

Full length Research Paper

# Prevalence of abortion in kundhi buffalo in district hyderabad, sindh – Pakistan

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Fifty dairy farms 5660 animals were examined between 10-12-2009 and 17-01-2011. Samples collected and examined during present investigation included milk, blood and vaginal secretion (swab) and placenta from the aborted dam along with liver, lungs and stomach contents from aborted fetus. In addition, information regarding the general health, vaccination history of the animal and management of the dairy farm was collected through the questionnaire based on the interview of farm owner. The study revealed an overall abortion rate of 3.53% in Kundhi buffalo in District: Hyderabad. The organisms identified as single infection included: *Brucella abortus* (16.3%), *Trichomonas fetus* (2.14%) and *Listeria monocytogenes* (1.82%). In case of mixed infection, *Brucella abortus* along with *Salmonella* spp: occurred in highest frequency (7.5%), followed by *Brucella abortus* along with *Staphylococcus aureus* (7.34%) and *Proteus vulgaris* along with *Escherichia coli* (3.4%). Based on the above observations, it was concluded that: (a) *B.abortus* appears to be a major single causal agent causing abortion in buffalo. (b) *Brucella abortus* along with *Staphylococcus aureus* (7.34%) appears to be major mixed cause of abortion in buffalo. (c) The abortion rate of 3.53% in Kundhi buffalo was lower than previously reported indicating availability of good professional services and better management in the dairy farms. The fetal stomach contents from aborted fetus appeared to be a better sample type for the isolation and identification of different organism responsible for the abortion in Kundhi buffalo.

**Keywords:** Milk, placenta, fetal stomach contents and bacteria.

## INTRODUCTION

Pakistan has 30.8 million buffalo heads (GOP 2009-10) that account for approximately 18.01 percent of the total world buffalo population. Pakistan is home to great biodiversity of buffalo, including the world famous Nilli-Ravi and Kundhi buffalo breeds renowned for their high milk production. The buffalo (*Bubalus bubalis*) is usually referred as “black gold of South Asia”. It plays an important role in the overall economy of Pakistan by contributing milk, meat, hides and draft power for various agricultural operations. Abortion leads to heavy economic loss, brucellosis was a costly disease of water buffaloes (Borriello *et al.*, 2006). Knowledge for the possible causes of early abortion in buffalo was yet limited few reports. Infectious abortion in buffalo was also a public health concern because the infectious agents also infect

the humans. An infection rate of 63.3% brucellosis in the butchers; and a combined infection rate of 31.82% in humans were also noted (Cadmus *et al.*, 2006).

Various studies have been carried out to identify the causes of abortion in cattle, which suggest that most frequently isolated agents, *Brucella* spp., *Campylobacter* spp., *Leptospira* spp. *Listeria* spp., *Salmonella* spp., *Chlamydophila* spp., *Mycoplasma* spp., and a variety of viruses and mycotic agents (Mccausland *et al.*, 2008). Similar organisms were also identified in sheep, goats and buffaloes (Sharma *et al.*, 2008). In Pakistan, very few studies have been carried out on the identification of infectious agents causing abortion in cattle and buffalo (Hussain *et al.*, 2008). However, such studies have not been carried out on buffaloes in Sindh province. The present study was therefore planned to determine the prevalence of abortion in Kundhi buffalo in district Hyderabad, to document the prevalence of abortion in Kundhi Buffalo in the area of study and to investigate

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the causes of abortion in Kundhi buffalo.

## MATERIALS AND METHODS

The study was carried out between 10-12-2009 and 17-01-2011. The present study included 50 dairy farms with a total number of 5660 animals in the vicinity of Distt: Hyderabad. The owners and dairyman of farms were requested for the quick report of abortion in buffaloes. The farmers were interviewed for the case history and other observations on special hand made questionnaire for final data interpretation. A total of 180 milk, 200 vaginal swabs, 200 bloods, 129 placenta, 105 aborted fetus samples as; lungs, liver and stomach contents from fetal were collected from aborted Kundhi buffalo and brought to the Microbiology section of Central Veterinary Diagnostic Laboratory (CVDL) Tandojam, Sindh, for further bacteriological investigations.

