Microbial risk assessment with special focus on antibiotic residues in mastitic buffalo milk

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Abstract

Fifty buffaloes(healthy and mastitic udders) were milked aseptically from separate regions of Kaliobia governorate .California mastitis test (CMT) and bacteriological examination were achieved .Upon susceptibility testing (12) antibiotics were used to explain different susceptibility patterns of the isolated bacteria

The objective of this study was the detection of oxytetracycline, sulphadimidine, penicillin G and ampicillin residues in raw buffalo's milk. 45 samples of raw milk (13 for oxytetracycline ,11 for sulphadimidine , 11 for penicillin G and 10 for ampicillin) were collected from private farms in Banha at Kaliobia Governorate. The period of exiperiment was January- March 2015 and the sample was analyzed with high performance liquid chromatography (HPLC) method . Out of the samples examined for oxytetracycline 30.76% (4 /13) were found to contain oxytetracycline residues and 54.5% (6/11) were found to contain sulfonamide residue . The amount of oxytetracycline in positive milk samples were found 452 ng/ml, 560 ng/ml, 1475 ng/ml and 2833 ng/ml which are much higher than WHO and FD recommended level. While, sulphadimidine residue was detected in the range of 3-22 ng/ml in 2 samples and 28 - 44 ng/ml in 4 samples which were lower than WHO and FD recommended level. Penicillin G and ampicillin residues did not found in the examined milk samples. This study indicates the presence of oxytetracycline residues more than allowed amount. Regulatory authorities should ensure proper withdrawal period before milking the animals and definite supervisions are necessary on application of these drugs.

INTRODUCTION

Bovine mastitis is the most costly disease for the dairy industry worldwide. Although a wide variety of pathogens have been isolated as causative agents of this disease, Staphylococcus aureus (S. aureus) is considered as one of the most important pathogens due to its resistance to certain antibiotics and its propensity to recur chronically. Recently, coagulase-negative staphylococci (CoNS) have been considered as opportunistic pathogens that cause bovine mastitis in many countries and could be

therefore described as emerging mastitis pathogens.Increasing attention has been paid to CoNS in both subclinical and clinical mastitis cases throughout the world(**Frey,2013).** Mastitis,is one of the most common problems in dairy production ,may be clinical presenting symptoms or subclinical with no visible signs. Both clinical and subclinical mastitis cause changes in milk composition. Subclinical mastitis is more problematic one, being non-symptomatic and consequently contribute to decreased milk quantity and quality **(Leitner et al.,2008)**. Mastitis is not only decrease milk yield but also alter its composition **(Shuster et al., 1991).** Mastitis influences the total milk output and modifies milk composition and technological usability (**Coulon et al., 2002**) subclinical mastitis is one of the most serious diseases of buffaloes, as the influenced animal shows no obvious symptoms and secrets apparently normal milk for a long time, during which causative organisms spread infection in the herd, this represents an important feature of the epidemiology of many forms of bovine mastitis **(Bakken and gudding, 1982).** Early diagnosis of mastitis is important for production losses and for enhancing the prospects of recovery, also the identification of subclinically infected gland is urgently required for successful control of mastitis in dairy animals **(Ahmed et al., 2008).** This European study shows that bacteria associated with acute clinical mastitis are susceptible to most antibiotics with the exception of penicillin G against S. aureus, and erythromycin and tetracycline against S. uberis. (**Valerie,2015).** Contagious pathogens such as S.aureus and S.agalactiae can be transmitted from dairy animal to another, where as environmental pathogens, such as S.dysgalactiae, S.uberis, Enterococcus spp.,coagulase negative staphylococci (CNS) and gram negative enteric bacilli (pseudomonas spp. and E.coli) can be transmitted during milking from the contaminated environment **(Bradley, 2002)** Staphylococcus species (46.3%) occupied the prime position among the bacterial isolates followed by Streptococcus species (9.76%), Escherichia coli (6.1%), mixed growth (32.96%) and sterile growth (4.88%). Antibiotic susceptibility test revealed highest sensitivity towards enrofloxacin. However, antibiotics showing higher rate of resistance patterns were streptomycin, penicillin G, ampicillin, cloxacillin, amoxicillin, kanamycin and lincomycin, (**Biswadeep,2015).** Highest resistance was observed against clindamycin and ampicillin. Coliform bacteria (E. coli and Klebsiella pneumoniae) showed resistance to most of the antimicrobials used. Detailed investigation is needed to identify the interplay of managemental and environmental risk factors to design appropriate control measures,(**Ragbe,2012).** The causes of mastitis are almost entirely infectious and mostly are bacterial infections, at least 137 biological infectious agents causing bovine mastitis are known to date and the commonest pathogens are staphylococcus species, streptococcus species and coliforms **(Radostits et al.,2000).** The conventional drugs used for treatment of mastitis are of limited values in most of the districts and due to this and other factors causative agents have showed variable degree of resistance. Some of the bacteria like S.aureus, streptococcus species and some other pathogens have already developed resistance to

