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STUDY ON ENUMERATION, CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY OF COAGULASE POSITIVE STAPHYLOCOCCI ISOLATED FROM HAND PUMP WATER

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ABSTRACT: *A study was conducted on bacteriological analysis of hand pump water for determining the quality and extent of contamination. A total of 100 hand pump water samples were studied for enumeration of typical staphylococci (TS) and Coagulase positive staphylococci (CPS). On m-staphylococcal agar 82% samples yielded typical staphylococcal colonies, 79% of which was in the range of 1-20 cfu/100ml, 15% in the range of 20-40 and 2% were between 100-500 staphylococci/100ml. Coagulase positive staphylococcus were found in 25 samples, of which 76% had a range of 1-20 and remaining 24% had count less than 100 cfu/100ml of hand pump water tested. Minimum value was 1 and maximum 364 for TS while for CPS minimum value was 2 and maximum was 86 cfu/100ml, with a mean value of 80.5 and 15.86 respectively. From 82 samples, 106 colonies randomly selected among 1733 golden to orange colonies of TS obtained on m-staphylococcal agar, were subjected to coagulase positive test. Thirty two coagulase positive strains from 25 positive samples were isolated and characterized. Twenty five were Mannitol fermentation positive while seven were negative. In our study 30% typical staphylococci were found pathogenic i.e. coagulase positive. A varying degree of resistance and susceptibility was found against twelve antibiotics. Ampiclox was found 100% effective for these CPS strains.*

KEYWORDS: Ground water, *Staphylococci*, Coagulase positive *Staphylococci*, antibiotic resistance.

INTRODUCTION

Water is essential to sustain life. It should be free from pathogenic agent and toxic chemicals. Ground water long been considered to be unquestionable excellent because of the soil barrier, considered as living filter, is often effective in providing isolation of this high quality source water from surface pollutant (, 2015). But increasing urbanization and increased use of recreational facilities have exerted extreme pressure on the microbial quality of both ground and remote surface water sources. Unfortunately much of the developing world's drinking water supplies are contaminated with enteric and other pathogens owing to inadequate sewage treatment and water purification facilities (Abbas *et al.*, 2007). According to WHO consumption of sewage contaminated drinking water account for 80% of all reported diseases and 40% of all deaths in Pakistan.

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Bacteria of genus staphylococcus have received a great deal of attention over the past century, and for good reason. Staphylococci are ubiquitous with widespread distribution in the environment, and their presence in aquatic environments is well established (Percival et al., 2004). The most notorious species; *Staphylococcus aureus* (*S aureus*), causes a wide variety of common, annoying, as well as serious, infections in man and other animals. *S aureus* is an important human pathogen. It is one of the most common contaminant in human food (Sylejmari et al., 2015) and agents of food poisoning. It causes fatal septicemia, meningitis, endocarditis, puerperal sepsis, toxic shock syndrome, pneumonia, destructive abscesses, osteomyelitis, septic arthritis and many others. The widely distributed pathogen has been isolated from nasal mucosa and skin of healthy humans, clinical cases, human and animal feaces, digestive tract of flies, dust & moisture droplets, clothing, foodstuffs, food-producing animals, food-catering and aquatic environments (Mahmood et al., 2015; Acco *et al*., 2003; Normanno et al., 2007; Kluytmans, 2010; Abulreesh and Organji, 2011).

Coagulase is a soluble enzyme like product of *staphylococci* that contribute to pathogenesis by protecting the cocci with a fibrin barrier against phagocytosis. *S aureus* in drinking water may also serve a source for colonizing residents exposed to contaminated water. As a result of their great resistance to stress and their adaptation to survival outside the human host, *staphylococci* have been suggested as alternative to coliform as indicators of pollutions in swimming pools, recreational waters hydrotherapy pools and water that might be added to food. *Staphylococci* are not in normal flora but their presence is associated with certain types of gastrointestinal diseases, hence can be used as indicator of pollution (Brock and Brock, 1978). Resistance to antimicrobial agents is a major public health concern as resistant bacteria can disseminate in the environment with possible transmission to human through contaminated food and water (McCarthy et al., 2015; Holmes et al., 2012; Kimberly et al., 2005).