### Preparation of glassware

New glassware was immersed in 1N HCl solution overnight to remove the surface alkali. The glassware was then taken out from 1N HCl solution and rinsed with 4 changes of normal tap water followed by 4 changes of distilled water. Used glassware, Petri dishes, conical flasks, bijoux bottles, universal bottles and all other glassware containing media and chemicals were autoclaved. Used glass Pasteur pipettes were soaked in antiseptic detergent solution of low concentration (1% chromic acid) to remove any greasy material that may have been deposited on pipette surface. After overnight dipping, the pipettes were removed for washing in tap water and distilled water. All the glassware were then drained, placed upside down in drying oven at 55°C and left to dry before capping/plugging for sterilization.

### Sterilization

Oven dried Petri dishes were placed in sterilizing box, while the small Petri dishes were wrapped in grease proof brown paper. Test tubes and glass pipettes were plugged with cotton plug and then wrapped in brown paper in batches of 5 and 10. The other glassware like conical flasks, measuring cylinders and centrifuge tubes were sealed individually with aluminum foil. All the materials were then placed in sterilizing oven at 165°C for 3 hours. Furthermore, the glassware with plastic or aluminum caps and all those which could not resist dry heat in oven were sterilized in an autoclave at temperature of 121°C with 15lb pressure for 15 minutes. Most commercially available general media ready to use were rehydrated in distilled water using standard procedures (Difco, 1960). The following media was

prepared and used during study (Table- 1).

### Brucella agar

It was rich in nutrients and growth factors and is very suitable to grow and isolate fastidious microorganisms. It was used to successfully isolate *Brucella* from diverse specimens contaminated with microflora, both saprophytes and commensals, in clinical samples as well as in foods. The quantity (43.1grams) was rehydrated in 1000ml of distilled water. Mixed well with frequent agitation and boiled for one minute or until the medium dissolved completely. The medium was then sterilized at 121°C for 15 minutes and poured into Petri dishes. Once solidified, the plates were inverted to dry up excess moisture.

### Brucella broth

It was prepared according to the formula described by Isenberg, (1992). The quantity of ingredients (28.1 grams) was suspended in one liter of distilled water. Mixed well with frequent agitation and boiled for one minute until completely dissolved. Then the medium was dispensed into tubes and sterilized at 121°C for 15 minutes.

### Listeria agar base palcam

It is recommended for the isolation of *Listeria monocytogenes* in food products and environmental samples. It allows the easy differential diagnosis of *Listeria monocytogenes* using a double system indicator: Esculin/Iron and Mannitol/Phenol red. Inoculate sample and incubate at a temp: of 35±2°C and observe after 24-48 hours. All (34.4gm) was suspended in 500ml of distilled water. Mixed well and heated with frequent agitation until complete dissolution then sterilized at 121°C for 15 minutes. Cooled to 50°C and aseptically added 1 vial of the reconstituted Palcam *Listeria* selective supplement. Mixed well and poured into Petri dishes.

### Cultural and staining characteristics of bacteria

Samples from the original solution were streaked over on different plates of tryptose agar, blood agar, MacConkey's agar and nutrient agar and incubated at 37°C for 24 hours to obtain primary bacterial growth. On day next, colonies were examined and counted by colony counter for calculating Colony Forming Units (CFU) to get quantitative measurement of mixed culture. Then individual colony, on the basis of its morphological and cultural characteristics, from blood, MacConkey's and