many antibiotics **(Kerro, 2003).** Macrolide-lincosamide-streptogramin (MLS) antibiotics, including erythromycin, clindamycin and spiramycin, are frequently used for treatment of bovine mastitis , Thus, results from an in vitro susceptibility testing are an important tool to guide a veterinarian in selecting the most efficacious antimicrobial agent(s) for therapeutic and prophylactic intervention,(**Longping,2015).**Risk assessment is an integral procedure in the control of(Lab Aquired Infection) LAIs.The interrelated notions of hazard and risk are part of this process.The hazard presented by a substance is the potential to cause harm in some way (e.g.,to cause an infection).The risk is the likelihood that it will cause harm in the circumstances under consideration(e.g.,give rise to an infection in a microbiological laboratory worker).In terms of harm presented, a risk can be perceived as vanishingly small and acceptable or as being so severe as to make it totally unacceptable.Deciding what constitutes an acceptable risk is a management task ,a risk that is acceptable in one set of circumstances may be unacceptable in another **(Collins ,1999**).Facility management is responsible for the development and institution of safety procedures and employee training programs that minimize the employee risk from a laboratory –associated infection on the basis of present or anticipated infectious hazards .The strategy for minimizing the occupational exposure of laboratory workers ,other facility employees,and the surrounding environment to infectious agents is based on the concept of microorganism containment,which includes physical factors (e.g.,facility design and safety equipment),standard microbiological practices, and administrative controls **(Fleming et al.,1995).**

Antibiotic residues are the most present inhibitory substances in milk because of their frequent usage in prevention or treatment of various diseases of bacterial etiology, including udder inflammation, the most common disease of dairy animals in intensive milk production **(Bruun et al., 2003; Petrović et al., 2008).**

Mastitis is the most prevalent disease in cattle which requires antimicrobial treatment(**Suhren, 2002** , **Mohsenzadeh and Bahrainpour, 2008).** The presence of antimicrobial substances in raw milk could have serious toxicological and technical consequences **(Kang et al., 2005).**

Antibiotic residues are of concern due to their possible adverse effects on people allergic to antibiotics, potential build up of antibiotic-resistant organism in humans and inhibition of starter cultures used to produce cultured milk products such as yogurt and cheeses **(Jones and Seymour, 1988).**

The excretion time of antibiotics varies from animal to animal, and depends on: type of used antibiotic, quantity of given antibiotic and the way of applications, but also it depends on age, health status, lactation stage and individual features of dairy animals **(Samaržija and Antunac, 2002).**

Antibacterial drugs such as oxytetracycline, penicillin G (benzylpenicillin) and sulphadimidine are routinely used in veterinary medicine for prevention and control of disease , and consequently, the most commonly found type residues in milk .

Oxytetracycline (OTC) is known as a broad-spectrum antibiotic with a bacteriostatic effect on the wide range of gram negative and gram positive bacteria. These antibiotics are widely used for the treatment of bovine mastitis and added at subtherapeutic levels to cattle feeds for prophylaxis(**Smilack et al., 1999)** .The mode of action lies in its binding to 30S ribosomal subunits of bacteria, thus inhibiting the protein synthesis(**Jevinova et al., 2003**) . Due to entero-hepatic circulation, a small amount of administrated dosage may persist in the body for a long time after administration.**(Botsoglou and Fletouris, 2001**)

Sulfadimidine (SM2) is an antibiotic which is widely used in human and veterinary medicine for effective treatment and prevention of diseases, or as growth promoter of farm animals, e.g., cows (**Msagati, and Nindi ,2004).**Its extensive use and high rate of pharmaceutical consumption can lead to the appearance of residues in milk via the effluents and products of animal origin. It causes serious side effects such as hypersensitive allergic reactions, drug-resistance problems in human and, even carcinogenic effects (**Huang et al., 2009**) .

Approximately 5-10 percent of the populations is hypersensitive to Penicillin at a concentration as low as 1 ppb and suffers allergic reactions (skin rushes, hives, asthema, anaphylactic shock). Concentration of 1 ppb delay starter activity during butter and yoghurt making.(**Khaskheli et al., 2008**)

Aim of the work:

Identification of pathogens associated with buffalo mastitis ,describtion of antimicrobial susceptibility patterns of bacteria from normal and mastitic buffaloes, risk assessment including lab workers and coworkers who should be well trained on all biosafety requirements using the necessary personal protective equipment(PPE) , evaluating the risk arised from working on the isolated bacteria taking into account the existing control measures on the lab., and analyzing residues of oxytetracycline, sulfadimidine , penicillin G and ampicillin by using HPLC method that are frequently seen in raw milk

MATERIAL AND METHODS

Standard techniques:

(**Stern et al.,1974**) found considerable bench-top and instrument surface contamination associated with all procedures .A more in depth discussion of some standard techniques that may produce aerosols in the laboratory follows.

1)Streaking plates

The use of a microbiologist loop is a common source of aerosol generation and subsequent contamination of laboratory surfaces . procedures that generate aerosols and

contaminate surfaces include the spontaneous discharge of liquid from a loop ,the streaking of media (particularly media with a rough surface),cooling a loop on the inoculated portion of culture media and heating a loop in an open flame **Sewell(1995)** mentions that the use of a biosafety cabinet (BSC) can decrease this risk when working with hazardous microorganisms.

2)Biochemical identification

A number of procedures are routinely used for the biochemical identification of enteric microorganisms.The following is a short ,but not exhaustive, discussion of procedures that have been found to cause aerosols in the laboratory and subsequent contamination of the laboratory environment and workers.

• Pipetting: Pipetting is a time honored laboratory technique that is a potential hazard **(Collins ,1993)** The risks associated with pipetting include ingestion via mouth pipetting ,inhalation via aerosols produced by mixing a microbial suspension or spilling drops on hard surfaces ,contamination of bench tops and fingers ,and injuries from broken glass pipettes .

