1. INTRODUCTION

Diarrhoea is one of the major cause of mortality in newborn calves. The incidence of diarrhoea in calves less than one month ranges between 15 to 20 per cent, signifying that the greatest risk occurs during the first two weeks of life (Vandeputte et al., 2010).

Salmonella are one of the most important micro-organisms that cause disease in man and animals and among the most common causes implicated in outbreaks of food born infectious disease around the world. Animals are mainly infected through feed, drinking water or environmental sources. The risk of Salmonella infection has been heightened by the globalization of trade in food, feed and live animal and changes in production, processing and handling of foods.

Bovine salmonellosis is of worldwide public health concern and is an economically important disease. Although cattle of all ages can be infected with Salmonella bacteria, serious infections and deaths are most often seen in calves up to 10 weeks of age (Lanzas et al., 2008). According to a study 56 per cent of 16 herds tested had at least one cow with a positive manure culture and about 10 per cent of almost 1000 samples cultured positive (Callaway et al., 2005). Rarely considered part of the normal flora of the intestinal tract, asymptomatic cattle can shed Salmonella for prolonged periods after infection and, in the case of Salmonella Dublin infection some cattle may be intermittent shedders for life. Dairy cattle ingest feed or water that has been contaminated with faeces from animals shedding the organism. It may be difficult to tell which cows are shedding bacteria because asymptomatic and sub clinically affected cows can shed as many organisms in their manure as the cows that are sick with salmonellosis.

Calves in raising operations are at high risk of suffering salmonellosis (Reynolds et al., 1986). In raising operations the continual admission of new calves from mixed sources and the high density confinement of a large number of susceptible animals favor the transmission of Salmonella. As a result, infection in a limited number of calves can spread very rapidly through the herd. Salmonella transmits predominantly by a faecal-oral cycle (Wray et al., 2000).
Salmonellosis is endemic in India and its importance, as potential zoonosis needs no emphasis *Salmonella* has more than 2,400 serovars (Sachan et al., 2013).

Some of these *viz.*, *S. typhi*, *S. gallinarum*, *S. dublin* and *S. choleraesuis* are host specific, the majority are non adapted and can cause infection in man and animals alike.

Detection of *Salmonella* is possible using international standard conventional methods that have been determined by accepted associations, such as the Association of Official Agricultural Chemists (AOAC), the Bacteriological Analytical Manual (BAM) and the International Organization for Standardization (ISO). These methods used selective broth to increase the number of *Salmonella* which provides accurate results. The isolation of *Salmonella* from the faecal sample is quite difficult and takes approximately 4-7 days (Verma et al., 2011). As the isolation of *Salmonella* is tedious and time consuming, use of simple, rapid serological tests like slide micro-agglutination test is convenient method for detecting the prevalence of *Salmonella* infection in animals (Sachan et al., 2013).

Keeping in view the above facts the present study was designed with the following objectives:

1. To investigate the infection of calves with *Salmonella* in organized dairy farms.
2. To investigate the infection of adult diarrhoeic bovines with *Salmonella* in above farms.
3. To study the enteric pathology in calves with salmonellosis.
2. REVIEW OF LITERATURE

Enteric *Salmonella* infection is a global problem both in man and animals, and has been attributed to be the most important bacterial etiology for enteric infections worldwide. The available literature on calf mortality and salmonellosis in cattle with special reference to isolation of *Salmonella* from bovines and pathology of salmonellosis in animals is reviewed. Literature has also been consulted to review the enteric pathology associated with salmonellosis.

2.1 Incidence of calf mortality due to enteric infections

Mortality patterns of two Zebu cattle breeds, Sahiwal and Tharparkar, and two crossbred strains, Karan Swiss and Karan Fries, maintained at the National Dairy Research Institute, Karnal were studied by Prasad *et al.* (2004). Nine-year (1989–90 to 1997–98) data on mortality were analyzed for year, season, age and cause effects on mortality rate. The overall mortality was 14.17 per cent. The mortality in Sahiwal, Tharparkar, Karan Swiss and Karan Fries averaged 14.35 per cent, 7.21 per cent, 17.12 per cent and 13.46 per cent, respectively. The mortality up to 2 months of age accounted for a major share (50–60% or higher) in different breed groups. Digestive problems followed by respiratory disorders together accounted for 70–80 per cent of total deaths.

Sharma *et al.* (2004) conducted study to determine mortality rate in buffalo versus cow calves. The workers reported the overall mortality rate of the cow calves to be 10 per cent, which got reduced to the tune of almost nil after Neem oil treatment. The workers reported the morality rate of the buffalo calves to be 30 per cent. Administration of Neem oil reduced the mortality rate to 4.1 per cent. A higher mortality of the buffalo calves was recorded during winter (41%) than during summer (20%). The mortality of the calves was more pronounced within a week of age.

Islam *et al.* (2005) identified the cause of calf mortality and its consequences on the reduction of milk yield. Calf mortality was found to be highest in monsoon (36.4%) followed by winter (34.6%) and summer (29.0%). Mortality was higher in male calves (55.6%) than female (44.4%). Calf
mortality rate was decreased with the increase in age of the calves and it was found highest in first month of life (35.2%).

Khan et al. (2007) collected data regarding buffalo calves rearing and calf mortality from 111 buffalo farms. The mortality rate for calves in these farms averaged 17.98 per cent. Colostrum feeding to newly born calves was practiced in all farms. In addition to suckling, solid feeding to calves was started during the second week of age. The weaning age ranged from 24 to 48 weeks. Calf mortality was influenced (P < 0.05) by total number of animals but did not vary due to number of buffaloes, number of cows, total calves, age and season.

Tiwari et al. (2007) conducted study on ninety commercial dairy owners in the Bareilly district of Uttar Pradesh by selecting 30 livestock owners each from small, medium and large size dairy farms and their dairy calf management practices were studied in detail. The workers observed that the calf health care practices in the commercial dairy farms are very poor. The poor care of calves in the commercial dairies was revealed by the fact that the mortality rate in buffalo calves in these dairies was 81.09 percent. This was mainly due to the poor management of calves which are not even given the minimum care of naval cord disinfection, timely colostrums feeding, deworming, appropriate space, proper milk feeding and timely treatment.

A survey was conducted in 25 organized dairy farms of Jabalpur, Madhya Pradesh by Jain et al. (2008) to explore the cause of heavy mortality of neonatal buffalo calves. The population of crossbred cows in the selected area was only 10 per cent of buffalo population. The surveillance study indicated that certain adversaries do exist in the buffalo colostrum and gastrointestinal tract of buffalo calves which are responsible for poor immune status and higher mortality rate leading to severe depletion of the best milch genotype and to a huge economic loss to Indian farmers.

In an outbreak of hemorrhagic septicemia in buffalo calves in Pakistan, 31.48 per cent mortality was recorded by Khan et al. (2011). Mortality significantly peaked on 8th day (37%). Elevated body temperature
(40.63+0.70°C), profuse salivation, dyspnoea, respiratory sound “rales”,
ocular discharge, mucus nasal discharge and restlessness were observed.

Naag, D. (2012) recorded an incidence of calf mortality at
livestock farm to be 61 per cent for buffalo calf and for cow calf as 40 per
cent.

2.2 Salmonellosis in bovines

Johnston and Jones (1976) isolated a lactose fermenting strain
of *Salmonella typhimurium* from two calves which died during an outbreak of
acute enteritis. The organism was biochemically typical in all other respects.
In one calf, uncomplicated by treatment before death, the autopsy findings
were those of a severe fibrinous enteritis which was reproduced in another
calf dosed orally with culture.

Petrie et al. (1977) described the clinical and epidemiological
features of an outbreak of salmonellosis due to *Salmonella enteritidis* in a
group of calves. The major clinical signs were dullness, pyrexia and
diarrhoea. Five of the 15 calves died but deaths were mainly confined to the
younger members of the group. The recovery of *Salmonella* organisms from
rectal swabs was maximal shortly before four of the five deaths occurred and
dropped rapidly thereafter. Only two of the surviving 10 calves developed
significant flagellar agglutination titres.

Wray and Sojka (1978) described clinical, bacteriological and
pathological findings in experimental *Salmonella typhimurium* infection in
calves. Jersey calves appeared more susceptible to infection than Friesian
calves. The clinical signs in most calves were pyrexia and characteristic
diarrhoea that lasted for up to 11 days; more severe symptoms were seen in
the calves that received the higher doses. Following infection, all calves
excreted *S. typhimurium* in their faeces, the highest counts being observed in
the calves that died. Necrotic enteritis in the ileum and large intestine was the
most striking lesion; lesions were uncommon in other organs.

Smith et al. (1979) introduced a highly virulent strain of
*Salmonella typhimurium* orally to produce disease experimentally in 21, 3 to 9
weeks old normal colostrum-fed calves. The disease was characterized by
fever, depressed attitude, and decreased appetite. Many calves given larger challenge dose levels also had diarrheic faeces containing mucus, fibrin, and blood. Fecal cultures were positive for *Salmonella*. Septicemia occurred in some calves (9 of 15 calves cultured were positive). Eleven calves died and 10 calves survived challenge exposure. Survival was inversely related to the size of the challenge inoculum and directly related (although to a lesser degree) to age of the calf.