**Table 1.** List of general culture media used in the study

S.#	Name of media	Used for the isolation of bacteria
1	Nutrient agar	<i>Staphylococcus</i> spp. <i>Escherichia coli</i> and <i>Streptococcus</i> spp.
2	Nutrient broth	<i>Escherichia coli</i> ; <i>Salmonella</i> spp. And <i>Streptococcus</i> spp.
3	Nutrient agar slant	Stock media for <i>Escherichia coli</i> ; <i>Salmonella</i> Spp and <i>Streptococcus</i> spp.
4	Tryptose agar	<i>Brucella</i> spp. <i>Corynebacteria</i> spp. <i>Listeria</i> spp; <i>Pasteurellala</i> spp. and <i>Vibrio</i> spp.
5	Tryptose broth	<i>Rubella</i> spp. <i>Corynebacteria</i> spp. <i>Listeria</i> spp. <i>Pasteurellala</i> spp. and <i>Vibrio</i> spp.
6	Peptone water	<i>Salmonalla</i> spp. <i>Escherichia coli</i> and <i>Staphylococcus</i> spp.
7	MacConkey's agar	<i>Enterobacteria</i> spp. <i>Proteus</i> spp. And <i>Staphylococcus</i> spp.
8	MacConkey's broth	<i>Coliforms</i> spp. <i>Salomonella</i> spp. And <i>Staphylococcus</i> spp.
9	Sorbitol MacConkey's agar (CM813)	<i>Escherichia coli</i> spp. and <i>Staphylococcus</i> spp.
10	Salmonella-Shigella agar (SS AGAR)	Enterobacteriaceae spp.
11	Blood agar	<i>Staphylococcus</i> spp. <i>Streptococcus</i> spp. and <i>Escherichia coli</i>
12	Bismuth sulphate agar (CM 210)	<i>Escherichia coli</i> ; <i>Salmonella</i> spp. <i>Shigella</i> spp. and <i>Streptococcus</i> spp.
13	Kligler iron agar (KIA)	<i>Proteus vulgaris</i> , <i>Echerichia coli</i> , <i>Salmonella</i> spp. And <i>Shigella</i> spp.
14	Endo agar base (CM479)	<i>Coliforms</i> spp. <i>Salmonella</i> spp. <i>Shigella</i> spp. and <i>Escherichia coli</i>
15	Simmon's citrate agar	<i>Enterbacteriaceae</i> spp. <i>Shigella</i> spp. And <i>Salmonella</i> spp.
16	Dextrose phosphate broth	<i>Shigella</i> spp. and <i>Escherichia coli</i>
17	Bismuth sulphate agar (CM 201)	<i>Salmonella</i> spp.
18	Cystine-Lactose-Electrolyte Deficient (CLED medium)	<i>Proteus mirabilis</i> , <i>Escherichia coli</i> and <i>Staphylococcus</i> spp.
19	Triple sugar iron agar (TSI)	<i>Escherichia coli</i> , <i>Proteus vulgaris</i> and <i>Salmonella</i> spp.

nutrient agars were picked-up by sterilized inoculating loop and cultured on nutrient agar plates and this process of sub culturing was continued until pure growths were obtained. Purity of isolated bacterial species was determined on the bases of their morphology and cultural characteristics. The pure colonies were then incubated at 37C° for 24 hours. After examination their growth, the stock culture of various purified isolates obtained were stocked in slants using refrigerator at 4C° for further tests.

### Staining characteristics of bacteria

Smears of the isolated colonies of pure culture were prepared and stained with Gram's Method and the behavior of the organisms was recorded as its being gram-negative or gram-positive. For Gram's staining, solutions were prepared such as; Ammonium oxalate

crystal violet, Lugol's iodine solution (mordant) and Dilute Carbol fuchsine (counter stain).

#### a). Ammonium oxalate Crystal violet

Two grams of crystal violet or methyl were dissolved in 20ml methyl alcohol. About 0.8 g of ammonium oxalate was dissolved in 80ml distilled water. The two solutions were mixed and filtered through 2 layers filter paper. After that the reagent was then stored in tightly capped bottle at room temperature.

#### b). Lugol's Iodine solution (mordant)

Two grams of potassium iodide were added with sufficient amount of distilled water in a beaker and

dissolved. Then 1g of iodine was added to the KI (potassium iodide) solution, mixed well until the iodine was completely dissolved and other quantity of distilled water was added and made up to 100ml. The solution was stored in dark bottle and kept at room temperature till used.

### **c). Dilute Carbol fuchsin (counter stain)**

A strong solution of carbol fuchsin was made by dissolving 1g of basic fuchsin in 10ml isopropyl or methyl alcohol. Five grams of phenol were dissolved in 100ml of distilled water and mixed both solutions very well, filtered and stored in a screw capped bottle at room temperature. The dilute of carbol fuchsin was made by mixing 10ml of strong carbol fuchsin with 90 ml distilled water.

#### **Staining procedure**

A drop of distilled water was placed on the glass slide and a pure colony was picked-up from dish and mixed with water and fixed by Bunsen burner's flame passed for three times. Few drops of ammonium oxalate crystal violet were applied for 30 seconds. The slide was then washed in running tap water then slide was covered with Lugol's solution for 30 seconds and also washed in running tap water. The slide was decolorized with 95% alcohol by hanging the slide in steep angle. As soon as the stain ceased to pour out (2-3 seconds), washed immediately in running tap water for further 30 seconds and the Carbol fuchsin was applied for 30 seconds. Carbol fuchsin was washed in running tap water then blot dried on paper and left at room temperature for the residual moisture to evaporate. A drop of oil immersion was placed on stained part of the slide, and then the slide was examined under the oil immersion objective. Photographs of the bacteria were made under oil immersion objective x100, by using camera fitted microscope.