The aim of the study was to investigate the incidence of typical Staphylococci & coagulase positive Staphylococci and their resistance to different antibiotics in hand pump water used for human consumption in Multan, Pakistan.

MATERIALS AND METHODS

Sampling procedure

A total of 100 hand pump water samples from various localities were collected in 300ml wide-mouth sterile bottle. Before collecting each sample, the water was allowed to spill by working the hand pump until fresh water started flowing. Temperature and pH of flowing sample was noted at the time of collection. The sample was examined within two hours or refrigerated at 5-7 °C not more than 12 hours. Ample air space was left in the sample bottle to facilitate thorough mixing of the sample at the time the water sample was subjected to bacteriological examination.

Enumeration of typical and coagulase positive staphylococci

Staphylococci were enumerated by the membrane filtration technique (APHA, 1975) on m-staphylococcal agar g/L tryptone 10.0, yeast extract 2.5, lactose 2.0, Mannitol10.0, Dipotassium hydrogen phosphate 5.0, sodium chloride 75.0, agar 15.0, and pH 7.0.

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Membrane Filtration Technique
Glass filtration apparatus was sterilized, assembled and attached to the vacuum pump by means of a rubber tube. Millipore membrane filter (Type HA) with a mesh of 0.45µm, 47mm diameter, was used for all the samples. Appropriate amount of water sample (50-300 ml), depending upon the water quality of the sample was filtered through the apparatus to attain 40-100 colonies on the membrane filter paper. As a precautionary measure the inner side of the funnel was rinsed by 20ml of sterile water, so that all the bacteria were accumulated on the membrane filter. The filter was removed with ethanol-flamed forceps and placed on m-staphylococcal agar plate, keeping grid side up, with a rolling motion to avoid entrapment of air. Plates were incubated at 37°C for 48 hours.

Baird Parker Agar: Pigmented colonies from m-Staphylococcal agar were isolated, purified and grown on Baird Parker agar (BPA) plates at 35±0.5°C After 48 hours brownish black, dark black and grey colonies were subjected to further tests.

Mannitol Salt Agar: - Grey to black colonies form BP agar were streaked onto Mannitol Salt agar (MSA), incubated at 35±0.5°C for 48Hours. Colonies with yellow zone and pink zone were subjected to coagulase test to determine virulence of the strain. Coagulase positive strains were Gram Stained to confirm the bunch like Gram positive cocci.

Coagulase Test: - A small amount of fresh 24hrs incubated culture from the MSA or nutrient agar was emulsified in the drop of sterile water on glass slide. Blood serum (30 µl) was added to the bacterial emulsion. Negative and positive controls were set with known negative and positive *Staphylococcal* strain. The positive reaction was indicated by rapid (or slow) fibrin clumping of the emulsion within 5-15 seconds on the slide. To avoid any false result each culture was tested three times.

Antibiotic Susceptibility Tests:
Twelve antibiotics discs; Penicillin, Methicillin, Orbenin, Augmentin, Trimethoprim, Cefiriaxone, Cefaclor, Velosef, Ampiclox, Tetracycline, Lincomycin and Erythromycin were used for test.

Purified culture of Coagulase positive Staphylococci from BPA was refreshed on nutrient agar. Few (2-3) colonies from fresh culture plate were emulsified in 1ml sterile 0.1 % peptone water (Gibco lab cat no. M381DO) in test tube to have a turbid suspension. Inoculum was applied to the overnight checked nutrient agar plates with a sterile cotton swab so that the bacteria were uniformly distributed in the form of lawn on the agar surface. Antibiotic disks (3-4) were applied on an inoculated plate and incubated at 37° C. The diameter of inhibition zone was noted with 12-18 hours to avoid false result due to bacteriostatic action of any antibiotic and the results were interpreted according to the table provided with antibiotics.