Van Zijderveld *et al.* (1982) studied epidemiological features of salmonellosis in veal calves caused by *S. dublin* and *S. typhimurium*. To trace the *Salmonella* organisms to their origin, faecal samples were collected from one week old calves in markets, in lorries and within 24 hours after their arrival on veal-calf units, and subsequently examined for the presence of *Salmonella*. *Salmonella* (47.5 per cent of *S. typhimurium*, 20 per cent of *S. dublin* and 32.5 per cent of other serotypes) was isolated from 3.5 per cent of the individual faecal samples of 1,143 calves on seven veal-calf farms.

Hinton *et al.* (1983) obtained swabs of rectal faeces daily for 28 days from 90 calves reared in five batches during the summer of 1983. The calves were purchased unweaned in markets and fed a milk-substitute diet. *Salmonella typhimurium* phage type DT204c was isolated from calves in four batches and *Salmonella newport* from one *Salmonella* was isolated from 55 (61%) calves; 30 were positive on up to four occasions while 21 and 4 animals respectively were positive between 5 and 11 and 15 and 20 times. In the majority of animals infection was probably subclinical since treatment with antibacterial drugs and excretion of *S. typhimurium* coincided in four calves only.

The fluorescent antibody test (FAT) was used by Wray and Callow (1989) for the direct detection of *Salmonellas* in 182 lymph nodes from adult cattle and from calves submitted for autopsy or used for experimental purposes. *Salmonella* were detected in 22 samples by the FAT and in 18 by culture examination. The predictive value of the FAT was 68% and it was concluded that the test could be used for the rapid presumptive diagnosis of bovine salmonellosis.
Tsolis et al. (1999) conducted studies to determine whether the *Salmonella enterica* serovar Typhimurium SPI-1 effector proteins SspA and SptP are important for enteropathogenicity. Strains lacking these proteins were tested during oral infection of calves. Calves infected with a SptP mutant or its isogenic parent developed diarrhoea and lethal morbidity. In contrast, calves infected with an SspA mutant developed diarrhoea, which resolved within 10 days but did not result in mortality. The SspA mutant was recovered from bovine intestinal tissues at numbers similar to those obtained for its isogenic parent and caused marked intestinal lesions. Thus, the severity of pathological changes caused by serovar Typhimurium strains or their ability to cause diarrhoea were not predictive of their ability to cause lethal morbidity in calves. It was concluded that factors other than or in addition to bacterial colonization, intestinal lesions, or electrolyte loss contribute to lethal morbidity in calves infected with serovar Typhimurium.

Houston et al. (2002) monitored patterns of *Salmonella* faecal shedding in naturally infected dairy herds, to determine the association between faecal shedding and individual animal production measures, and evaluate potential risk factors for shedding of *Salmonella* organisms among cattle in dairy herds. Results suggested that subclinical faecal *Salmonella* shedding can persist in dairy herds for up to 18 months with no measurable effects on health or production of individual cows.

Seleim et al. (2002) collected faecal samples from calves suffering from diarrhoea and isolated *Salmonella* in 17.5 per cent of the cases while in contact apparently healthy calves had 3.4 per cent of *Salmonella*. Four serovars were elucidated namely, *S. typhimurium*, *S. dublin*, *S. enteritidis*, and *S. anatum* from diarrhoeic and apparently healthy calves, respectively.

Fecteau et al. (2003) determined the efficacies of ceftiofur for treatment of experimental salmonellosis in neonatal calves. 36 calves were orally challenged with *Salmonella enteritica* serovar Typhimurium (6.5 x 10⁸ colony-forming units). Six additional calves were retained as non medicated non challenged control calves. Four days following *Salmonella* challenge, surviving calves were randomly allocated to ceftiofur-treated (5 mg/kg, IM, q
24 hr) or non medicated control groups. Ceftiofur treatment was associated with a significant decrease in rectal temperature and diarrhoea. 3 of 15 medicated calves and 4 of 14 non-medicated calves died or were euthanized between days 4 and 18. A significant decrease in faecal shedding of *Salmonella* organisms was observed in treated calves, compared with non-medicated calves. *Salmonella* organisms were isolated from all 10 non-medicated calves at necropsy, whereas no *Salmonella* organisms were isolated from 5 of 12 medicated calves.

McEvoy *et al.* (2003) determined the prevalence, serotype and antibiotic resistance profile of *Salmonella* isolates in cattle and on carcasses at a commercial abattoir. *Salmonella* was isolated from 2 per cent of faecal, 2 per cent of rumen and 6 per cent of carcass samples. *Salmonella* was most frequently isolated from samples taken during the period August to October. *S. dublin* was isolated from 72 per cent of positive samples. *S. agona* and *S. typhimurium* definitive type were each isolated from 14 per cent of positive samples. All *S. typhimurium* isolates were resistant to ampicillin, chloramphenicol, streptomycin, sulphafurazole and tetracycline.

Pender (2003) reported salmonellosis in a herd of beef cows. The worker reported that two post parturient beef cows in a herd of 30 developed acute enteritis, with pyrexia and bloody diarrhoea containing intestinal casts. *Salmonella typhimurium* phage type 66 was isolated from faeces of both animals; both recovered after treatment with tetracycline. A third cow had died without treatment after showing similar signs.

Murugkar *et al.* (2005) reported the isolation along with the serotypes, phage types and antibiogram pattern of *Salmonella* among man, livestock and poultry in the northeastern India. A total of 13 (9.7%) *Salmonella* isolates were recovered from cattle diarrhoeic rectal swabs comprising *S. enteritidis*, *S. typhimurium* and *S. bareilly*.

Berge *et al.* (2006) detected *Salmonella enterica* on > 50 per cent of farms and in 7.5 per cent of 3,686 faecal samples. Many isolates (33%) were resistant to multiple antimicrobials. Shedding of *Salmonella spp.* was negatively associated with increasing calf age, herds being closed to
incoming cattle, and antimicrobial supplementation of milk replacer; prophylactic antimicrobial treatment in day-old calves increased shedding. No association between farm management and presence of multiple-antimicrobial-resistant *S. enterica* or between calving management and presence of *S. enterica* in calves < or 1 week old was detected.

Nagal *et al.* (2006) isolated *Salmonella enterica*, subspecies enterica serovar Berta from three out of five blood samples of 3-6-month-old ailing calves. The isolates were sensitive to ciprofloxacin, enrofloxacin, ofloxacin and moderately sensitive to gentamicin, lomefloxacin, oxytetracyclines, tetracyclines, but refractory to ampicillin and penicillin. Treatment with enrofloxacin resulted in cent per cent recovery. The authors were of the opinion that the infection might have been picked up by calves from poultry birds reared in the same premises.

Bouchrif *et al.* (2009) studied the prevalence and antibiotic-resistance of *Salmonella* isolated from food. In total, 11,516 food samples collected from 2002 to 2005 were investigated. Isolated *Salmonella* were characterized by serotyping and susceptibilities were determined for 15 antimicrobial drugs using the disc diffusion assay. The overall percentage of *Salmonella* prevalence (*n* =105) was 0.91 per cent with rates of 71 per cent for slaughterhouses and 9 per cent for seafood. Sixteen different serotypes were identified among 104 *Salmonella enterica* isolates. Twenty-nine percent of isolates (*n*=30/105) were resistant to at least one antimicrobial. Resistance to tetracycline was the most common finding (21%), followed by resistance to ampicillin (13%), amoxycillin+clavulanic acid (9%), streptomycin (7%), chloramphenicol (4%) and nalidixic acid (3,8%). None of the isolates was resistant to ciprofloxacin.

Singh *et al.* (2009) screened serum samples of 333 cows (aborted 19, healthy breeding cows 194, cows with pyrexia 120) and 200 buffalo (healthy breeding buffaloes 80, slaughtered for meat 120). A total of , 48.3 per cent cows with pyrexia of unknown origin (PUO), 47.4 per cent cows those aborted within last 15 days of serum collection, 6.2%healthy breeding cows, 10.7% breeding buffaloes and 8.3% buffaloes slaughtered for meat were positive for anti *Salmonella* cytotoxin-I immunoglobulins in indirect
enzyme linked immuno-sorbent assay (ELISA). Significantly high prevalence of anti-
*Salmonella* antibodies in cows either with PUO or abortion indicated the important role of the organism in health and production of cattle in northern India.

A study was conducted by Singh *et al.* (2010) to determine the plasmid profile and drug resistance pattern of zoonotic *Salmonella* isolates from Indian buffaloes. The workers observed that multiple drug resistance was common among *Salmonella* isolates of buffalo origin, particularly against aminoglycosides, oxytetracyclin, ampicillin and cephalexin. Presence of plasmids is not mandatory for occurrence of multiple drug resistance in *S. enterica* strains.

Izzo *et al.* (2011) conducted studies to identify the common serotypes and antimicrobial resistance patterns of *Salmonella* spp. associated with diarrhoea in Australian dairy calves under the age of 6 weeks. *Salmonella* serovar Dublin, *S. serovar* Typhimurium and *S. serovor Bovismorbificans* were the three most common *Salmonella* serotypes isolated.

Jadidi *et al.* (2012) collected 1124 fecal samples and conducted biochemical and molecular assays to detect and diagnose *Salmonella* spp. in cattle. *Salmonella* bacteria were identified with biochemical test. The antimicrobial susceptibility test with disc diffusion method was performed on samples of *Salmonella* by using a molecular based approach, and it was possible to identify *Salmonella* sp by amplifying specific genes "16s rRNA" as a step for identification. The study showed that the molecular-based approaches are more rapid for initial detection of *Salmonella* spp.