#### **Motility of bacteria**

The organisms isolated were transferred in nutrient broth and allowed at 37°C for overnight to grow. A drop of this growth was placed over a cover slip and then inverted on the grooved slide and examined microscopically. The motility of the bacteria was clearly distinguished by drifting of the bacteria and Brownian movement. A ring of petroleum jelly was applied around the concavity of the depression slide. Using sterile technique, a pure isolated colony was transferred into nutrient broth, mixed well and a drop of this was placed in the center of a clean cover slip. The depression of slide, with concave surface facing down was covered with cover-slip, so that the depression covers the drop of culture, the slide was gently pressed to

form a seal between the slide and the cover-slip. The slide was then quickly turned right side up so that the drop continues to adhere to the inner surface of the cover-slip. For microscopic examination, the drop of culture was first focused under the low-power objective with reduced light. A drop of oil was then placed on the cover-slip and kept under oil immersion objective for detailed observation. The motility of the bacteria was clearly observed by drifting of the bacteria and Brownian movement. Finally, data were subjected to analysis of variance to ascertain difference among the sample types on the software packages (USA, SXW 8.1) Student Edition of Statistics.

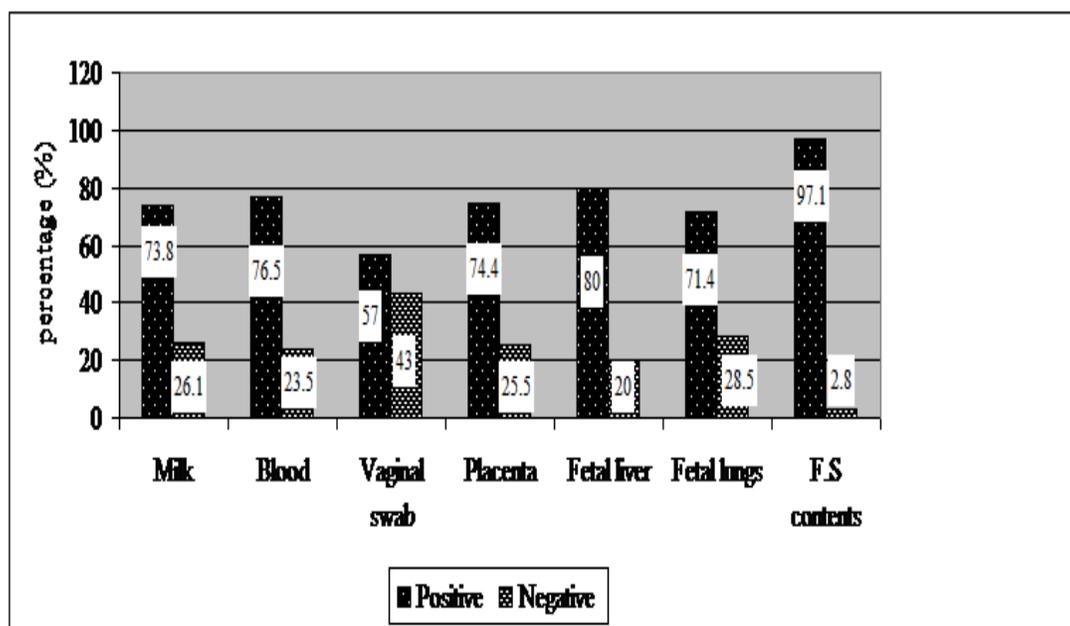
## **RESULTS**

The overall prevalence of abortion was recorded as 3.53% and the following samples were collected for microbial investigation during the study.

### **Organisms identified**

Total milk samples from aborted Kundhi buffaloes 180 were collected; 133 (73.8%) were found positive for the presence of different bacteria after culture and 47 (26.1%) were negative for bacterial culture, as no any bacterial growth was observed upon culturing on general media as well as specific media. While from 200 blood samples collected from aborted Kundhi buffaloes; 153 (76.5%) were observed positive for bacterial growth and 47 (23.5%) were negative for any bacterial growth. Whereas 114 (57.0%) vaginal swabs were identified as positive for bacterial load and 86 (43.0%) were negative for bacterial growth from a total of 200 vaginal swabs collected from aborted Kundhi buffaloes (Figure 1). During present study, samples about 129 of placenta's were also collected from aborted buffaloes. 96 (74.4%) of the collected samples revealed positive bacterial growth and 33 (25.5%) showed no bacterial growth on culturing. During present study 105 aborted fetuses were also collected and fetal (liver, lungs and stomach contents) were processed for the presence and identification of microbial agent. 84 (80.0%) fetal liver, 75 (71.42%) fetal lungs and 102 (97.14%) fetal stomach contents were identified as positive for bacterial isolation while 21 (20.0%) fetal liver, 30 (28.5%) fetal lungs and 03 (2.8%) fetal stomach contents were found negative for bacterial growth (Table -2).