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RESULTS

Enumeration of Typical and Coagulase positive staphylococci:

On m-staphylococcal agar 82% water samples yielded typical staphylococcal colonies, 79% of which was in the range of 1-20 cfu/100ml, 15% in the range of 20-40 and 2% were between 100-500 staphylococci/100ml. Coagulase positive staphylococcus were found in 25 samples, of which 76% had a range of 1-20 and remaining 24% had count less than 100 cfu/100ml of hand pump water tested (Table 1). Minimum value was 1 and maximum 364 for TS while for CPS minimum value was 2 and maximum was 86 cfu/100ml, with a mean value of 80.5 and 15.86 respectively.

Table 2 indicates the details of typical *staphylococci*. These 82 samples contained 1733 colonies of yellow, orange or golden colour. White colonies were not counted on the membrane filter. From these 1733 strains 106 were picked up at random but representing all the samples and these were streaked and purified on baird-parker agar. All produced brownish black, dark black or gray colonies on this agar. Gram staining showed that all the isolates were gram positive cocci in bunch shape. These were again streaked on mannitol salt agar to confirm the mannitol fermentation reaction. 65 (61.3%) were found to be mannitol negative and 41 (38.7%) mannitol positive. All the 106 mannitol positive and mannitol negative strains were them subjected to coagulase test. Of the 65 mannitol negative strains, 7 (11%) were observed to be coagulase positive. 58 (89%) proved to be coagulase negative. Among 41 mannitol fermentation positive staphylococcal strains, 25 were coagulase positive while 16 were not showing coagulase activity. Therefore, in all 32 strains among 106 tested were found coagulase test positive i.e. 25 strains were both mannitol and coagulase-positive and 7 were mannitol-negative but coagulase-positive (Table 2). Hence it was observed that 25% samples had coagulase-positive *Staphylococus* species.

Susceptibility of coagulase positive Staphylococci

Coagulase positive staphylococci isolated from hand pump water showed varying degree of susceptibility to twelve antibiotics (Table 3). Susceptibility decrease among the isolates in the order; ampiclox > velosef > augmentin > orbenin > erythromycin > cefaclor > tetracyclin > methicillin > trimethoprim > penicillin > lincomycin > cefiriaxone. Ampiclox exhibits 100% and velocef 93% sensitivity for CPS isolated.

DISCUSSION

More than 70% of the population in Pakistan lives in rural areas. Ground water particularly hand pump water is the sole source of drinking water in majority of rural areas. Low microbiological quality of hand pump water is due to poor sanitation in the surrounding areas. Because water is consumed in large quantity it may be infections even if it contains only a small number of pathogens. Staphylococcal diseases are caused by both coagulase positive and coagulase negative staphylococci however coagulase positive staphylococci are more pathogenic (Geldreich 1991).

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Lechevallier & Seidler (1980) recovered 6.25 % *S. aureus* form 114 samples from private & private & public supply drinking water which had typical staphylococci forming. 45% of the total samples tested while our survey revealed much greater recovery of coagulase-positive staphylococci (30%) while typical staphylococci were detected in 82% water samples. The reasons for this high recovery rate are uncertain but it may be due to poor sanitary condition of the under ground water or higher rates of survival of staphylococci in our warm under ground waters. Hussein in 2014 reported that 46% of potable water samples in Makah, Saudi Arabia collectively yielded staphylococci, with 65% of these isolates confirmed as *S. aureus*.

Similar and higher percentages of S. aureus occurrence in water were reported worldwide (Harakeh et al., 2006; Faria et al., 2009), including Makkah, Saudi Arabia (Mihdhdir, 2009; Abulreesh and organji, 2011). Although S. aureus can be found among the genera that normally exist in potable water as HPC bacteria (Allen et al., 2004), *Staphylococcal* species are primary and opportunistic pathogen, there are many reasons for potential concern when S. aureus are present in drinking water supplies. Common food preparation practices such as washing boiled potatoes, pasta and cooling of boiled eggs could possibly leave these food items contaminated with S. aureus. If these food items used for preparation of salads are left at room temperature, or improperly refrigerated, the possibility of staphylococcal food intoxication certainly exists if these S. aureus contaminants were toxigenic.