Abdullah *et al.* (2013) examined a total of 114 faecal samples for the isolation of bacteria, of which 44 (38.6%) samples were positive for *E. coli*, 25 (21.9%) samples were positive for *Salmonella* spp. 15 (13.2%) samples were positive for *Staphylococcus*, 18(15.8%) samples accounted for mixed infection and 12 (10.5%) samples were negative for bacteria. The antibiogram study revealed that most of the *E. coli, Salmonella* spp. and *Staphylococcus* spp. were resistant to penicillin, ampicillin, amoxycillin and bacitracin.
However, most of the *E. coli, Salmonella spp.* and *Staphylococcus spp.* were susceptible to azithromycin, gentamicin and ciprofloxacin.

Sachan *et al.* (2013) collected a total of 1500 serum samples from cattle, buffalo, goats and dogs from five districts of Uttar Pradesh, India and screened them for presence of *Salmonella* agglutinins employing slide microagglutination tests using two coloured antigens of *S. typhimurium* and *S. enteritidis*. Species-wise distribution of positive agglutinins was highest in dogs (25.2%), followed by cattle (11%) and buffalo (9.2%).

Sychanh *et al.* (2013) examined total, 269 animals (225 buffaloes and 44 cattle) for the presence of *Salmonella* in mesenteric lymph nodes, caecum, abdominal and diaphragmatic muscles. The proportion of slaughtered buffaloes and cattle identified as positive for *Salmonella* was 6.69 per cent, with 7.11 per cent in buffaloes and 4.54 per cent in cattle.

Ansari *et al.* (2014) carried out studies to isolate, identify and detect the antimicrobial resistant profile of *E. coli* and *Salmonella* from diarrhoeic calves. Of the samples collected 35 (25%) and 11 (8.8%) was found positive for *E. coli* and *Salmonella*, respectively. Antimicrobial resistance of these two isolates was found against amoxycillin and tetracycline whereas a high sensitivity was found towards ciprofloxacin, levofloxacin, azithromycin and cefotaxime.

### 2.3 Pathology of Salmonellosis

Malasaki *et al.* (1987) experimentally infected calves, aged 20 days with *Salmonella dublin*. After sacrificing the animals *Salmonella dublin* organisms were found in all parenchymal organs and in the lymph nodes. Morphologically, the prevalence was established of inflammatory necrobiotic processes in the liver, with activation and proliferation of the sinus endothelium of the lymph nodes, and catarrhal inflammation of varying manifestation in the small intestine.

Zhang *et al.* (2003) observed that in *S. enterica* infection the resulting acute inflammatory response is associated with an increase in vascular permeability resulting in mucosal edema. Furthermore, the influx of neutrophils is associated with necrosis of the upper most ileal mucosa. The
injury to the intestinal epithelium leads to leakage of extravascular fluids and massive transmigration of neutrophils into the intestinal lumen, a process normally prevented by the epithelial permeability barrier.

Frizzo et al. (2012) studied the pathogen translocation and histopathological lesions in an experimental model of *Salmonella dublin* infection in calves receiving lactic acid bacteria and lactose supplements. The oral entry of *Salmonella*, proliferation of this microorganism in the small intestine, and its rapid penetration into the lamina propria cause edema, macrophage and lymphocyte proliferation, and polymorphonuclear (PMN) recruitment. This is accompanied by expansion of the central lacteals, and generates a sharp decline in apical enterocytes while provoking a proliferative reaction in the bottom of the crypts (enteritis regenerative). The bacteria also invade regional lymph nodes, leading to macrophage and PMN recruitment. The same happens in submucosa veins, producing phlebitis and thromboembolism. Circulatory disorders lead to irreversible damage of the villi with apical necrosis, hemorrhage, and fibrin exudation (fibrinous necrotic enteritis). Bacteria spread through blood and the lymphatic system cause septicemia with involvement of the mesenteric lymph nodes, liver, and spleen.

Snider et al. (2014) orally inoculated ten-week-old calves with increasing doses of either *Salmonella enteric* serovar Typhimurium or Newport. Clinical illness scores were assigned based on rectal temperature, fecal consistency, attitude and hydration. Gross and microscopic pathology findings were also evaluated. These older calves exhibited clinical and pathologic signs of severe gastroenteritis without death losses with effective dose of $1 \times 10^8$ CFUs for *S. typhimurium* and $1 \times 10^7$ CFUs for *S. newport*.
3. MATERIALS AND METHODS

3.1 Location of work

The work was conducted in the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Jabalpur Madhya Pradesh.

3.2 Metrological data and features of place

Jabalpur is situated at 231.7° latitude and 79.57° E longitudes at 410.87 MSL (mean sea level) in the southern part of second agro-climatic zone, including Satpura plateau and Kymore hills. It has tropical climate having average rainfall of 1241mm.

3.3 Study period

The study was conducted for a period of seven months from September to March. (2013-2014).

3.4 Materials

3.4.1 Animals

Cow and buffalo calves as well as adult animals, of either sex or different breeds, in and around Jabalpur were included in the study.

3.4.2 Equipments

- Vertical Laminar Flow : Make: Vaiometra
- Bacterial Incubator: Make SE 105 Scientech
- Deep Freezer (-24°C) : Make Elanpro
- Autoclave : Make Scientech
- Mini Centrifuge machine: Make Spinwin India. MC- 01
- Ultra pure water assembly:Make: Milipore

3.4.3 Media

Ready to use Nutrient agar plate of Hi-media (MP001), Xylose-lysine deoxycholate agar plate of Hi-media (MP031) and Mueller Hinton agar plates of Hi-media (MP1084) were used for culture of organisms and antibiogram test.

3.4.3.1 Buffer peptone water (Pre-Enrichment media)

To prepare the buffer, peptone water (HiMedia, Code no. M920) was procured and the powder was suspended in sterilized distilled water.
3.4.3.2 Tertrathionate broth (Enrichment media)

Tetrathionate broth containing brilliant green in the final concentration of 1:1,00,000 was used as a selective enrichment broth for primary isolation of *Salmonella*.

3.4.3.3 Brilliant green agar

Brilliant green agar (BGA) was used as a selective medium for primary isolation. BGA was prepared by suspending 59.09 grams of powdered media in 1000 ml distilled water. The suspension was heated till boiling to dissolve the powder completely. Sterilization was done by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3.4.3.4 Brain heart infusion broth

Brain heart infusion broth (HiMedia) was prepared as an enriched medium by dissolving 37 grams of Brain heart infusion broth in 1000 ml distilled water. The solution was dispensed in to tubes and sterilized by autoclaving at 15 lbs/sq inch pressure, 121°C for 15 minutes. Ready to use Brain heart infusion broth of Himedia (LQ077v) were also used.

3.4.3.5 Gelatin agar

To prepare the media gelatin agar (HiMedia, Code no. M920) was procured and the powder was suspended in sterilized distilled water. Sterilization was done by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3.4.4 Biochemical kits

Hi *Salmonella* Identification kit (KB001), Hi motility Biochemical kit for *Salmonella* (KBM002) and Hi-carbohydrate Kit(KB009) were used for biochemical characterization of the isolates.

3.4.4.1 Indole Test

Indole nitrate broth LQ 081 was used for performing the test.

3.4.4.2 Hydrogen Sulphide production on triple sugar Iron agar

The triple sugar Iron agar slants were prepared using the powder supplied by Hi-Media.
3.5 Methodology

3.5.1 Collection of samples

3.5.1.1 Blood

Blood (5ml) for serum was collected aseptically from the external jugular vein in sterile container for separation of serum.

3.5.1.2 Faecal swab

Faecal contents/swabs collected per rectal using sterilized gloves and samples were placed in polythene bags which were labeled and sealed properly for identification of calves.

For bacteriological examination samples were collected from total 82 calves (16 diarrhoeic cow calves, 22 diarrhoeic buffalo calves, 16 non diarrhoeic cow calves, 28 non diarrhoeic buffalo calves) and 50 adult animals (05 diarrhoeic adult buffalo, 05 diarrhoeic adult cattle, 20 non diarrhoeic adult buffalo and 20 non diarrhoeic adult cattle). The monthly collection of faecal samples is as shown in table 01.

Diarrhoea was defined as an abnormally loose consistency of faeces, and was noted along with the clinical signs present in the calves (anorexia, depression, weakness), and observations including the colour and consistency of faeces were also noted.

Table 01: Monthly collection of faecal swabs from bovines

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<th>Months</th>
<th>Cow calves</th>
<th></th>
<th>Buffalo calves</th>
<th></th>
<th>Adult cattle</th>
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<td>20</td>
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</table>

D- Diarrhoeic, ND- Non-diarrhoeic
3.5.1.3 Intestinal contents

Swabs /contents from different parts of the intestine were also collected from 41 postmortem cases having gross lesions of enteritis.

3.5.1.4 Tissue

Dead calves at Livestock Farm Adhartal, College of Veterinary Science & A. H. Jabalpur and those received for necropsy examination from organised dairies in and around Jabalpur were subjected to detailed post mortem examination. Gross pathological changes were noted in all major viscera mainly liver, mesenteric lymph nodes and different part of intestine (duodenum, jejunum, ileum, caecum, colon and rectum). If gross findings were indicative of enteritis then the affected tissue samples were collected from liver, mesenteric lymph nodes and different part of the intestine in 10% buffered formalin for histopathological examination following the method of Gridley (1960). Intestinal content were collected aseptically from duodenum, ileum and jejunum for bacteriological study.

3.6 Processing of samples

3.6.1 Microbiological examination

3.6.1.1 Examination of Direct Smear

The direct smears were prepared on microslides from the 173 samples and stained with Gram’s staining for the identification of bacteria.

