microorganisms which affect the quality of milk.Given growing public awareness about food safety and quality, information about the microbial contamination of milk is of great significance. Until now information on such aspects in Pakistan is scant and scattered. This study was performed to check the microbial contamination of milk in different areas of Faisalabad, Punjab, Pakistan. In this study, *Streptococcal* species that are adversely affecting the quality of milk are isolated. Because these microorganisms also cause food-borne diseases in the population so their susceptibility to different antibiotics is also determined. This test revealed that the organism is multidrug resistance (MDR).This MDR organism is one of the maincauses of the emerging problem of antibiotics resistance in humans. These bacteria become resistant to antibiotics when these antibiotics are overused in livestock(Azevedo *et al.* 2015). This resistant organism enters into humans through the milk of these organisms.

Materials and methods

Sample collection

A total of 90 raw milk samples were collected from different Dairy farms in Faisalabad city. The farms were chosen randomly and samples were collected in the early morning. Approximately 100-200 ml was aseptically collected from containers of bulk milk from each farm into a sterile Scotch bottle. The samples were collected immediately after milking at ambient temperature (28-30°C). After collection, samples were placed in an ice-box and delivered to the laboratory within 1-2 hours.

Microbiological analysis

Samples were analyzed for their microbial quality and safety and the prevalence of selected bacterial

pathogens. Nutrient agar and 5% Sheep blood agar was prepared according to the manufacturer's instructions. The agar was left to set and stored in the refrigerator. After diluting the samples serially, 100 µl was inoculated on the agar and spread using a sterile glass spreader.

The plates were incubated at 37°C for 24-48 hrs and pure culture was prepared by streaking colonies on separate plates using a sterile platinum loop. After incubation, pure bacterial colonies were isolated and smear was prepared on a clean glass slide and stained according to Gram's Method of staining and observed under a microscope for determination of Morphological characteristics. The isolates were mainly identified based oncultural, morphological and biochemical characteristics. Different biochemical tests were performed including Catalase, Urease, Oxidase, VP and CAMP.

Antibiotic susceptibility testing

Antibiotic susceptibility test was performed using the disc diffusion method(Hudzicki 2016). Mueller-Hinton agar was inoculated with inoculums of the test organism. Sixteen locally available antibiotics were selected for antibiotic susceptibility testing in the study.

The antibiotic disc used were Amikacin (30 µg), Cefoxitin (30µg), Tazobactam (110µg), Levofloxacin (5µg), Tobramycin (30µg), Ampicilin-sulbactam (20µg), Tigecycline (15µg), Cefipime (30µg), Ampicilin (10µg), Cefotoxime (10µg), Trimethoprim (25µg), Cefuroxime (30µg), Chloromphenicol (30µg), Cefixime (5µg), Tetracycline (10µg), Gentamycin (30µg). The discs were placed on agar surface. The plates were incubated at 37°C for 24 hrs. After incubation, zones of inhibition were measured and results were recorded as resistant or susceptible to specific antibiotics as shown in Fig 1 and 2.

Minimum Inhibitory concentration

MIC was performed according to the standard method(Wiegand, Hilpert, and Hancock 2008). Mueller-Hinton broth with 5% sheep blood broth was prepared and supplemented with different concentrations of antibiotics. Direct colony suspension from overnight incubated sheep blood agar plate, equivalent to 0.5 McFarland standard was used(Catarina *et al.,* 2018). Minimum inhibitory concentration was performed according to CLSI guidelines for further confirmation of susceptibility of the isolate to antibiotics.MIC was performed for seven antibiotics which include Cefotaxime, Cefuroxime, Ampicillin, Tazobactam, Amikacin, Erythromycin, and Tetracycline. After 24 Hrs of incubation, results were recorded as resistant or susceptible to a specific antibiotic.

Results

In the present study, the activity of different antibiotics against gram positive *Streptococcus pyogenes* isolates were measured both qualitatively and quantitatively by presence or absence of zones of inhibition and MIC.

Table 1. Biochemical tests.