The frequency of different organism's pure culture isolated from Milk, Blood, Vaginal swab, Placenta and Fetus (fetal liver, lungs and stomach contents). Analysis of variance showed the highly significant (DF=6, 117, F=0.39, P>0.05) difference in the frequency of pure organisms isolated from the culture of milk, blood, vaginal swab, placenta and fetus. Whereas; LSD showed the



**Figure 1.** The percentage of positive and negative samples with regard to the identification of organisms in aborted fetus and the dam

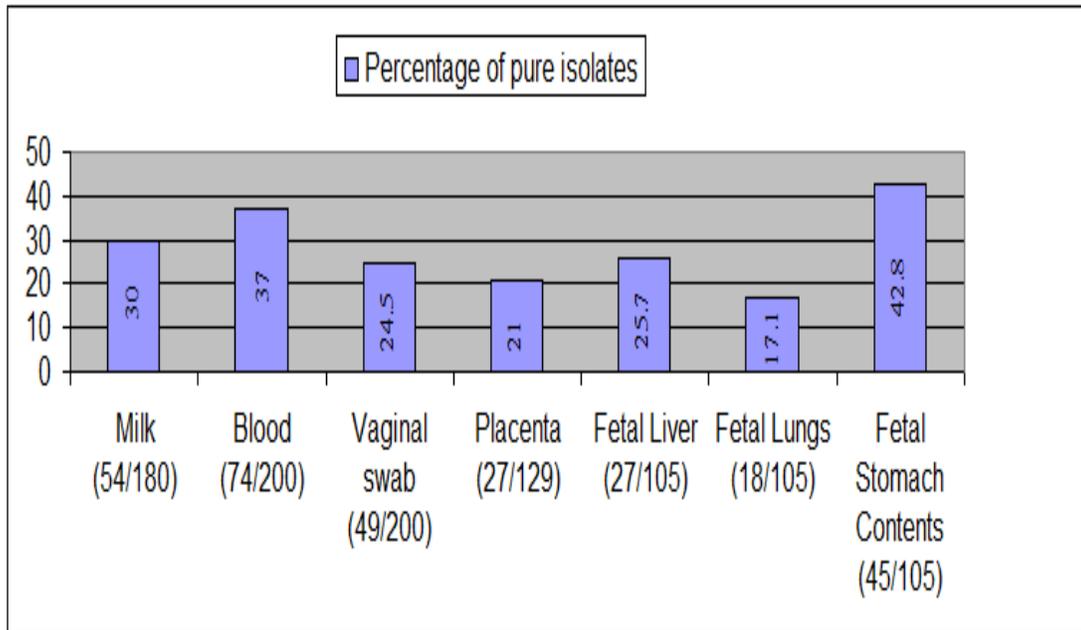
**Table 2.** Percentage of positive and negative samples for isolation of organisms in aborted dam and fetus

S. No.	Samples	Positive samples	Percentage of +ve samples	Negative samples	Percentage of -ve samples
Milk	180	133	73.8	47	26.1
Blood	200	153	76.5	47	23.5
Vaginal.swab	200	114	57.0	86	43.0
Placenta	129	96	74.4	33	25.5
Fetal .liver	105	84	80.0	21	20.0
Fetal lungs	105	75	71.4	30	28.5
Fetal stomach contents	105	102	97.1	03	2.8
<b>±SE</b>		<b>10.38</b>		<b>9.84</b>	