3.6.1.2 Bacterial Culture

The faecal swabs were suspended in 10% phosphate buffer 37°C for 24 hr. The supernatant was taken and poured in the tetrathionate broth (Enrichment media) and incubated at 41°C for 24 hour. Thereafter, the supernatant was taken and streaked on both xylose lysine deoxycholate (XLD) agar and brilliant green agar (selective media) and incubated at 37°C for 24 hours. Preliminary identification of suspected Salmonella cultures was made on the basis of morphology. The characteristic colony presumptively considered to be Salmonella were picked up and re-streaked on Nutrient agar and incubated at 37°C for 24 hours. The colonies appearing on Nutrient agar after 24 hours were picked with inoculation loop and plunged in brain heart infusion broth as shown in flow chart.
3.7 Growth on XLD plates

A typical *Salmonella* colony had a slightly transparent zone of reddish colour and a black centre, a pink-red zone was seen in the media surrounding the colonies.

3.8 Growth on BGA Agar plates

Typical *Salmonella* colonies on a BGA agar plate caused the colour of the medium to be red/pink (phenol red was the indicator). The colonies were grey-reddish/pink and slightly convex.

3.8.1 Examination of smear

Smear on microslide were prepared from the isolated colonies on nutrient agar and stained by Gram’s method of staining.
3.9  Biochemical Identification kit

3.9.1  KB001 HI *Salmonella* Identification Kit

3.9.1.1  Principle

Each kit is a standardized colorimetric identification system utilizing seven conventional biochemical tests. The tests are based on the principle of pH change and substrate utilization. On incubation *Salmonella* exhibit metabolic changes which are indicated by spontaneous colour change in the media that can be interpreted visually.

3.9.1.2  Preparation of the inoculum

A single well isolated colony was picked and inoculated in 5 ml brain heart infusion broth and incubated at 35°C-37°C for 4-6 hours until the inoculums turbidity was > 0.10D at 620 nm or 0.5 Mcfarland standard. The density of the suspension should be adjused to 0.1D at 620nm or Mcfarland standard.

3.9.1.3  Inoculation of the kit

1. The kit was opened aseptically by peeling off the sealing tape
2. Each well was inoculated with 50 µl of the above inoculums by surface inoculation method.
3. Alternatively the kit can also be inoculated by stabbing each individual well with a loopful of inoculums.

3.9.1.4  Incubation

The preparation is incubated at 37°C for 18-24 hours.

3.9.1.5  Interpretation of the result

The results were interpreted as per the standard given in the result interpretation chart. Addition of reagents in well 1 and 2 should be done at the end of incubation period that is after 18-24 hours.

3.9.2  Methyl red test  (well no.1)

2-3 drop of methyl red reagent (1007) was added to well no.1.
If the reagent remained red in colour the test was considered positive whereas if the reagent decolorized and became yellow the test was considered negative.

3.9.3 Voges Proskauer’s test (well no 2)

2-3 drops of the baritt reagent A (R029) and 1 drop of Baritt reagent (R030) were added in well no.2.

Pinkish red color development within 5-10 minutes indicated a positive test no change in colour or a slight copper colour (Due to reaction of baritt reagent A and baritt reagent B) indicates a negative reaction.

Table 02: Interpretation Chart for KB001 HI Salmonella Identification Kit.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test</th>
<th>Reagent to be added after incubation</th>
<th>Principle</th>
<th>Original colour of the medium</th>
<th>Positive reaction</th>
<th>Negative reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methyl red</td>
<td>1-2 drops of methyl red reagent</td>
<td>Detect acid production</td>
<td>Colourless</td>
<td>Red</td>
<td>Yellowish – orange</td>
</tr>
<tr>
<td>2</td>
<td>Voges Proskauer’s</td>
<td>1-2 drops of the baritt reagent A and 1-2 drops of the baritt reagent B</td>
<td>Detect acetoin production</td>
<td>Colourless / light yellow</td>
<td>Pinkish red</td>
<td>Colourless / slight copper</td>
</tr>
<tr>
<td>3</td>
<td>Urease</td>
<td>-</td>
<td>Detect urease activity</td>
<td>Orangish yellow</td>
<td>Pink</td>
<td>Orange yellow</td>
</tr>
<tr>
<td>4</td>
<td>H₂S production</td>
<td>-</td>
<td>Detect H₂S production</td>
<td>Orangish yellow</td>
<td>black</td>
<td>Orangish yellow</td>
</tr>
<tr>
<td>5</td>
<td>Citrate utilization</td>
<td>-</td>
<td>Detect capability of organism to utilize citrate as sole carbon source</td>
<td>Green</td>
<td>Blue</td>
<td>Green</td>
</tr>
<tr>
<td>6</td>
<td>Lysine utilization</td>
<td>-</td>
<td>Detect lysine decarboxylation</td>
<td>Olive green to light purple</td>
<td>Purple/dark purple</td>
<td>yellow</td>
</tr>
<tr>
<td>7</td>
<td>ONPG</td>
<td>-</td>
<td>Detect galactosidase activity β</td>
<td>colourless</td>
<td>Yellow</td>
<td>colourless</td>
</tr>
</tbody>
</table>

3.10 KBM002 Hi motility biochemical test kits

3.10.1 Principle

A combination of 12 tests for confirmation of *Salmonella* based on motility and other biochemical tests.
3.10.2 Inoculation of the kit
1. The kit was opened aseptically by peeling off the sealing tape.
2. The first well was inoculated by stabbing. The second well was left uninoculated.
3. The remaining kit wells (well no. 3-12) were inoculated by stabbing each individual well with a loopful of inoculums.

3.10.3 Incubation:
The preparation was incubated at 37°C for 18-24 hours

3.10.4 Interpretation of the result
The results were interpreted as per the standard given in the result interpretation chart.

3.10.5 Motility: well no .1
Motility is seen as movement of pink growth from 1st well to 2nd well.

3.10.6 Arginine utilization:well no .5
Arginine utilization was interpreted as a change of olive green colour to dark purple in positive case and to yellow colour in negative case.

**Table 03: Interpretation Chart for KBM002 Hi motility biochemical test kits**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Test</th>
<th>Principle</th>
<th>Original colour of the medium</th>
<th>Positive reaction</th>
<th>Negative reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Motility</td>
<td>-</td>
<td>Light pink</td>
<td>Dark pink growth</td>
<td>Light pink</td>
</tr>
<tr>
<td>2</td>
<td>Motility</td>
<td>Detect motility</td>
<td>Light pink</td>
<td>Movement of dark pink growth from 1st well to 2nd well</td>
<td>Light pink</td>
</tr>
<tr>
<td>3</td>
<td>Arginine Utilization</td>
<td>Detects Arginine decarboxylation</td>
<td>Olive green to light purple</td>
<td>Purple /Dark Purple</td>
<td>Yellow</td>
</tr>
</tbody>
</table>
3.11 KB009 Hi carbohydrate kits

3.11.1 Principle:

Each Hi carbohydrate kits is a standardized colorimetric identification system utilizing thirty five carbohydrate utilization tests. The test are based on the principle of pH change and substrate utilization. On inoculation organisms undergo metabolic changes which are indicated by spontaneous colour change in the media. Fermentation reactions of eight sugars viz. glucose, mannitol, maltose, sorbitol, adonitol, sucrose, salicin, and lactose were studied.

3.11.2 Inoculation of the Kit

The kit was opened aseptically by peeling off the sealing foil. Each well was inoculated with 50 µl of the inoculums prepared in brain heart infusion broth and incubated at 37°C for 24 hours. In positive cases the original pinkish red colour changed to yellow.

3.12 Indole test

The culture in nutrient agar was added to Indole nitrate broth and incubated for 24 hours at 37°C. To the turbid broth 0.2ml of Kovac’s reagent. (Paradimethyl amino benzaldehyde) was added. Development of deep red color at the surface of the reagent and ether indicated a positive result.

3.13 Gelatin liquefaction test

Gelatin butt was stabbed and incubated at 37°C for 48 hrs and transferred to refrigerator till the medium of the control tube solidified. If the liquefied gelatin did not solidify on refrigeration a positive result was indicated.

3.14 Confirmation of *Salmonella* spp. by Latex agglutination test

Colonies grown in selective agar plates were tested with latex slide agglutination test for the confirmatory identification of presumptive *Salmonella* colonies.

3.14.1 Principle of the test

Latex particles are coated with polyvalent antisera raised against a wide range of *Salmonella* antigen. When mixed with a suspension of
Salmonella organism, the latex particles rapidly agglutinate to form visible clumps. HiSalmonella Latex Test Kit (LK02) detects >99% of motile Salmonella species and early investigations have indicated that specific non motile species may also be detected. The latex agglutination test used is designed to identify S. typhimurium, S. enteretidis, S. typhi, and S. cholerasuis.

3.14.2 Reagents and Materials Provided

I. Salmonella Latex reagent: 2.5 ml Latex particles coated with rabbit antiserum against Salmonella antigens. Preserved with 0.099% sodium azide.

II. Positive Control: 0.5ml Inactivated preparation of Salmonella antigens preserved with 0.099% sodium azide. Sample Diluent 0.85% Isotonic Saline: 5.0ml. Preserved with 0.099% sodium azide.

III. Disposable agglutination cards: 25 cards, each with 6 black agglutination areas.

IV. Mixing sticks: 50 disposable mixing sticks.

3.14.3 Test Procedure

1. One drop sample diluents were dispensed into the circle of an agglutination slide.

2. Using an inoculating loop, a colony from the selective agar plate was removed and emulsified with the drop of sample. Suspensions were made only from colonies with morphologies resembling Salmonella spp.