M-staphylococcal agar is a selective medium for isolation and differentiation of pathogenic staphylococci on the basis of salt tolelence, pigmentation and manitol fermentation. Pathogenic staphylococci (coagulase positive) are able to grow on the high salt manitol medium to form orange, yellow, or golden colonies which give positive reaction for acid production. Non pigmented colonies are white are non pathogenic (Oxoid, 1982).

Results of a number of workers point out that only typically dark black shiny colonies on Baird parker may be considered as coagulase positive. But according to our survey brownish black and grey staphylococcal colonies were also found coagulase positive. The reason for utilizing varing degree of tellurite, may be that media (water) deprived of essential nutrients, may cause sub-lethal damage to the cell physiology that may affect their potential to reduce tellurite present in baird parker agar medium. It was also seen that grey colonies usually produce slow coagulase reaction. Coagulase negative species particularly *Staphylococcus epidermidis* is responsible for as many as 10% of all cases of human bacterial endocarditis and exhibit varing degree of antibiotic resistance (Mehdinejad et al., 2008)

So it can be concluded that untreated underground water of individual private supplies is a cause of grave public concern because it is not possible to give technical advice to individuals and it is also difficult to monitor the private water supplies on regular basis. It is also not possible to give health education to individual consumers, because they do not pay head to such advice, as they do not have the resources to improve the water supply system.The extensive use of antibiotics in medicine over several decades has led to many studies on the occurrence of resistance in environmental bacteria. Resistance to antimicrobial agents is a major public health concern as resistant bacteria can disseminate in the environment with possible transmission to

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human through contaminated food and water (Filali, et al 2000; Faria et al., 2009; Holmes et al., 2012).

Beta-lactum antibiotics have held a central position in antimicrobial therapy and staphylococcal resistance to them is important for this reason alone. Methicillin resistant S. aureus (MRSA) is currently the most commonly identified antibiotic-resistant pathogen worldwide (Culos et al., 2013), S. aureus that were recovered from water exhibited resistance to penicillin G, while resistance to oxacillin was 76.2% (Ippolito et al., 2010). Of the twelve antimicrobial drugs in this investigation ampiclox and augmentine were the most effective drugs against isolates tested showing 100% sensitivity.

In conclusion, it can be said that the presence of staphylococci in waters should be considered hazardous and their presence should regularly be sought even in drinking waters. There is a need to develop convenient and easy methods for their isolation and identification. Criteria as to the portability of water should also be established.

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Table 1: Incidence of Typical Staphylococci and Coagulase positive Staphylococci in water from Hand Pump

Organism	Number	Positive	No. of samples with CFU/100ml		
			1-20	20-100	100-500
Typical Staphylococci	100	82	65	15	2
Coagulase positive staph	100	25	19	6	-

Table 2: Details of Typical Staphylococci on m-Staphylococcal agar

Colony colour	*No. of Typical colonies*	*Colonies tested*	**Mann +ve ▪Coag +ve*	*Mann -ve Coag +ve*	*Mann+ve Coag -ve*	*Mann -ve Coag -ve*
Golden	462	34	13	3	9	9
Yellow	275	35	35	9	1	20
Orange	996	37	3	3	2	29
Total	1733	106	25	7	16	58

*Mannitol fermentation ▪Coagulase positive

Table 3: Susceptibility of 30 Coagulase positive Staphylococcal strains to various Antibiotics

Antibiotics	*Resistant*	*Moderate Resistant*	*Susceptible*
Penicillin	18	4	8
Methicillin	6	13	11
Orbenin	3	1	26
Augmentin	0	3	27
Trimethoprim	15	6	9
Cefiriaxone	4	20	6
Cefaclor	4	8	18
Velosef	1	1	28
Ampiclox	0	0	30
Tetracycline	9	4	17
Lincomycin	15	8	7
Erythromycin	4	1	25

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