• Centrifugation :Centrifuge accidents cause relatively few laboratory- associated infections ,but a single accident often exposes a large number of individuals. Unrecognized releases of aerosols during centrifugation may be responsible for laboratory-acquired infections without an identifiable source.The centrifuge safety cup must be opened in a BSC after centrifugation**(Collins,1993).**

• Other procedures:Other hazardous procedures are also routinely performed in the microbiology laboratory. For instance, if a film of liquid exists between two surfaces that are separated (e.g., when removing a petri dish cover or test tube cap) an aerosol may result.Liquids hitting a hard surface (breakage or spillage) create large aerosols and contamination of the environment.

3)Slide agglutination and microscopic preparations

When a loopful of a liquid culture is spread on a slide or a suspension is made on a slide from a solid culture ,small droplets may be broadcast, particularly if the loop is wielded energetically.When the loop is withdrawn from the drop,more small droplets may be scattered and aerosols may be formed.

Animals & milk samples:

A total of 50 buffaloes collected from separate regions of Kaliobia governorate.Diagnosis was made on the basis of history,clinical examination of the udder,macroscobic evaluation of secretions,the California mastitis test (CMT)and bacteriological examination of milk.Milk samples were taken aseptically and transported to the laboratory as the following:

Teats were washed thoroughly and dried with a seprate towel .Teat ends were cleaned with 70% alcohol before sampling.The first three streams of milk from each teat were discarded.Then quantities of 20 to 50 ml of milk were collected aseptically into two

sterile vials.Milk samples were transported on ice to the laboratory and kept at 4˚C until diagnosis of bacteriological assays.15 pooled samples of mammary secretions from clinically inflamed udders ,25 from subclinically inflamed udders and 10 from normal udders of buffaloes were used as material for this study .Several methods for detection of mastitis are available for detecting somatic cells in milk ,including the California mastitis test (CMT,a buffalo side test) The CMT detect formation of a gel when DNA in somatic cells react with a detergent .The reaction occurs on a paddle (CMT) and is graded subjectively (neg,trace,1,2,3).CMT result can be used as rough estimates of the number of somatic cells in milk according to (**Schalm et al.,1971**).

Isolation and identification of bacterial isolates :The different species of bacteria were isolated from mastitic milk by traditional ways for isolation and identification.Loopfull of milk sample was streaked onto 5% sheep blood agar ,MacConkey agar ,mannitol salt agar and Edward agar plates(Oxoid),then incubated at 37˚C for 24 h. Colonies were initially assessed by their morphology and hemolysis patterns ,followed by Gram staining and motility tests.The isolates were identified according to (**Quinn et al.,2002).** Biochemical tests,specifically,catalase,coagulase,growth on mannitol salt agar, growth in 40% ox bile,esculin hydrolysis,sodium hippurate hydrolysis ,carbohydrate fermentation tests (glucose,mannitol,ribose,sorbitol and trehalose),biochemical reaction on MacCkonkey agar,indole reduction,methyl red tests,urease production and citrate utilization tests, triple sugar iron agar(TSI)were performed as required.In cases where no growth was detected,plates were reincubated at 37˚C for an additional 24 h.

Antimicrobial suscebtibility testing of bacterial isolates: (**Koneman et al.,1997)**

The following antimicrobial discs (Oxoid) were used:Oxytetracycline(30mg),penicillin(10mg),ofloxacin(5mg),kanamycin (30mg),chloramphenicol(30mg),polymixin.B,sulphamethoxazol/trimethoprim(1.25+23.75) ,ampicillin (10mg),gentamycin(10mg) and vancomycin(30mg).

A study was focused on 45 buffalos located in Banha at Kaliobia Governorate. Raw milk samples were collected in 6 months from different dairy farms. After collection of milk samples from different locations of cattle farms they were kept in the refrigerator (4°C) and the collected milk samples were rapidly transferred to laboratory (Animal Health Research Institute-Dokki-Giza) in an ice box for detection of antibiotic residues (oxytetracycline , sulphadimidine penicillin G and ampicillin) by using high-performance liquid chromatography (HPLC) (Shimadzu, LC-10AT) with a photo-diode array (Shimadzu, SPD-M10A) detector . Penicillin G standard (procaine salt) was obtained from Sigma (St. Louis, MO, USA), oxytetracycline HCl and sulphadimidine sodium standards were obtained from Sanofi-DIF (Istanbul, Turkey).

A simple method for determination of residual oxytetracycline in milk by HPLC was developed, according to the procedure described by (**Furusawa ,1999)** . Extraction was made with 20% (v/v) trichloroacetic acid (TCA) from milk and filtered through a 0.45-μm disposable syringe filter unit. A C-8 column (Biochemmock, 7μm, 250x 4 mm I.D.)

and a mobile phase of acetonitrile- acetic acid-water (28:4:68, v/v/v) with a photo-diode array (UV detection at 354 nm) detector was used. The mobile phase was set at a flow-rate of 1.0 ml/min at ambient temperature. The average recovery was 68%, and the limit of detection was 0.10μg/ml. The injection volume was 20μl.