3. The slide was gently rocked for up to 2 minutes and observed for autoagglutination or clumping.

4. If the suspension remained smooth, the reagent test 1 was mixed by gently inverting and one drop was added near to the bacterial suspension.

5. The latex reagent and the bacterial suspension were mixed with a clean mixing stick and the slide was gently shaken two or three times.

6. The preparation was examined for agglutination within a maximum of 2 minutes.
3.14.4 Interpretation

Agglutination within 2 minutes was considered a positive result and indicated the presence of *Salmonella* in the sample. Absence of agglutination indicated that the four species of *Salmonella* were not present in the test culture.

3.15 Serological Examination

Slide micro agglutination test was employed to assess the sero-prevalence of *Salmonella* agglutinins in different animals. Lyophilised culture of *S. typhimurium* (ATCC, 25241) was procured for performing the test. Suspension of the organism was prepared in sterile saline taking care to prepare a smooth milky suspension. The test was performed as per the method described by Muktaruzzaman *et al.* (2010).

About 2-3 drops of milky suspension were placed on a clean grease free glass slide. Then 2-3 drops of test serum were placed adjacent to the antigen. By using a match stick or toothpick, antigens and sera were mixed and observed for 2-3 minutes. Appearance of thick clump (agglutination) was taken as a positive result whereas absence of clump formation was indicative of negative test.

3.16 Antibiotic sensitivity test

The bacterial isolates were subjected to *in vitro* antibiotic sensitivity test as per the method of Bauer *et al.* (1966). The antimicrobial agents (concentration in mg) included: ampicillin (Ap, 25), amoxycillin (Am, 10), cephalexin (Cp, 30), chloramphenicol (Ch, 30), chlortetracycline (Ct, 30), ciprofloxacin (Cf, 30), doxycycline (Do, 10), enrofloxacin (Ex, 10), gentamicin (Gm, 30), kanamycin (Km, 10), nalidixic acid (Na, 30), nitrofurantoin (Nf, 100), norfloxacin (Nx, 10), tetracycline (Tc, 30), and trimethoprim (Tr, 30) (Hi- Media Laboratories, Mumbai, India).

Isolates were grown in Brain Heart Infusion (BHI) broth for 6-8 hrs until there was a moderate turbidity. Plates of Mueller Hinton (MH) agar No.2 were seeded with about one ml of inoculums. The inoculum were
allowed to dry. Antibiotic discs were placed on inoculated agar surface at about two cm apart. The plates were incubated at 37°C overnight and diameter of the zones of inhibition were measured. The measurements were compared with zone size interpretative chart furnished by the manufacturer and the zones were graded as sensitive and resistant.

3.17 Pathological Examination

The processed tissue samples of ten postmortem cases with history of diarrhoea, gross lesions of enteritis and found positive for *Salmonella spp.* were studied to determine the histopathological changes in intestine, mesenteric lymph nodes and liver using routinely stained sections. Sections from different parts of Intestine were observed in all ten cases and histological lesions were noted.

3.18 Special staining techniques

3.18.1 Demonstration of connective tissue

Special staining (Masson's trichrome) was done for the demonstration of collagen fibre (Gridley, 1960).

Masson's trichrome stain

The tissue was mordent by keeping them in Bouin's fluid for overnight their after the section were cooled and washed in running water until yellow color disappeared. The sections were stained in Weigert's hematoxylin solution for 10 minutes and in Biebrich scarlet- acid fushin solution for 15 minutes. Thereafter they were kept in phosphomolybdic acid – phosphotungstic acid solution for 10 to 15 minutes. Counterstaining was done by aniline blue solution for 5 – 10 minutes.

3.18.2 Demonstration of bacteria

For demonstration of organisms, tissue sections were also stained with Mac Callum Good Pasture staining method described by Gridley (1960).
Mac Callum Good Pasture staining

The tissue sections were placed in Good Pasture stain for 10 min. After differentiating in the full strength formalin for few minutes counterstaining was done using saturated aqueous picric acid. The sections were then stained in Sterling’s Gentian Violet solution and Gram’s Iodine.
4. RESULTS

4.1 Herd composition and calf management practices

The samples for present study were collected from animals at five different organized dairy farms in and around Jabalpur. However, the details of herd composition were available only for the organized dairy farm located at College of Veterinary Science & A.H. Jabalpur. At the start of study period in the month of September, 2013, the farm comprised 12 buffalo calves and 2 cow calves. During the study period 25 buffalo calves and 11 cow calves were born as well as 26 cow calves and 27 buffalo calves were purchased. In the seven month duration of study period, 29 buffalo calves and 9 cattle calves died (Table 04).

Table 04: Calf herd composition of Adhartal dairy farm during the period September 2013-March 2014

<table>
<thead>
<tr>
<th>S.No</th>
<th>Animal</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Initial number of buffalo calves</td>
<td>12</td>
</tr>
<tr>
<td>2.</td>
<td>Initial number of cow calves</td>
<td>02</td>
</tr>
<tr>
<td>3.</td>
<td>Buffalo calves born during the period</td>
<td>25</td>
</tr>
<tr>
<td>4.</td>
<td>Cow calves born during the period</td>
<td>11</td>
</tr>
<tr>
<td>5.</td>
<td>Buffalo calves purchased during the period</td>
<td>27</td>
</tr>
<tr>
<td>6.</td>
<td>Cow calves purchased during the period</td>
<td>26</td>
</tr>
<tr>
<td>7.</td>
<td>Buffalo calves died during the period</td>
<td>29</td>
</tr>
<tr>
<td>8.</td>
<td>Cow calves died during the period</td>
<td>09</td>
</tr>
</tbody>
</table>

At Livestock Farm Adhartal the weaning is done immediately after birth and the cow and buffalo calves are housed together. The calves are hand fed with colostrums during the initial 0-5 days, @ 1/10\textsuperscript{th} of body weight. Thereafter milk is given 1/10\textsuperscript{th} of body weight, 1/15\textsuperscript{th} of body weight and 1/20\textsuperscript{th} of body weight till 5-30 days, 30-45 days and 45-60 days, respectively. The starter ration is introduced from 15-20 days and is increased gradually. Regular monitoring for parasitic infections is done and whenever required the calves are given piperazine, if below 21 days of age, or albendazole if above 21 days of age for deworming. No vaccination is done till 6 months of age.
The herd composition for the other organised dairy farms could not be assessed. The history provided and available data revealed similar managemental practices followed by the dairy owners. However in two dairy farms colostrums feeding of calves were not followed.

4.2 Calf mortality

The number of calves received for post mortem examination during the study period (September 2013-March 2014) is shown in table 05. Total 45 cases of calf postmortem were received of which 10 were cow calves, and 35 were buffalo calves. More number of female (n= 26) were received for post mortem examination in comparison to male animals (n= 19). Highest numbers of calf postmortem were received in the month of November. Enteric lesions were observed in 41 cases during gross examination.

**Table 05: Calf mortality pattern**

<table>
<thead>
<tr>
<th>Month</th>
<th>Average ambient temperature</th>
<th>Number of post mortem</th>
<th>Cow calf mortality</th>
<th>Buffalo calf mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>September 2013</td>
<td>27.50 ºC</td>
<td>01</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>October 2013</td>
<td>24.75 ºC</td>
<td>01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>November 2013</td>
<td>19.50 ºC</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>December 2013</td>
<td>17.15 ºC</td>
<td>05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>January 2014</td>
<td>16.45 ºC</td>
<td>11</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>February 2014</td>
<td>18.00ºC</td>
<td>08</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>March 2014</td>
<td>22.20ºC</td>
<td>06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>45</strong></td>
<td><strong>06</strong></td>
<td><strong>04</strong></td>
</tr>
</tbody>
</table>

4.3 Incidence of Salmonellosis in calves

To investigate the infection of calves with *Salmonella* in organized dairy farms faecal samples were collected from 82 calves of 05 organized dairy farms in and around Jabalpur.

To determine the presence of *Salmonella spp* in diarrhoeic as well as non diarrhoeic buffalo and cow calves the samples were collected directly from the rectum of each calf by using sterile swabs (Plate 01).
In direct smear examination of rectal swab, after staining with Grams method, Gram negative bacilli were seen in few cases of diarrhoeic buffalo and cow calves (Plate 02).

The micro-organisms were cultured on xylose lysine deoxycholate agar and brilliant green agar medium for isolation.

In xylose lysine deoxycholate agar slightly transparent zone of reddish colour and a black centre with a pink-red zone in the media surrounding the colonies were preliminary considered positive for *Salmonella spp.* (Plate 03).

In brilliant green agar *Salmonella* colony was observed as grey-reddish/pink and slightly convex growth (Plate 04).

For further confirmation smears were prepared from the growth observed in both the media and stained with Grams stain. Short plump Gram negative rods were identified as *Salmonella spp* (Plate 05).

Out of the 82 samples collected from calves, 03 were found positive for *Salmonella* on the basis of bacterial growth in xylose lysine deoxycholate agar and brilliant green agar. Of these 02 samples were of diarrhoeic cow calf and 01 was from diarrhoeic buffalo calf. None of the faecal samples collected from non diarrhoeic animals were found positive for *Salmonella* (Table 06, Figure 01).