Biochemical test	Results
Catalase	Negative
Oxidase	Negative
Urease	Negative
VP	Negative
CAMP	Negative

Microbiological analysis

After inoculation and incubation of samples, smears were prepared on a clean glass slide and stained with Gram's Method of staining. Microscopy of these smears revealed gram-positive cocci which were present mainly in short chains. For identification of *Streptococcus pyogenes,*isolates were streaked on 5% sheep blood agar which after incubation showed β-Hemolysis.Further biochemical testing was performed and results are shown in Table 1.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the disc diffusion method and results were interpreted using CLSI guidelines. A total of sixteen antibiotics that are commonly used against

Streptococcus pyogenes were selected for this test and results are summarised in table 2.

The isolates were susceptible to only seven antibiotics which were Amikacin(30 μg), Tazobactam(110 μg), Levofloxacin(5 μg), Tobramycin(30 μg), Ampicilin-sulbactam(20 μg), Chloromphenicol(30 μg), Gentamycin(30 μg).

Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) was performed using CLSI guidelines for further confirmation of the susceptibility of isolates to different antibiotics. For this test, seven antibiotics were selected and results are shown in table 3. The organism was susceptible to only three antibiotics which were Ampicillin, Amikacin and Erythromycin.

Table 2. Antibiotic susceptibility testing.

Serial No.	Antibiotics	Standard (mm)		Results (mm)
		Susceptible	Resistant	
01	Amikacin(30 μg)	≥ 19	≤ 15	25
02	Cefoxitin(30 μg)	≥20	≤19	15
03	Tazobactam(110 μg)	≥22	≤21	26
04	Levofloxacin(5 μg)	≥17	≤13	23
05	Tobramycin(30 μg)	≥15	≤12	19
06	Ampicilin-sulbactam(20 μg)	≥15	≤11	15
07	Tigecycline(15 μg)	≥29	≤23	21
08	Cefipime(30 μg)	≥24	≤21	0
09	Ampicilin(10 μg)	≥26	≤22	0
10	Cefotoxime(10 μg)	≥28	≤25	15
11	Trimethoprim(25 μg)	≥16	≤15	0
12	Cefuroxime(30 μg)	≥23	≤13	0
13	Chloromphenicol(30 μg)	≥21	≤17	21
14	Cefixime(5 μg)	≥19	≤15	0
15	Tetracycline(10 μg)	≥23	≤18	0
16	Gentamycin(30 μg)	≥15	≤14	18

Table 3. Minimum inhibitory concentration.

Antibiotics	MIC(μg/ml)		Results (μg/ml)
	Susceptible	Resistant	
Cephalosporin			Group of antibiotic
Cefotaxime	≤0.5	≥2	8
cefuroxime	≤1	≥4	32
Penicillins			Group of antibiotic
Ampicillin	≤0.25	≥8	2
Tazobactam	≤0.25	≥4	16
Aminoglycosides			Group of antibiotic
Amikacin	≤1	≥16	0.125
Macrolides			Group of antibiotic
Erythromycin	≤0.25	≥1	0.25
Tetracycline			Group of antibiotic
Tetracycline	≤2	≥8	64

Discussion

Our present work was designed to perform the study on antimicrobial activity of different antibiotics on gram positive *Streptococcus pyogenes* isolates. This bacterium mainly presents on animal skin from where it contaminates the milk. *Streptococcus* species are also the pathogen that causes clinical and sub-clinical mastitis(Tardy and Bouveron 2002). Milk and milk product gets contaminated with pathogenic bacteria mainly due to processing, handling and unhygienic environment(Thaker, Brahmbhatt, and Nayak 2013). Results showed that the organism was resistant to the great number of antibiotics. For antibiotic susceptibility testing we chosed different commonly used antibiotics and zones of inhibition were measured and results were interpreted using CLSI guidelines. Our study showed that the isolates were susceptible to very small number of antibiotics and results revealed that the organism is Multi-Drug resistant (MDR). The bacterium that is resistant to at least one antibiotic in three or more drug classes is termed as Multidrug resistance bacteria(Basak, Singh, and Rajurkar 2016).