The method for determination of residual sulphadimidine sodium in milk by using HPLC with a photo-diode array detector (UV detection at 266 nm) was presented, described by (**Furusawa, 2000**) . A C-18 column (Nucleodur, 5μm, 250x 4 mm I.D.) and a mobile phase of 25% (v/v) ethanol solution (in water) was used. The mobile phase was set at a flow-rate of 0.8ml/min. at ambient temperature. The average recovery was 93%, and the limit of detection was 0.02μg/ml.The injection volume was 10μl.

A rapid ion-pairing liquid chromatographic method was developed for the determination of penicillin G in milk described by(**Takeba ,1998**) . Extraction was made with acetonitrile from milk and clean up solid-phase extraction with C18 cartridge.

Penicillin G was separated on a C-18 column (Nucleodur, 5μm, 250x 4 mm I.D.) with a mobile phase (1ml/min) of acetonitrile-methanol-0.05 M potassium dihydrogenphosphate (20:10:80, v/v/v) mixture containing 5 mM of sodium 1-decanesulfonate adjusted to pH 3.5 and UV detection at 210 nm. The average recovery was 65%, and the limit of detection was 0.05μg/ml. The injection volume was 10μl.

RESULTS AND DISCUSSION

Results of the present study showed prevalence of clinical ,subclinical mastitis,30%,50% respectively and 20% as healthy animals (**Table ,1**).Out of 200 teats,85 were non milk producing.

A total of 115 milk samples were screened for mastitis by California mastitis test (CMT) out of which 75 milk samples showed positive for mastitis.

The bacteriological analysis of the present study showed 75 bacteriologically positive samples from which 90 bacterial isolates were recovered **.(Table, 2)** . The predominant species were Staphylococcus aureus 13.33% followed by E.coli 12.22%, Streptococcal species 10%, coagulase negative Staph. 8.88 %, Klebsiella spp. 7.77% , Micrococcus species 7.77% and Corynebacterium spp. 6.66% (**Table, 3).**

The ideal means of dealing with mastitis is to prevent it from happening. However, even under the best prevention and control programs, mastitis will occur. Remember that mastitis is an inflammation of the mammary gland. Detection of mastitis is generally based upon some indicators of the inflammation. However, treatment of mastitis works best if there is some information on the particular bacterium causing the problem(**Walter ,2010).**

Detection of the inflammation is based upon the response of the animal to the infection. Several significant changes occur in the tissue and in the milk in response to infection. These include infiltration of leukocytes (referred to as somatic cells) (**Walter ,2010) ..**In

practice,composite milk samples(from all four quarters) of less than 200,000 cells/ml are taken as indicating the absence of infection.As cell counts increase so does the chance that mastitis is present **.(Walter ,2010)**

the prevalence of subclinical mastitis in buffalo (on animal basis) in the present study was 50%(**Table 1**) higher than (**Anirban et al.,2012**) and(**Saini et al.,1994**) and coordinate with (**Chavan et al. ,2007).** The dissimilarity might be due to the differences in the managemental practices, genetic divergence and climatic conditions (**Ramprabhu and Rajeswar, 2007**).From (**Table, 5**) it is evident that the most prevalent etiological agent was Staphylococcus aureus (24.44%) followed by Escherichia coli (20%),Klebsiella spp.(17.77%)then Streptococcus spp. (12.22%) and as single and mixed infections which was attributed to abundance of the organisms in the atmosphere . Similar observations were found by (**Chavan et al. ,2007**) and were lower than(**Anirban et al.,2012**). The prevalence of SCM(subclinical mastitis) was observed more for hind quarters than the fore quarters. Similar observation was reported by(**Sharma et al. ,2007**). The hind quarters are closer to the legs and thus more exposed to dung and urine, that is, unhygienic condition than the front quarters. In addition, more turn over of milk in the hind quarters make them more susceptible to wear and tear, hence, prone to inflammatory reactions (**Ramprabhu and Rajeswar,2007**).Out of 115 milk samples,75 positive for mastitis by California mastitis test. A positive value indicates the proportions of animals with positive tests which are really diseased.The likelihood ratio of a positive test result expresses how much more likely the animal is to have a positive test result when actually diseased than if disease free (that is, it is the ratio of the likelihoods of having and not having the disease) (**Petrie and Watson, 2008**).The present study revealed a predominance of Staphylococcus aureus which is similar to the finding by(**Khan and Muhammad ,2005).** Staphylococcus aureus and Streptococcus spp. are the most common contagious pathogens of bovine mammary gland. S. aureus is a major pathogen responsible for subclinical mastitis while Streptococcus spp. is still a significant cause of chronic mastitis where control measures for contagious mastitis are not used (**Keefe, 1997**).Thus, the present study reveals the predominance of contagious form of subclinical mastitis at the farm that needs to be controlled with appropriate measures to prevent further spread. On the other hand, a high prevalence of subclinical mastitis due to E. coli and other streptococci infection which are considered environmental pathogens (**Radostits et al., 2007**). Although resistance to antibacterial drugs among mastitis pathogens has been well documented for nearly four decades, evidence has not been presented to suggest that this is either an emerging or progressing phenomenon. Controlled studies have not determined, on a pharmacodynamic basis, which drug therapeutic regimens may increase this risk, or for that matter, help to decrease it. Monitoring should be continued, preferably by studies that follow data over a course of time and not one point in time**.(Ron et al.,2004)** Staph.aureus strains showed high sensitivity to ofloxacin (100%),oxytetracycline & gentamycine (90.90%) each such a result coordinated   with(**Fazlani et al.,2011)**,E.coli   strains   were   highly   sensitive   to