**Table 06: Isolation of *Salmonella spp.* from faecal swabs of calves**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total number</th>
<th>Positive</th>
<th>Positive per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhoeic cow calves</td>
<td>16</td>
<td>02</td>
<td>12.50</td>
</tr>
<tr>
<td>Diarrhoeic buffalo calves</td>
<td>22</td>
<td>01</td>
<td>04.50</td>
</tr>
<tr>
<td>Non diarrhoeic cow calves</td>
<td>16</td>
<td>00</td>
<td>00.00</td>
</tr>
<tr>
<td>Non diarrhoeic buffalo calves</td>
<td>28</td>
<td>00</td>
<td>00.00</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>03</td>
<td>03.65</td>
</tr>
</tbody>
</table>
4.4 Incidence of *Salmonella* in adult bovine

To investigate the infection of adult bovine (above 6 months) with *Salmonella* in organized dairy farms, faecal samples were collected from 50 adult animals belonging to 05 organized dairy farms in and around Jabalpur as shown in table 07. The same procedure as outlined for calves was followed for isolation of *Salmonella Spp.*

**Table 07: Isolation of *Salmonella spp.* from faecal swabs of adult bovines**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total number</th>
<th>Positive</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhoeic cow</td>
<td>05</td>
<td>00</td>
<td>0.0</td>
</tr>
<tr>
<td>Diarrhoeic buffalo</td>
<td>05</td>
<td>00</td>
<td>0.0</td>
</tr>
<tr>
<td>Non diarrhoeic cow</td>
<td>20</td>
<td>01</td>
<td>5.0</td>
</tr>
<tr>
<td>Non diarrhoeic buffalo</td>
<td>20</td>
<td>00</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>01</td>
<td>2.0</td>
</tr>
</tbody>
</table>

In direct smears stained with Grams method, Gram negative bacilli were seen in few cases (Plate 06).

Out of the 50 samples collected from adult animals, 01 sample collected from adult non-diarrhoeic cattle was found positive (Figure 02) for *Salmonella* on the basis of bacterial growth in xylose lysine deoxycholate agar and brilliant green agar.

4.5 Isolation of *Salmonella spp.* from bovine post mortem cases:

Intestinal contents from 41 postmortem cases of cattle and buffalo calves were processed as outlined earlier for isolation of *Salmonella sp.* Out of 09 samples collected from cattle calf 04 (44.44%) were positive for *Salmonella spp.* Out of 32 samples collected from buffalo calf 06 (18.75%) were positive for *Salmonella spp.* (Table 08).
Table 08: Isolation of *Salmonella* spp. from the post mortem cases

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total number</th>
<th>Positive</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle calves</td>
<td>09</td>
<td>04</td>
<td>44.44</td>
</tr>
<tr>
<td>Buffalo calves</td>
<td>32</td>
<td>06</td>
<td>18.75</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>10</td>
<td>24.39</td>
</tr>
</tbody>
</table>

4.6 Characterization of bovine *Salmonella* spp.

The biochemical characteristics of the isolates obtained from bovines both adult and calves are summarized in the table 09 (Plates 07 & 08).

Table 09: Biochemical characterization of *Salmonella* spp.

<table>
<thead>
<tr>
<th>S No.</th>
<th>Biochemical reaction</th>
<th>No. of isolates</th>
<th>Per cent of positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive isolates</td>
<td>Negative isolates</td>
</tr>
<tr>
<td>1. IMViC Reaction</td>
<td></td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>a) Indole</td>
<td></td>
<td>5</td>
<td>09</td>
</tr>
<tr>
<td>b) MR</td>
<td></td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>c) VP</td>
<td></td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>d) Citrate Utilization</td>
<td>14</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>2. H2S Production</td>
<td>14</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>3. Urease</td>
<td>06</td>
<td>08</td>
<td>42.85</td>
</tr>
<tr>
<td>4. Lysine Utilization</td>
<td>14</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>5. O.N.P.G</td>
<td>03</td>
<td>11</td>
<td>21.42</td>
</tr>
<tr>
<td>6. TSI</td>
<td>09</td>
<td>05</td>
<td>64.28</td>
</tr>
<tr>
<td>7. Gelatin liquefaction</td>
<td>-</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>8. Carbohydrate fermentation tests</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Lactose</td>
<td>02</td>
<td>12</td>
<td>14.28</td>
</tr>
<tr>
<td>b) Arabinose</td>
<td>07</td>
<td>07</td>
<td>50.00</td>
</tr>
<tr>
<td>c) Maltose</td>
<td>07</td>
<td>07</td>
<td>50.00</td>
</tr>
<tr>
<td>d) Sorbitol</td>
<td>06</td>
<td>08</td>
<td>42.28</td>
</tr>
<tr>
<td>e) Dulcitol</td>
<td>05</td>
<td>09</td>
<td>35.71</td>
</tr>
<tr>
<td>f) Glucose</td>
<td>09</td>
<td>05</td>
<td>64.28</td>
</tr>
<tr>
<td>g) Adonitol</td>
<td>02</td>
<td>12</td>
<td>14.28</td>
</tr>
<tr>
<td>h) Salicin</td>
<td>02</td>
<td>12</td>
<td>14.28</td>
</tr>
<tr>
<td>i) Manitol</td>
<td>02</td>
<td>12</td>
<td>14.28</td>
</tr>
</tbody>
</table>

IMViC: Indole, Methyl red, Voges-Proskauer, Citrate utilization test
ONPG: Ortho-nitrophenyl-b-D-galacto-pyranose
TSI: Triple sugar Iron agar
All the isolates were Indole and VP negative and positive for citrate utilization and H₂S production. Five isolates were found positive for MR whereas six isolates were determined positive for urease test. Positive tests were observed for 14, 03 and 09 isolates of *Salmonella* for Lysine utilization, ONPG and TSI, respectively. All the isolates tested negative for gelatin liquefaction. Sugar fermentation tests revealed that 12 isolates tested negative for fermentation of lactose, adonitol, salicin and mannitol. Nine isolates tested negative for dulcitol fermentation, eight for sorbitol, seven each for arabinose and maltose and five for glucose fermentation.

4.7 **Motility of *Salmonella* isolates**

The 14 isolates of *Salmonella* recovered from calves were tested for motility using a commercially available Kit. The test revealed that 10 isolates (71.42%) were motile whereas 04 were found to be non motile (Plate 09).

4.8 **Confirmation of *Salmonella spp* by Latex Agglutination Test**

The 14 isolates of presumptive colonies of *Salmonella spp.* were further confirmed by using latex agglutination test. The latex agglutination test used in the present study was designed to identify *S. typhimurium*, *S. enteretidis*, *S. typhi* and *S. cholerasuis*. The test has a sensitivity of 99.3 per cent and specificity of 100 per cent for the diagnosis of above four species of *Salmonella*. The results of latex agglutination test identified *S. cholerasuis* in 01 isolate only (Plate 10). The remaining 13 isolates were not found positive for any of the four species.

4.9 **Assay for in vitro antibiotic sensitivity test**

Six isolates obtained from intestinal content and rectal samples of calves having the biochemical characteristics of *Salmonella spp.* were subjected to *in vitro* antibiotic sensitivity test. The isolates were tested against commonly used antibiotics viz. ampicillin (Ap, 25), amoxycillin (Am, 10), cephalaxin (Cp, 30), chloramphenicol (Ch, 30), chlorortracycline (Ct, 30), ciprofloxacin (Cf, 30), doxycycline (Do, 10), enrofloxacin (Ex, 10), gentamicin (Gm, 30), kanamycin (Km, 10), nalidixic acid (Na, 30), nitrofurantoin (Nf, 100), norfloxacin (Nx, 10), tetracycline (Tc, 30), and trimethoprim (Tr, 30) (Hi-Media Laboratories, Mumbai, India). The results are as shown in table 10. (Plate 11)
Table 10: Antibiotic sensitivity pattern of isolates

<table>
<thead>
<tr>
<th>S No.</th>
<th>Antibiotic</th>
<th>No. of isolates sensitive</th>
<th>No. of isolates resistant</th>
<th>Per cent of sensitive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tetracycline (Tc, 30)</td>
<td>5</td>
<td>1</td>
<td>83.30</td>
</tr>
<tr>
<td>2</td>
<td>Ciprofloxacin (CIP, 5mcg)</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Norfloxacin (No, 5mcg)</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Amoxacillin (AMX, 10mcg)</td>
<td>3</td>
<td>3</td>
<td>50.00</td>
</tr>
<tr>
<td>5</td>
<td>Enrofloxacin (EX, 10mcg)</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Chloramphenicol (C, 10mcg)</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>Nitrofurantoin (Nf, 100)</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Gentamicin (GEN, 10mcg)</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>Trimethoprim (Tr, 30)</td>
<td>3</td>
<td>3</td>
<td>50.00</td>
</tr>
<tr>
<td>10</td>
<td>Kanamycin (Km, 10)</td>
<td>3</td>
<td>3</td>
<td>50.00</td>
</tr>
<tr>
<td>11</td>
<td>Nalidixic acid (Na, 30)</td>
<td>2</td>
<td>4</td>
<td>33.30</td>
</tr>
<tr>
<td>12</td>
<td>Chlortetracycline (Ct, 30)</td>
<td>3</td>
<td>3</td>
<td>50.00</td>
</tr>
<tr>
<td>13</td>
<td>Cephalexin (Cp, 30)</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>Ampicillin (Ap, 25)</td>
<td>0</td>
<td>6</td>
<td>0.00</td>
</tr>
<tr>
<td>15</td>
<td>Doxycycline (Do, 10)</td>
<td>3</td>
<td>3</td>
<td>50.00</td>
</tr>
</tbody>
</table>

The assay revealed that all the isolates were highly sensitive (100%) for cephalaxin, ciprofloxacin, norfloxacin, enrofloxacin, chloramphenicol, nitrofurantoin and gentamicin followed by tetracycline (83.3%), amoxyacillin, trimethoprim kanamycin, chlortetracycline and doxycycline (50%). Only 33.30 per cent isolates were found sensitive against nalidixic acid. All the *Salmonella* isolates were found resistant to ampicillin. (Figure 3)

4.10 Serological Examination

Slide micro agglutination test was done to assess the seroprevalence of *Salmonella* agglutinins in different animals. The slide agglutination test was performed in 101 samples. Amongst these 48 were
from diarrhoeic cases (22 cattle calf, 16 buffalo calf, 08 adult cattle and 02 adult buffalo) and 53 were from non-diarrhoeic apparently healthy animals (23 cattle calf, 16 buffalo calf, 07 adult cattle and 07 adult buffalo).