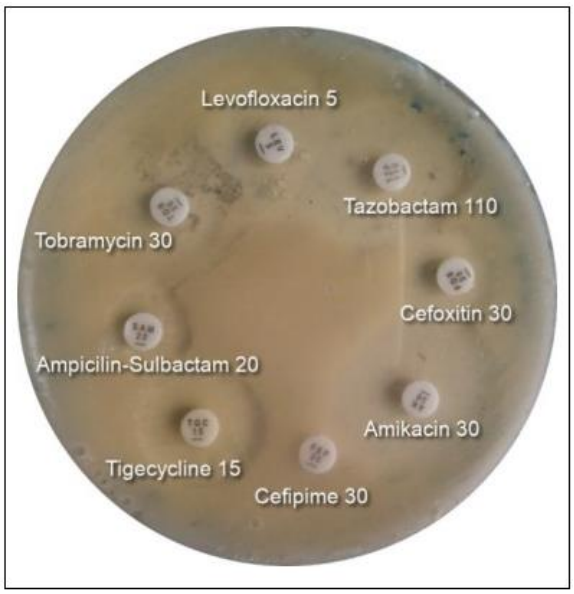

Fig. 1. Zones of inhibition after 24 hrs of incubation (Ref: Author).

The sensitivity of bacteria to the life-saving drugs was very low and these resistant bacteria contribute to the antibiotic resistance in humans which is an emerging problem worldwide. For further confirmation, MIC was performed according to CLSI guidelines. For this test we chosed seven antibiotics that are most commonly used and our results showed that the isolates were sensitive at much higher concentration of antibiotics. Our study is in complete agreement with previous studies such as (Mcdaniel *et al*. 2014) that the milk can serve as a source for transferring microorganisms from animals to humans and further studies can be made to understand the mechanism of resistance against antibiotics in animals. Antibiotic resistance development among bacteria poses a great problem that should be addressed.

Fig. 2. Zones of inhibition after 24 hrs of incubation (Ref: Author).

These resistant bacteria enter into a human through the food products of these animals. These organisms become resistant to multi drugs due to the overuse of antibiotics in livestock.

Conclusion

Food products serve not only the source of nutrition but also the substrate for the growth of microorganisms. The growth of microorganisms causes food spoilage. The spoilage may lead to foodborne illness. Milk and milk product gets contaminated with pathogenic bacteria mainly during processing and handling due to unhygienic environment. The study was limited to check only the microbial contamination and not the other contaminants like chemicals, adulterants, etc. Results indicate that the isolates were resistant to a large

number of life-saving drugs that are most commonly used. These bacteria become resistant due to overuse of these antibiotics in livestock. These resistant bacteria enters human through food products of these animals and are the main cause of antibiotic resistance in human.

To prevent microorganisms from contaminating milk proper hygienic measures must be implemented and milkmen should clean the udder of the animal before milking. The environment of animals, Utensils, Water and milkman's hands should be cleaned before milking to avoid the contamination of milk. Moreover, the animal should be periodically tested for mastitis as it could also contaminate milk.

Conflict of interest

The author and co-authors have declared no conflict of interest in this study.

References

Elmoslemany AM, Keefe JP, Dohoo IR, Dingwell RT. 2009. Microbiological quality of bulk tank raw milk in Prince Edward Island dairy herds. Journal of Dairy Science **92(9),** 4239-4248. http://dx.doi.org/10.3168/jds.2008-1751

Aijaz Hussain Smmoro, Arain MA, Khaskheli M, BachalBhutto. 2002. Isolation of Escherichia Coli from Raw Milk and Milk Products in Relation to Public Health Sold under Market Conditions at Tandojam, Pakistan. Pakistan Journal of Nutrition **1(3),** 151-152. http://dx.doi.org/10.3923/pjn.2002.151.152

Carla Maria Lopes de Azevedo, Diana Pacheco, Luisa Soares, Ricardo Romao, Monica Moitoso, Jaime Maldonado, Roger Guix, Joao Simoes. 2015. Prevalence of contagious and environmental mastitis-causing bacteria in bulk tank milk and its relationships with milking practices of dairy cattle herds in São Miguel Island (Azores). Tropical Animal Health and Production **48(2),** 451-459. http://dx.doi.org/10.1007/s11250-015-0973-6