ofloxacin,polymixin B and vancomycin (100%) each followed by cefotaxime and ceftriaxone (94.44%). Such results coincided with(**Fazlani et al.,2011)** found moderate sensitivity to ofloxacin (84.6%),Klebsiella exhibited high sensitivity to kanamycin, polymyxin B, ceftriaxone , Cefotaxime (93.75%) a result coordinated with **(Urmi et al.,2014)**who found (100%) sensitivity to polymixin B.Strept. spp. were 100% sensitive to gentamicin &vancomycin according to **(Shakuntala, 2003)**, which is in agreement with the current findings**.(Shakuntala ,2003)** also indicated (75%) sensitivity to chloraphenicol, which is comparable with the present finding (72.72%).Micrococcus spp.showed more sensitivity to chloramphenicol ,polymixin B, sulphamethoxazole/trimethoprim ceftriaxone & Cefotaxime (88.88%) each which coincided with **(Rind and Khan ,2000)** who showed that highly effective drugs against the organism were sulphamethoxazole/trimethoprim, chloramphenicol, tetracycline and ampicillin..Coagulase negative Staph. showed (100%) sensitivity to ofloxacin,sulphamethoxazole/trimethoprim and vancomycin. Kanamycin, chloramphenicol,&polymixin B.(87.5%) which coordinated with the results of(**Belayneh et al., 2014)** who stated that CNS was highly sensitive to Chlramphenicol (100%) and Vancomycin (81%). Finally Coryne.spp. showed high sensitivity to ofloxacin , polymixin B & vancomycin (100%) each, almost similar results were also recorded by(**Rind and Shaikh ,2001)..** Staph.aureus is multi-drug resistant i.e resistance to 3 or more of antimicrobials were found to be resistant to ceftriaxone,cefotaxime(100%),penicillin,polymixin B.(82.82%) each(**David et al.,2013)**noticed the same for penicillin resistance among Staph.&E.coli showed (100%)resistance to sulphamethoxazole/trimthoprim(1.25+23.75mg),moderate (83.34%) to penicillin and (72.23%) to ampicillin ,on another hand ,**(David et al.,2013)** found E.coli less resistant strains ,difference in animal husbandry,management practices as well as enforcement of antimicrobial regulations could account for this .Klebsiella showed (100%) resistance to penicillin&vancomycin,moderate to chloramphenicol (81.25%), such results coordinated with **(David et al.,2013)** who describe Klebsiella as multidrug resistance.Strept.spp.(100%)resist ampicillin and (90.91%) to polymixin B.CNS resist ceftriaxone,cefotaxime(100%) and penicillin (87.5%).Finally,Corynebacterium spp.resist penicillin and gentamycin (83.34%) and Micrococcus spp.resist them also (77.78%) and (88.89%) respectively.However, antibiotics showing higher rate of resistance patterns reflect the poor quality of milk available to the consumers, lack of adequate hygienic practices, pre-emptive prophylactic regimen and indiscriminate use of antimicrobials.

This work also highlights the urgent need to set additional clinical breakpoints for antibiotics frequently used to treat mastitis. These findings provide dairy producers with more information on which pathogen-specific clinical &subclinical cases should receive treatment and how to manage these buffaloes, thereby reducing masiitis impact on buffalo well being and profitability.

The examined microorganisms were characterized according to material safety data sheet as risk group no. 2 which needs containment biosafety level 2. Laboratory practices were one

of the most important items implemented in the laboratory including: authorized access, biosafety posted signs, working in the biosafety cabinet class 2, wearing the appropriate personal protective equipment (PPE) which including: gloves, over shoes and laboratory coats with no need to goggles as well as masks. After bacteriological examination, proper disinfectant should be used and decontamination of the samples takes place using autoclave according to standard operating procedures (SOPs). It is worthy to mention that all the activities for biosafety implementations were carried out according to training programmes held in the institute focused on using biosafety lab practices, biosafety cabinet,donning and doffing, using proper disinfection and waste disposal   .

.Of great importance to believe that in this risk assessment, most routine procedures that are carried out in the Biosafety Level 2 laboratory can be expected to cause aerosols and therefore contaminate the laboratory and the worker. Most of hazards are minimized by enclosing the opening of the container with a disinfectant-soaked pledget or placing the containers in a BSC before opening them **(Sewell,1995).**

  Primary containment provides physical separation of the infectious agents from the laboratory worker.Primary barriers include strict adherence to microbiological practices and techniques and use of safety equipment such as biological safety cabinets, safety centrifuge containers,and personal protective equipment (e.g.,gloves,masks,face shields and glasses,coats,and gowns).Secondary containment refers to the facility design and acts as a secondary barrier to protect all workers within the facility and to protect the outside environment **(Sewell,1995)..**

  Biosecurity – the protection of livestock from exposure to disease causing organisms.

One of the greatest disease threats to buffalo is from another whether through direct contact or through surfaces, equipment or people contaminated by diseased animals. This threat is greatest when buffaloes are brought together into housing, particularly at calving, when stress reduces the effectiveness of the animal's immune system. Proper vaccination plays an important role in disease prevention. Medication can also be used once animals are seen to be sick. However neither of these can offer complete, effective and economical protection against the wide range of disease organisms that threaten buffalo. Biosecurity completes the aim of protection, excluding disease organisms from the animal's environment. This is the only way that the cycle of disease can be broken.