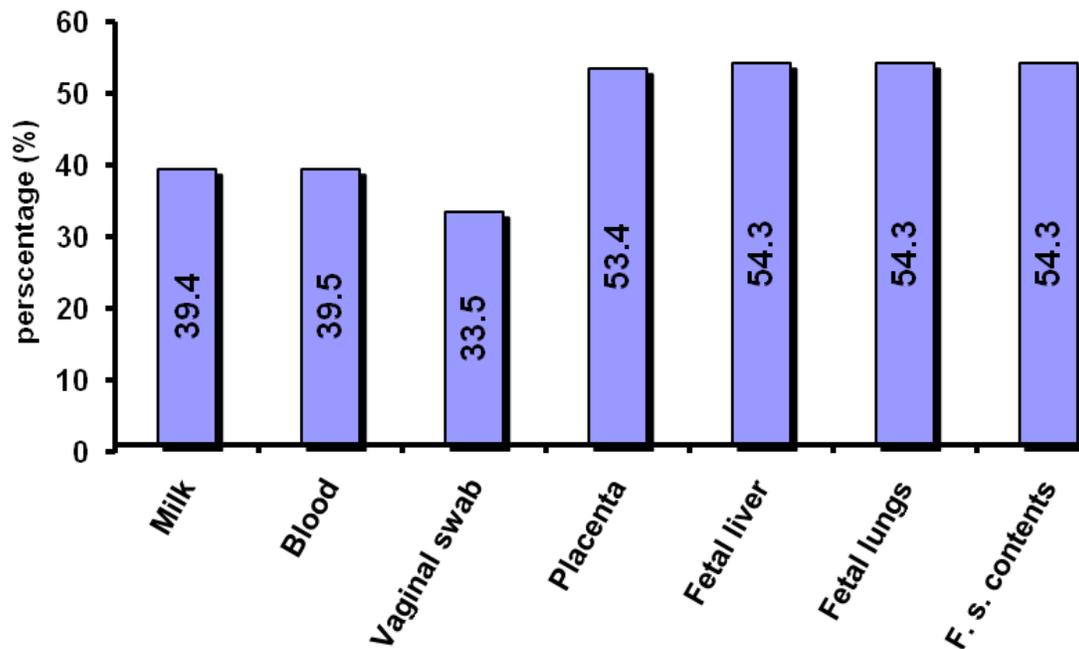
non-significant (DF=2, 118, F=12.61, P=0.001, R<sup>2</sup>=0.179) difference. During the present study; from 54 (30.0%) milk samples pure culture of different bacteria were isolated. Blood samples 74 (37.0%) revealed pure bacterial isolations on culturing upon different general as well as specific growth media. Whereas, vaginal swabs 49 (24.5%) were found positive for pure bacterial isolates 27 (21.0%) were also found positive for pure bacterial isolation. From a total of 105 collected fetuses 27 (25.7%) fetal liver, 18 (17.1%) fetal lungs and 45 (42.8%) fetal stomach content gave a pure bacterial growth upon culture on the general and specific media (Figure. 2).

During the present study different mixed bacterial cultures were also identified from the samples collected from aborted Kundhi buffalo. Analysis of variance showed the significant (DF=7, 98, F=0.38, P>0.05) difference in the frequency of mixed organisms isolated from the

culture of milk, blood, vaginal swab, placenta and fetus during the study period. Whereas; LSD showed the highly significant difference (DF=1, 103, F=3.01, P=0.096, R<sup>2</sup>=0.0284). The frequency of different mixed organism isolated from the culture of milk, blood, vaginal swab, placenta and aborted fetus (fetal liver, lungs and stomach contents). In the present study, from 180 collected milk samples 77 (39.4%) from aborted Kundhi buffalo which were identified positive for mixed bacterial infection as mix bacterial growth produced on culturing the sample over general and specific growth media. Whereas, 79 (39.5%) blood samples from 200 collected samples were found positive for mix isolations. While, 65 (33.5%) out of 200 collected vaginal swabs from aborted Kundhi buffalo produced mixed isolations upon culturing the samples on the growth media. During the study, the 129 placenta were also collected and investigated for the presence of