4.10.1 Seroprevalence of *Salmonella* agglutinins in diarrhoeic bovine

The sero-agglutination was observed in 14.5 per cent diarrhoeic cases comprising 02, 03, 01 and 01 cases from cattle calf, buffalo calf, adult cattle and adult buffalo, respectively. Maximal seroprevalence was detected in diarrhoeic adult buffalo (50%) (Table 11).

**Table 11: Seroprevalence of *Salmonella* agglutinins in diarrhoeic bovine**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhoeic cow calves</td>
<td>22</td>
<td>02</td>
<td>20</td>
<td>9.00</td>
</tr>
<tr>
<td>Diarrhoeic adult cattle</td>
<td>08</td>
<td>03</td>
<td>05</td>
<td>37.50</td>
</tr>
<tr>
<td>Diarrhoeic buffalo calves</td>
<td>16</td>
<td>01</td>
<td>15</td>
<td>6.25</td>
</tr>
<tr>
<td>Diarrhoeic adult buffalo</td>
<td>02</td>
<td>01</td>
<td>01</td>
<td>50.00</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>07</td>
<td>41</td>
<td>14.50</td>
</tr>
</tbody>
</table>

**Association between diarrhoea and serum antibody**

Association between clinical finding of diarrhoea and presence of *Salmonella* antibody in serum of animal was determined by using the Chi Square test. The results are as shown in tables 12 & 13. The findings indicated that the occurrence of diarrhoea and *Salmonella* antibody in serum are not associated with each other.

**Table 12: Association between diarrhoea and presence of antibody in calf serum**

<table>
<thead>
<tr>
<th>Diarrhoeic sample</th>
<th>Present</th>
<th>Absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agglutination test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>03</td>
<td>09</td>
<td>12</td>
</tr>
<tr>
<td>Negative</td>
<td>35</td>
<td>30</td>
<td>65</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>39</td>
<td>77</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 0.00805 \text{; Insignificant at } P < 0.05 \]
Table 13: Association between diarrhoea and presence of antibody in adult bovine serum

<table>
<thead>
<tr>
<th>Agglutination test</th>
<th>Present</th>
<th>Absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>04</td>
<td>03</td>
<td>07</td>
</tr>
<tr>
<td>Negative</td>
<td>06</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>14</td>
<td>24</td>
</tr>
</tbody>
</table>

\( \chi^2 = 0.05186 \); Non significant at 5 \% level (P < 0.05)

4.10.2 Seroprevalence of *Salmonella* agglutinins in non diarrhoeic bovine

Agglutination was observed in 22.6 per cent non diarrhoeic apparently healthy animals comprising 06, 02, 03 and 01 cases from cattle calf, buffalo calf, adult cattle and adult buffalo, respectively. Maximal seroprevalence was detected in non diarrhoeic adult cattle (28.5\%) (Table 14).

Table 14: Seroprevalence of *Salmonella* agglutinins in non diarrhoeic bovine

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non diarrhoeic cow calves</td>
<td>23</td>
<td>06</td>
<td>17</td>
<td>26.00</td>
</tr>
<tr>
<td>Non diarrhoeic adult cattle</td>
<td>07</td>
<td>02</td>
<td>05</td>
<td>28.50</td>
</tr>
<tr>
<td>Non diarrhoeic buffalo calves</td>
<td>16</td>
<td>03</td>
<td>13</td>
<td>18.70</td>
</tr>
<tr>
<td>Non diarrhoeic adult buffalo</td>
<td>07</td>
<td>01</td>
<td>06</td>
<td>14.20</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>12</td>
<td>41</td>
<td>22.60</td>
</tr>
</tbody>
</table>
4.11 Enteric Pathology in Calves with Salmonellosis

During the study period gross and histopathological studies were done in total 10 cases from which *Salmonella spp.* was isolated. These included 04 cattle calf and 06 buffalo calf. (Plate 12) All the animals were below one month of age and belonged to two organized farms and had history of diarrhoea and anorexia of 2-3 days duration before death. The animals had more or less similar gross and microscopic pathology.

4.11.1 Gross Pathology

Body condition was poor with rough skin coat. Carcass was septicaemic (Plate 13) with dilated anus and cuddled milk in the abomasums (Plate 14). Mucous membranes were congested. The entire gastrointestinal tract was heavily congested and intestinal lumen contained bloody exudates with excessive mucous (Plate15). In maximum cases small intestine had blood mixed watery contents and mild erosions were present on mucosa. In few cases the caecum, colon and rectum were haemorrhagic and mesentery was congested (Plate 16). Enlarged mesenteric lymph nodes, necrotic foci on liver and distended gall bladder were consistently observed (Plate 17). In one case there was haemorrhagic splenitis. No gross changes were observed in the kidneys in all ten cases.

4.11.2 Histopathology

Microscopic examination of duodenum revealed catarrhal enteritis with infiltration of polymorhonuclear cells in the lamina propria (Plate 18). The submucosa had congested vessels and in one case there were haemorrhagic foci.

Ileum showed consistent mucosal thickening with increased exudates and necrosis of villi tip in few cases (Plate 19). The lumen of ileum contained necrotic debris with degenerated neutrophils. (Plate 20). The submucosa had increased lymphoid tissue (Plate 21).

Jejunum showed degenerated villi, oedema of lamina propria and microhaemorrhages.( Plate 22)
Caecum and colon revealed villi atrophy with proliferative reaction in the crypts (Plate 23). The lamina propria was infiltrated with mixed population of inflammatory cells. In three cases areas of haemorrhages were also observed with erosions in the epithelial layer (Plate 24).

Mesenteric lymph nodes showed acute hyperplastic lymphadenitis with cortical follicular hyperplasia and increased macrophages and neutrophils (Plate 25).

Microscopic section of liver revealed degenerative changes with vacuolations and foci of hepatitis (Plate 26).

The intestinal sections were also stained with Massons trichrome blue to demonstrate the connective tissue. Increased fibrosis were observed in the ileum and caecum (Plate 27). Staining with Goodpasture stain for demonstration of bacteria but no Gram negative organism was found.
5. DISCUSSION

Salmonellosis has been studied very thoroughly by many workers but many details of its pathogenesis are still lacking and many researchers all over the world are engaged in solving this mystery. Wray (1995) rightly opined that Salmonellosis is a hundred years old and is still going strong. In 2003, approximately 2600 serotypes of Salmonella bacteria have been named and characterized, and the number is constantly growing thanks to new molecular techniques that facilitate differentiation between the different types of Salmonella bacteria. The two most frequently found Salmonella serotypes in cattle are Salmonella Dublin and Salmonella Typhimurium. (Rall et al., 2005) The first written reports about salmonellosis in cattle were probably from year 1865 when “calf paratyphoid” outbreaks were described in Germany, the Netherlands and Denmark. Exactly which bacteria and which serotype caused these outbreaks is unknown (Borriello et al., 2012).

Salmonellosis is an important endemic disease of calves. Enteric Salmonella infection is a global problem both in man and animals, and has been attributed to be the most important bacterial etiology for enteric infections worldwide. Borriello et al. (2012) documented the increase incidence of salmonellosis, associated with the development of intensive rearing system, especially that caused by Salmonella typhimurium in calves. The present work was undertaken with the primary objective of determining the infection of calves and adult bovines with Salmonella in organized dairy farms around Jabalpur and also to study the enteric pathology in Salmonellosis.

5.1 Isolation & Identification of Salmonella spp in bovine faecal samples

Salmonella has been widely reported in cattle and infected animals may shed the organism in their faeces without showing any clinical signs of disease (McEvoy et al., 2003). Keeping this in mind in the present study to investigate the infection of calves with Salmonella in organized dairy farms faecal samples were randomly collected from 82 calves of 05 organized...
dairy farms. Direct smears prepared from the faecal swabs and stained by Grams staining method revealed the mixed population of bacteria along with typical short plump Gram negative rods. Todar (2005) have described *Salmonella* as Gram-negative, non-spore forming, rod shaped, facultative intracellular anaerobic bacterium in the family Enterobacteriaceae trivially known as enteric bacteria.