Consideration must be given to the maintenance of the required standards in all area of the farm including cubicles, calving facilities, calf rearing, collection areas, milking parlor and dairy. Apart from the abvious need to protect the stock from disease either onto or within the farm, as with all food production chains, the safety of the consumer has to be a paramount consideration. Calves need the best possible start in life, and cows need the best possible care at calving to ensure a good profitable lactation. A crucial factor in achieving these aims is to make sure that the calving environment is as clean and pathogen free as possible. Calving should be in a designated building away from the rest of the herd which

should be thoroughly cleaned of all organic debris ,the surfaces should be pressure washed using detergent sanitizer and sprayed with a solution of broad spectrum disinfectant .

After each calving, remove all bedding, Clean and disinfect . There are many factors involved in the development of mastitis, including genetic predisposition and milking techniques. Of vital importance is the environmental control of mastitis pathogens. The reduction of bacteria in the immediate surroundings must reduce the opportunity for such bacteria to gain access to the udder so cow housing should be cleaned and disinfected when the building is empty with bedding renewal .Also all equipment, utensils, feed racks and drinkers should be cleaned and disinfected daily. Milking parlour and dairy, the area where the diary farmer would naturally practice high standards of hygiene with milk hygiene and mastitis prevention high on the agenda .

In regards to antibacterial residues in raw milk samples out of 13 analyzed raw milk samples, 4 or 30.76% were positive for oxytetracycline residues (**Table ,7**) and **Figure. 1**. It was revealed that the 4 milk samples contain oxytetracycline more than the maximum allowable level according to FDA and WHO (**Table, 8**) . Values of oxytetracycline found in all positive samples ranged from 452 -1475 ng/ ml. This somehow agrees with the result of (**Zahid Hosen et al. ,2010**) who detected oxytetracycline residue in 5 milk samples. The amount of oxytetracycline in milk samples were found 1800 ng/ml. 2700 ng/ml, 2800 ng/ml, 1700 ng/ml and 2000 ng/ml in samples 1 – 5, respectively which are much higher than WHO and FDA recommended level. , Another study carried out in Macedonia by(**Kamberi ,2014**) who noticed oxyteteracycline residues in 4.4% (6/135) of raw milk samples by using HPLC method. All positive milk samples confirming different values of oxyteteracycline per liter milk: 60ug/l, 90ug/l, 220ug/l, 260ug/l, 430ug/l, 1340ug/l (ppb). In Iran,(**Abbasi et al. ,2011**) : found that in114 raw milk sample, 14.5 % was positive for oxyteteracycline residue. Its levels were above WHO standards (100 ng/g) in mean level 154± 66.3 ng/g. But there are also some reports about no presence of these agents in milk samples, (**ORUÇ and SONAL,2005**) did not detect oxyteteracycline drugs in 25 raw cow milk samples by using HPLC method in Bursa..

In concern to Sulfadimidine residues out of 11 analyzed raw milk samples, 6 or 54.5% were positive for sulfadimidine residues (**Table , 7**)) and **Figure. 2.** . It was revealed that 6 milk samples contain sulfadimidine within the maximum allowable level according to FDA and WHO (**Table , 8**) . Values of sulfadimidine found in all positive samples ranged from 3 -44 ng/ ml. The prevalence of antibiotic residue in raw milk during the study is higher than that of(**Thapaliya et al., 2013**) who detected 5.3% (8/150) of raw milk samples were found to contain sulfonamide residue. sulfonamide residue was detected in the range of 0-1 ppb in 6 samples and 2-4 ppb in 2 samples in Thailand .The residues level detected were below their MRLs as set by the(**Codex Alimentarius Commission (CAC) ,2005**) and(**WHO ,2006).** While ,(**ORUÇ and SONAL,2005**) did not detect sulfadimidine drugs in 25 raw cow milk samples by using HPLC method in Bursa .

Lesser amount of residue in positive sample may be due to lesser use of antibiotics during late winter season (time when study was conducted) when disease occurrence is comparatively lower. According to**(Yamaki et al. ,2004)** ,the seasonal factor also affects the prevalence of the antibiotic residue.

We did not detect penicillin G in11 raw milk samples examined in our study. The results of this work were similar to those found by **(ORUÇ and SONAL,2005)** which also did not detect residue of penicillin G in 25 raw cow milk samples by using HPLC method in Bursa . Results of present study is not in line with the study conducted by**(Thapaliya et al. ,2013)** who detected 12%   (18/150) of raw milk samples were found to contain penicillin residue. Penicillin residue was found in the range of 0-1 ppb in 14 of the samples and 2-4 pbb in 4 samples in Thailand. While, in Pakistan(,**Khaskheli et al. ,2008)** detected 32 (64%) of raw milk samples were found to contain penicillin G residue by using HPLC method. Penicillin G residue was found in the range of 0.4 µg/ L to 400µg/ L.

Concerning the residue of ampicillin, out of the 10 samples of buffalo raw milk samples that have been analyzed none showed any residue of ampicillin. This result agrees with the that of **(ORUÇ and SONAL,2005)** which also did not detect residue of ampicillin in 25 raw cow milk samples by using HPLC method in Bursa . Other study carried out in Pakistan, by**(Khaskheli et al., 2008)** shows that out of 137 of the analysed raw milk samples 24 (48%) were found to be contaminated with ampicillin residues. Its concentrations ranged between 0.5 to 141.0µ.