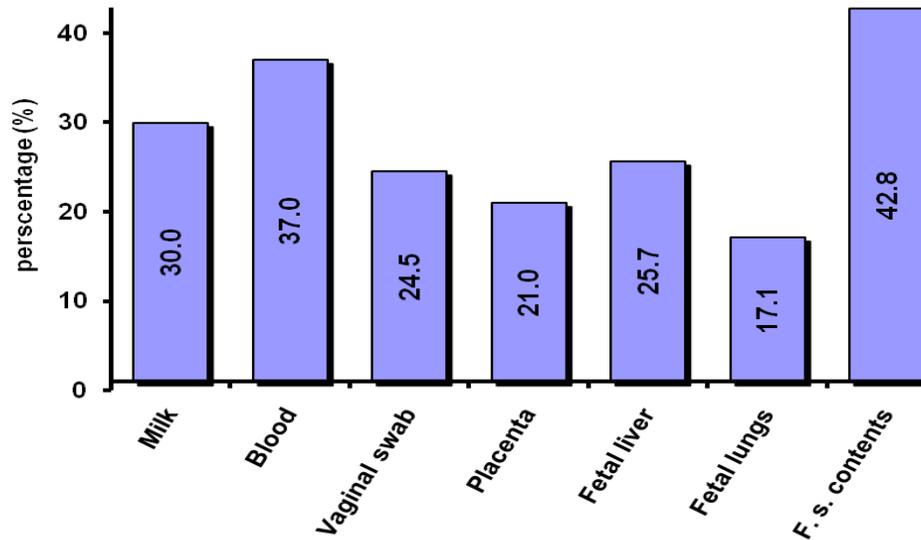
**Figure 2.** The summary of the frequency of pure bacterial isolation culture from milk, blood, vaginal swab, placenta and fetus



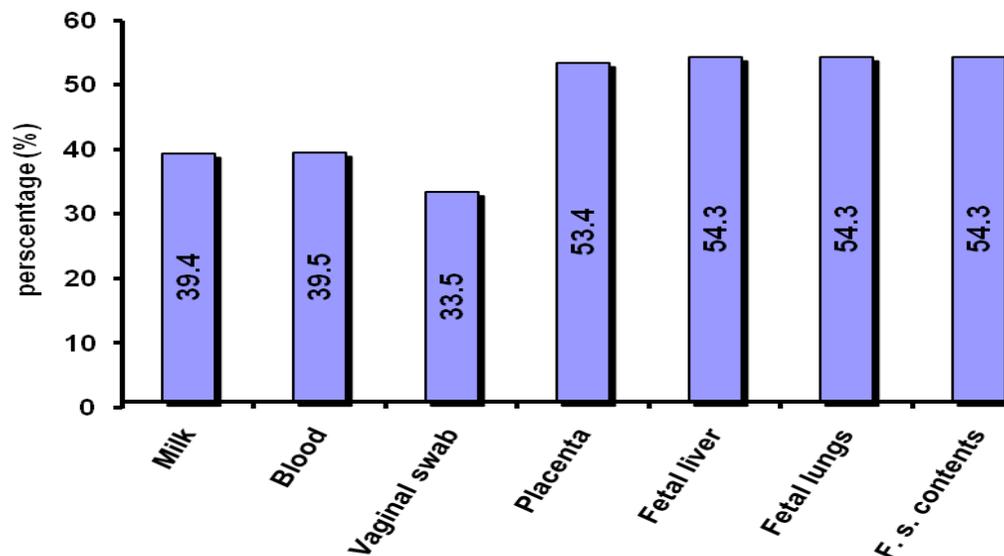
**Figure 3.** The frequency of mix organisms isolated from the culture of milk, blood, vaginal swab, placenta and fetus

bacterial agent 69 (53.5%) were found positive for mix bacterial infection. Aborted fetuses were also collected during present study (105) and necropsy of these aborted fetuses were performed fetal (liver, lungs and stomach contents) were collected and processed for the isolation of microbial agents. From 105 collected fetal liver; 57

(54.3%) were found positive for mix isolates. 57 (54.3%) fetal lungs were also identified positive for mix bacterial load after culturing on the general and specific growth media. Whereas, the 57 (54.3%) fetal stomach contents were found positive for mixed source of bacterial infection in this study shown in (Figure 3).



**Figure 4.** Percentage of samples with isolation of single organism in aborted fetus and the dam



**Figure 5.** Percentage of samples for the mixed isolation of organism in aborted fetus and the dam

During the present study; it was observed that milk samples were 30.0%, blood 37.0%, vaginal swab 24.5%, placenta 21.0%, aborted fetal (liver 25.7%, lungs 17.1% and stomach contents 42.8%) were reliable for the investigation of the microbial agents. During this study, highest pure culture was observed in aborted fetal stomach content 42.8%, followed by aborted dam's blood 37.0%, while the milk of aborted dam 30.0%, medium pure culture of bacteria was observed from aborted fetal liver 25.7% followed by vaginal swab 24.5% and the lowest pure culture was observed from placenta of aborted dam 21.0% followed by aborted fetal lungs 17.1% (Figure 4).