Cinthia Alves-Barroco, Catarina Rodrigues, Luis Raposo R, Catarina Bras, Mario Diniz, Joao Caco, Pedro Costa M, lida Santos – Sanches, Alexandra Fernandes R. 2018. Streptococcus dysgalactiae subsp. Dysgalactiae isolated from milk of the bovine udder as emerging pathogens: In vitro and in vivo infection of human cells and zebrafish as biological models, microbiologyOpen **8(17).** http://dx.doi.org/10.1002/mbo3.623

Clinton Mcdaniel J, Diana Cardwell M, Robert Moeller B, Gregory Gray C. 2014. Humans and Cattle: A Review of Bovine Zoonoses. Vector borne and zoonotic diseases **14(1),** 1-19. http://dx.doi.org/10.1089/vbz.2012.1164

Guerin-Faublee V, Tardy F, Bouveron C, Carret G. 2002. Antimicrobial susceptibility of Streptococcus species isolated from clinical mastitis in dairy cows. International journal of antimicrobial agents **19(3),** 219-226. http://dx.doi.org/10.1016/s0924-8579(01)00485-x

Guetouache Mourad, Guessas Bettache, Medjekal Samir. 2014. Composition and nutritional value of raw milk. Issues in Biological Sciences and Pharmaceutical Research **2(10),** 155-122. http://dx.doi.org/10.15739/IBSPR.005

Thaker HC, Brahmbhatt MN, Nayak JB. 2013. Isolation and identification of Staphylococcus aureus from milk and milk products and their drug resistance patterns in Anand, Gujarat. Veterinary World **5(12),** 10-13, http://dx.doi.org/10.5455/vetworld.2013.10-13

Humberto Guanche Garcell E, Guilarte Garcia Pedro Vazquez Pueyo I, Rodriguez Martin, Ariadna Villanueva Arias, Ramon Alfonso Serrano N. 2016. Outbreaks of brucellosis related to the consumption of unpasteurized camel milk. Journal of Infection and Public Health **9(4).** http://dx.doi.org/10.1016/j.jiph.2015.12.006

Jan Hudzicki. 2009. kirby-bauer disk diffusion susceptibility test protocol, American Society For Microbiology (ASM)(December 2009), 1–13, https://www.asm.org/Protocols/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Pro

M. Jeykumar, Vinodkumar G, Bimal P Bashir, Sudhakar Krovvidi. 2013. Antibiogram of mastitis pathogens in the milk of crossbred cows in Namakkal district, Tamil Nadu, Veterinary World **6(6),** 354-356. http://dx.doi.org/10.5455/vetworld.2013.354-356

Prejit Nanu E, Latha C. 2007. Microbial quality assurance of milk during production, processing and marketing. American Journal of Food Technology **2(3),** 136-144, http://dx.doi.org/10.3923/ajft.2007.136.144

Silpi Basak, Priyanka Singh, Monali Rajurkar. 2016. Multidrug resistant and extensively drug resistant bacteria: A Study. Journal of Pathogens 1-5, http://dx.doi.org/10.1155/2016/4065603

Stephen Oliver P, Bhushan Jayarao M, Raul Almeida A. 2005. Foodborne Pathogens in Milk and the Dairy Farm Environment: Food Safety and Public Health Implications. Foodborne pathogens and disease **2(2),** 115-29, http://dx.doi.org/10.1089/fpd.2005.2.115

Wiegand I, Hilpert K, Hancock RE. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols **3(2),** 163-175, http://dx.doi.org/10.1038/nprot.2007.521

Yasser Haggag N, Mohamed Nossair A, Alaa Mansour M, Amir Abd el Rahman. 2018. Streptococci in Dairy Farms: Isolation, Antibiogram Pattern and Disinfectant Sensitivity. Alexandria Journal of Veterinary Sciences **59(2),** 85-92, http://dx.doi.org/10.5455/ajvs.8031