CONCLUSIONS/SIGNIFICANCE:

This study was carried out to investigate the current antibiogram status of buffalo mastitis & the most predominant bacteria were assessed by the results of bacteriological evacuation of mastitic milk samples Staph. aureus,E.coli,Klebsiella spp.and Strept. spp.. While judjing the antimicrobial agents by high sensitivity and pansusceptible strains ,it was found that amongest the effective antimicrobials ofloxacin was sensitive against Staph.aureus,E.coli,CNS and Coryne spp.. followed by vancomycin was sensitive against Strept.spp., E.coli,CNS and Coryne spp. .Polymixin B. was sensitive against E.coli,Coryne spp. and Klebsiella spp..ceftriaxone and cefotaxime were sensitive against E.coli,Klebsiella spp. and Strept.spp..Finally,gentamycin was sensitive against Staph.aureus and Strept.spp.. So this work also highlights the urgent need to set additional clinical breakpoints for antibiotics frequently used to treat mastitis. These findings provide dairy producers with more information on which pathogen-specific clinical and subclinical mastitis cases should receive treatment and how to manage these buffaloes, thereby reducing their impact on buffaloes well being and profitability.Also referring to material safety data sheet for microbial risk assessment as biosafety measures and explaining biosecurity measures in the farm.

The present study also showed higher prevalence (30.76%) and amount of oxytetracycline residues in Kaliobia dairy farms. Therefore, the buffalo milk samples did not have desired conditions because of presence of tetracyclines residues more than

Maximum Residue Limits (MRLs). So that ,The regulatory authorities should ensure proper withdrawal period before milking the animals and definite supervisions are necessary on application of these drugs. The authorities should also adopt comprehensive strategy for ensuring a safe milk supply of good quality .

Table1:Incidence of clinical and subclinical mastitis buffaloe.

Examined buffaloes	Clinical cases		subclinical cases		Normal cases	
50	No.	%	No.	%	No.	%
	15	30	25	50	10	20

Percentage was calculated according to total number of examined buffaloes (50)

Table2: Incidence of CMT ,bacteriologically positive samples and bacterial isolates among the examined milk samples

Milk samples	CMT positive samples		Bacteriologically Positive samples		Bacterial isolates	
115	No.	%	No.	%	No.	%
	75	65.21	75	65.21	90	78.26

Percentage was calculated according to total number of milk samples(115)

Table3: Incidence of single bacterial isolates from buffalo mastitic milk sample

Single bacterial isolates	No. of isolates	%
Staph. aureus	12	13.33
E.coli	11	12.22
Strept. spp.	9	10
Coagulase negative Staph.	8	8.88
Klebsiella spp.	7	7.77
Micrococcus spp..	7	7.77
Corynebacterium spp.	6	6.66
Total	60	66.66

Percentage was calculated according to the total number of isolates(90)

Table4: Incidence of mixed bacterial isolates in mastitic buffalo milk samples

Mixed bacterial isolates	No. of isolates	%
Staph. aureus& E.coli	4	4.44
Staph. aureus&Klebsiella spp.	4	4.44
Staph. aureus& Strept. spp.	2	2.22
E.coli & Klebsiella spp.	3	3.33
Klebsiella spp.& Micrococcus spp..	2	2.22
Total	15	16.66

Percentage was calculated according to the total number of isolates(90)

Table5: Incidence of single and mixed bacterial isolates in mastitic buffalo milk samples

Bacterial species	Staph.aureus		E.coli		Klebsiella spp.		Strept.spp.		Micrococcus spp.		Coagulase _+ve Staph.		Coryne bacterim spp.	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Single strain infection 60(6.66%)	12	13.33	11	12.22	7	7.77	9	10	7	7.77	8	8.88	6	6.66
Mixed strain infection 30(3.33%)	10	11.11	7	7.77	9	10	2	2.22	2	22.22				
Total 90 (100%)	22	24.44	18	20	16	17.77	11	12.22	9	10	8	8.88	6	6.66

Percentage was calculated according to the total number of recovered isolates (90)

Table 6:In Vitro susceptibility pattern of the isolates against different antibiotics