The reliability of observed as milk samples were 39.4%, blood 39.5%, vaginal swab 33.5%, placenta 53.4%, aborted fetal (liver 54.3%, lungs 54.3% and stomach contents 54.3%) for the investigation and identification of the mixed microbial agents. Highest mix culture was observed in aborted fetal organs (liver, lungs and stomach content) 54.3% followed by aborted dam's placenta 53.4%, while medium mix culture of bacteria was observed from aborted dam's blood 39.5% followed by aborted dam's milk 39.4% and the lowest pure culture was observed from the vaginal swab of aborted dam 33.5% (Figure 5) in the present study.

## DISCUSSION

In this study, highest prevalence of abortions (11.11%) in buffalo was observed in only one farm; which were found to be lower than Khan, (1994) who reported 13.69 and 11.93% in some of the villages in the surroundings of Faisalabad, Pakistan with similar managerial conditions. The lower incidence may be due to difference in source of sample collection, hygienic conditions and management. The difference in abortion rates within animals may be due to several reasons, the higher incidence rate of abortion in present study may be due to presence of large number of younger buffalo cows in herds of studied area which may be due to less acquired immunity to infectious agents. Further, the number of lactations and stage of gestation may also influence the incidence as abortion rates were higher in the early stages of pregnancy than advanced stage as reported for cows. The same may also be true in buffaloes of the study area. Variation may be due to seasonal patterns, managerial differences, hygienic conditions practiced at the farm and vaccination program may also play a role in the incidence of abortions.

During present study, milk, blood, vaginal swab, placenta, fetal tissues, liver, lungs and stomach contents were studied. From aborted Kundhi buffalo were observed positive for the isolation of bacteria while; milk, blood, vaginal swab, placenta and fetal tissues (liver, lungs and stomach contents) were identified as negative for bacterial isolation. This was comparable to Cadmus *et al.*, (2006) who reported fetal tissues were positive for the bacterial load in infected aborted buffaloes, although in his study, placenta appeared to be less reliable. The best sample for the identification of causal agent responsible for the abortion in buffalo was to be established, the present findings indicate that fetal stomach contents and maternal blood may be more reliable as sample type. Results of the present study were in close agreement with Salihi *et al.*, (2009) who found up to 65.1% bacterial isolates and reported that fetal stomach contents were most satisfactory for the isolation and identification of bacteria.

With regard to the frequency of different pure bacterial isolates obtained from milk, blood, vaginal swab, placenta and fetus (fetal liver, lungs and stomach contents), *Brucella abortus* was recorded as the highest (16.3%) to reveal as a causal agent in the study area. This is an agreed with Aulakh *et al.*, (2008) who reported 16.41% prevalence of brucellosis in buffaloes in Punjab, India. In contrast of Durani *et al.*, (2009), who observed a prevalence of 23% of brucellosis in the surroundings of Lahore, Pakistan. The variation could be due to different study techniques employed by various workers. Additionally, in this study the whole population was taken as target population, whereas, earlier studies were either confined to some selected farms or based on conveniently selected samples. *Trichomonas fetus* was

the 2<sup>nd</sup> highest (2.14%) causative agent observed in present study. This was lower than in cattle in which 13% abortion associated with *Trichomonas fetus* in Iran (Razmi *et al.*, 2006). Mixed group bacterial isolates were also obtained from the collected samples of aborted Kundhi buffalo which are in agreement with (Sammartino *et al.*, 2006) who described that, *Brucella abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*. *B. abortus* and *Salmonella* spp: usually causes brucellosis and mastitis in cattle, bison and water buffalo. The second highest mixed bacterial isolated group *Brucella abortus* and *staphylococcus aureus* was identified in this study. The results of present study were higher than Anderson *et al.*, (1990) who reporting 6.8% mixed infection of *Brucella* spp; *Actinomyces pyogenes*; *Sterptococcus* spp; *Salmonella* spp: and *Escherichia coli* as a common cause of abortion in cattle it was further suggested that pyometra, metritis and mastitis results as a sequelae disease in the aborted bovine animals. A combined infection of 3.4% due to *Proteus vulgaris* and *Escherichia coli* were also isolated from samples of aborted Kundhi buffalo. A large scale vaccination may also help to lower the prevalence of this disease (brucellosis); therefore, in larger dairy herds, vaccination may be helpful to overcome this problem. A large scale campaign through media may also be helpful to create awareness among people to use boiled or pasteurized milk to lower the risk of this human health problem.

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