Isolated bacterial species Antibiotics	Staph. aureus. n.=22	E.coli n.=18	Klebsiella. spp. n.=16	Strept. spp. n.=11	Micrococcus. spp.n.=9	Coagulase. .–ve Staph. n.=8	Corynebacterium. spp.n.=6
Oxytetracyclin30mg	20/22 (90.90%)	14/18 (77.7%)	6/16 (37.5%)	9/11 (81.81%)	7/9 (77.77%)	5/8 (62.5%)	5/6 (83.33%)
Penecillin10mg	4/22 (18.18%)	3/18 (16.66%))	0/16 (0%)	6/11 (54.54%)	2/9 (22.22%)	1/8 (12.5%)	1/6 (16.66%)
Ofloxacin5mg	22/22 (100%)	18/18 (100%)	12/16 (75%)	6/11 (54.54%)	7/9 (77.77%)	8/8 (100%)	6/6 (100%)
Kanamycin30mg	15/22 (68.18%)	8/18 (44.44)	15/16 (93.75%)	10/11 (90.90%)	2/9 (22.22%)	7/8 (87.5%)	2/6 (33.33%)
Chloramphenicol 30mg.	12/22 (54.54%)	10/18 (55.55)	3/16 (18.75%)	8/11 (72.72%)	8/9 (88.88%)	7/8 (87.5%)	4/6 (66.66%)
Polymixin B	4/22 (18.18%)	18/18 (100%)	15/16 (93.75%)	1/11 (9.09%)	8/9 (88.88%)	7/8 (87.5%)	6/6 (100%)
Sulphamethoxazole/ Trimethoprim (1.25+23.75mg)	16/22 (72.72%)	0/18 (0%)	5/16 (31.25%)	9/11 (81.81%)	8/9 (88.88%)	8/8 (100%)	5/6 (83.33%)
Ampicillin10mg	9/22 (40.90%)	5/18 (27.77)	6/16 (37.5%)	0/11 (0%)	6/9 (66.66%)	3/8 (37.5%)	2/6 (33.33%)
Gentamycin10mg	20/22 (90.90%)	12/18 (66.66%)	9/16 (59.25%)	11/11 (100%)	1/9 (11.11%)	6/8 (75%)	1/6 (16.66%)
Vancomycin30mg	19/22 (86.36%)	18/18 (100%)	0/16 (0%)	11/11 (100%)	7/9 (77.77%)	8/8 (100%)	6/6 (100%)
Ceftriaxone	0/22 (0%)	17/18 (94.44%)	15/16 (93.75%)	10/11 (90.90%)	8/9 (88.88%)	0/8 (0%)	4/6 (66.66%)
Cefotaxime	0/22 (0%)	17/18 (94.44%)	15/16 (93.75%)	10/11 (90.90%)	8/9 (88.88%)	0/8 (0%)	3/6 (50%)

The chemical assays were performed on different collected milk samples to determine the presence of oxytetracycline , sulfadimidine , penicillin G and ampicillin residues.

Chemical assay for qualitative analysis:

Table (7) Antibiotic concentrations of buffalo's raw milk samples by using HPLC analysis

Antibiotic	No of samples	Positive sample	Residue%	Above MRL	MRL ppb)(
Oxytetracycline	13	4	30.76%	4 (30.76%)	100
Sulfadimidine	11	6	54.5%	-	100
Penicillin G	11	ND	-	-	4
Ampicillin	10	ND	-	-	4

Table (8) Oxytetracycline and sulfadimidine concentrations in buffalo's raw milk samples determined by HPLC method

Antibiotic	Concentrations of residue (ng/ml)	Above /below MRL	MRL(ppb)*
Oxytetracycline	452 560 1475 2833	Above	100
Sulfadimidine	3 22.8 28 29 32 44	below	100

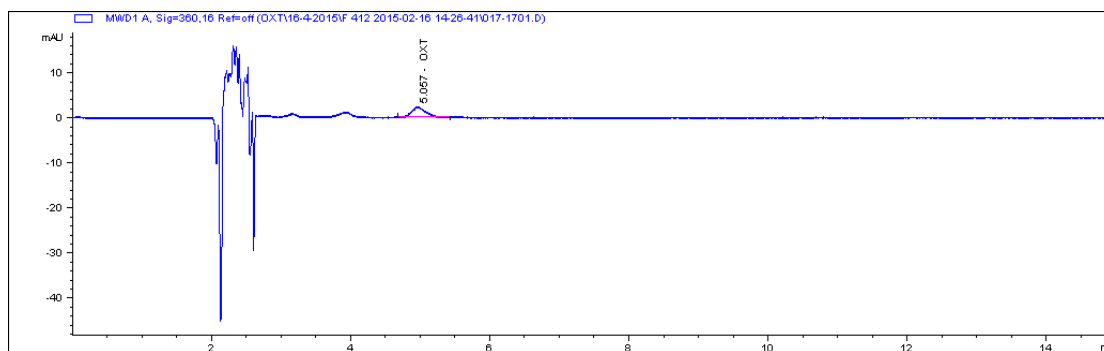MRL) : Maximum residual level referred by (**Nisha ,2008)***

Figure. 1 : The HPLC chromatograms of a milk sample containing oxytetracycline residues (2833 ng/ ml.(
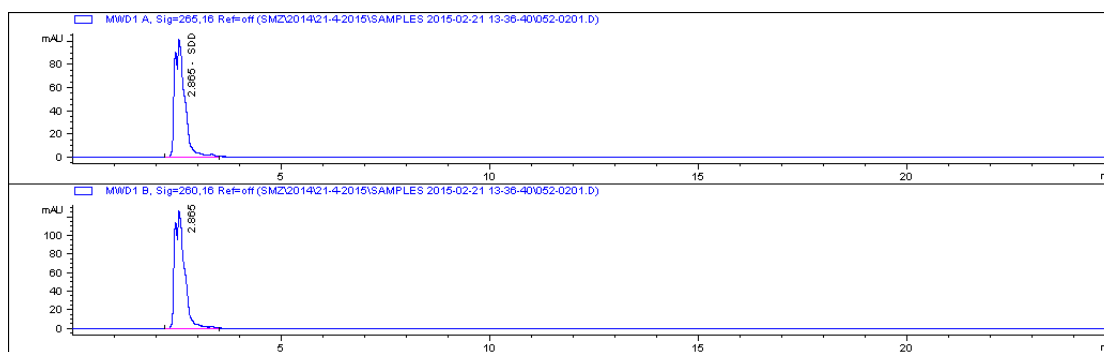

Figure2 : The HPLC chromatograms of a milk sample containing sulfadimidine residues (44 ng/ ml)

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