In our study both pre enrichment media (buffer peptone water) and enrichment media (tetrathionate broth) were used to isolate *Salmonella*. The faecal swabs were suspended in buffer peptone water at 37°C for 24 hr. Pre-enrichment may not be the best method for isolating less vigorous *Salmonella* strains, such as the host-adapted strains, from faeces because of overgrowth by competing organisms during non-selective pre-enrichment (D’Aoust and Purvis., 1998). Mallinson *et al.* (2000) opined that the number of *Salmonellae* in faeces from asymptomatic animals, environmental samples, animal feed and food is usually low, and it is usually necessary to use pre-enrichment media, such as buffered peptone water or universal pre-enrichment broth, to assist isolation. This may allow the small numbers of *Salmonellae*, which may otherwise be killed by the toxic effect of selective enrichment media, to multiply, or it may help to resuscitate *Salmonellae* that have been sub-lethally damaged, e.g. by freezing, heating, and exposure to biocides, organic acids, bacteriocins, phage or desiccation.

In our study the supernatant was taken from the pre enrichment media and poured in the tetrathionate broth (enrichment media) and incubated at 41°C for 24 hour. Earlier, Robertson *et al.*, (1983) also used tetrathionate broth as enrichment media for qualitative and quantitative demonstration of *Salmonella* in faecal samples of calves. International Organization for standardization (2002) have stressed the need to use selective enrichment media for growth of *Salmonella spp*. Enrichment media are liquid or semi-solid agar media that contain additives that selectively permit *Salmonellae* to grow while inhibiting the growth of other bacteria. There are numerous methods for isolation of *Salmonella* in use world-wide.

(Fricker, 1987; Gerhardt, 1981; Reissbrodt, 1995). Examples of selective enrichment media are sodium tetrathionate, as in Müller–Kaufman
broth, selenite F, selenite cysteine, brilliant green broth and Rappaport–Vassiliadis broths, or semi-solid Rappaport–Vassiliadis medium. The literatures indicate that selenite F broth (SFB) is frequently used as an enrichment medium for the isolation of *Salmonella*. Harvey and Price (1975) found that it was statistically more efficient than brilliant green selenite broth (BGSB), Muller-Kauffmann tetrathionate broth (TB) and Rappaport's magnesium chloride malachite green broth (RB).

Some, enrichment media however, are also relatively toxic to certain serovars of *Salmonella*, e.g. selenite inhibits *S. choleraesuis*, and brilliant green is toxic to many strains of *S. dublin*.

Elevated temperatures have also been used to increase the selectivity of enrichment medium, and a temperature of 43°C is used in some laboratories, although this may be inhibitory with some media, e.g. tetrathionate and Rappaport–Vassiliadis at 43°C inhibit temperature-sensitive strains, especially *S. dublin* and 41.5°C is now recommended for incubation of Rappaport–Vassiliadis broth-based media (Voogt et al., 2001). Following these guidelines in the present study also the incubation in enrichment media was done at 41°C.

As per recommendation of OIE (2010) in some cases it may be advantageous to use more than one selective broth or to culture by both pre-enrichment and direct selective enrichment/direct plating, although often the benefit does not justify the extra cost (OIE,2010). Keeping this in view for better isolation in the present study both pre-enrichment and enrichment media were used, we also agree with the observations of Mallision et al. (2000) that the culture techniques and media that may work best in a particular diagnostic situation depend on a variety of factors, including the *Salmonella* serovar, source and type of specimens, animal species of origin, experience of the microbiologist, and availability of selective enrichment and selective plating media.

In present study for selective isolation of *Salmonella spp.* streaking was performed on both xylose lysine deoxycholate (XLD) agar and brilliant green agar(selective media) and incubated at 37°C for 24 hours. The
use of BGA and XLD as conventional media was based on the AOAC(1995) and BAM (2005) recommendations. Shamy et al. (2008) evaluated the sensitivity and specificity of XLD and BGA as (100%, 93.71%) and (0 %, 100%), respectively.

Xylose-lysine-desoxycholate (XLD) agar is the most commonly used highly selective medium for the recovery of enteric pathogens from faecal specimens (Chang et al, 1999; Nye et al., 2002). It contains yeast extract as a source of nutrients and vitamins. It utilizes sodium desoxycholate as the selective agent and, therefore, is inhibitory to Gram-positive micro-organisms. Lysine is included to enable the Salmonella group to be differentiated from the non pathogens since without lysine, Salmonellae rapidly would ferment the xylose and be indistinguishable from nonpathogenic species. Falaco et al. (1979) showed that Xylose Lysine Agar was inferior to SS Agar and Brilliant Green Agar, slightly superior to EMB Agar, and superior to Bismuth Sulfite Agar for the isolation of Salmonella. Its great advantage is that it permits easy identification of the Salmonella colonies.

It is now well established that a single medium is only rarely able to recover all pathogens contained in a specimen. Therefore other media for the isolation of Salmonella and possibly for other enteric pathogens must be inoculated with the specimen (Bopp et al., 2003).

In our study for isolation of Salmonella from faecal samples simultaneous streaking was also done on Brilliant green agar. Kristensen et al. (1925) first described the use of Brilliant Green Agar as a primary plating medium for the isolation of Salmonella. The report described the medium as useful for the differentiation of “paratyphoid B” from other intestinal gram-negative bacilli. It was later modified and Brilliant Green Agar was used in addition to tetrathionate broth for the isolation of Salmonella from stool specimens. BGA is now recommended by many workers for use in testing clinical specimens (Isenburg, 1992). The outstanding selectivity of this medium permits use of moderately heavy inoculam, which should be evenly distributed over the surface. Many workers have also used Brilliant green agar for isolation of Salmonella spp. from faecal samples of animals (Moringo et al., 1989, Rall et al., 2005).
Koyuncu and Haggblom (2009) made a comparison between XLD plates and BGA-plates and observed that XLD is superior to BGA, due to higher selectivity. In our study also more numbers of *Salmonella* isolates were obtained in XLD as compared to BGA.

In the present study 3.65 per cent (3/82) of faecal swabs collected from calves were found positive for *Salmonella* on the basis of bacterial growth in xylose lysine deoxycholate agar and brilliant green agar. Of these 02 samples were of diarrhoeic cow calf and 01 was from diarrhoeic buffalo calf. The spread and persistence of *Salmonellas* on calf rearing units and on dealers premises has been the subject of previous studies. Although, *Salmonella* is very common on dairies world-wide there are limited national surveillance programs for *Salmonella* in cattle compared to poultry or swine. Literature reveals variable results in the prevalence of the faecal shedding of *Salmonella* by calves.

Hinton *et al.* (1983) reported 61 per cent excretion of *Salmonella* in the faeces of calves fed milk substitute. A study of 33 dairy calf farms in California indicated that approximately 20 per cent of day-old calves were shedding *Salmonella* in their faeces (Huston *et al.*, 2002). Seleim *et al.* (2002) collected faecal samples from calves suffering from diarrhoea and isolated *Salmonella* in 17.5 per cent of the cases while in contact apparently healthy calves had 3.4 per cent of *Salmonella*. Berge *et al.* (2006) detected *Salmonella enterica* on > 50 per cent of farms and in 7.5% of 3,686 faecal samples. Nagal *et al.* (2006) isolated *Salmonella enterica*, subspecies enterica serovar Berta from three out of five blood samples of 3-6-month-old ailing calves. Izzo *et al.* (2011) conducted studies to identify the common serotypes and antimicrobial resistance patterns of *Salmonella spp.* associated with diarrhoea in Australian dairy calves under the age of 6 weeks. *Salmonella* serovar dublin, S. serovar typhimurium and S. serovar Bovis morbificans were the three most common *Salmonella* serotypes isolated. Abdullah *et al.* (2013) collected 114 faecal samples directly from the rectum of diarrhoeic calves to characterize the bacterial pathogens and observed that 25 (21.9%) samples were positive for *Salmonella spp.* Contradictory to the finding of the earlier worker remarkably low number of animals were found
positive for *Salmonella*. This discrepancy can be attributed to difference in geographical location, epidemiology of the organism, and limitation of the present laboratory.

In our study none of the faecal samples collected from non diarrhoeic calves were found positive for *Salmonella*. This is contradictory to the findings of Acha *et al.* (2004) who reported the occurrence of *Salmonella* in both healthy as well as diarrhoeic calves. However the workers could not associate the faecal shedding of *Salmonella* with the occurrence of diarrhoea. Kaura (1988) postulated that *Salmonella* is both a major pathogen causing calf diarrhoea leading to early-age calf-mortality and a foremost pathogen transmitted through animal products. Khan and Khan (1991) reported that *Salmonella* in neonatal calves (28 days old) produces diarrhoea in 1 to 12 per cent calves and morbidity up to 20 per cent. However, based on our results it is hypothesized that in calf the faecal shedding of *Salmonella* can occur without any clinical feature of diarrhoea.

In the present study *Salmonella* spp. were isolated from 44.44 and 18.75 per cent of post mortem intestinal contents collected from cow and buffalo calves, respectively. More than 10 per cent of buffalo meat samples have been reported as contaminated with *Salmonella* in northern India (Sharma *et al*., 1989). Ploger *et al.* (1980) found *Salmonella* in 16.8 per cent of the necropsies of 726 calves up to 4 weeks old, as an only agent in 42.6 per cent of cases and associated to other bacterial and viral enteropathogens in the remaining percentage. Taoudi *et al.* (1983) also marked *Salmonella* in 27.6 per cent of samples of intestinal content of calves. Other authors consider Salmonellosis as important cause of calf mortality and reported variable isolation frequencies from 1 to 10.6 per cent (Langoni *et al*., 2004). However, Tsolis *et al.* (1999) opined that factors other than or in addition to bacterial colonization, intestinal lesions, or electrolyte loss contribute to lethal morbidity in calves suffering from salmonellosis.

In preweaned calves, the most important factors associated with decreased likelihood of fecal shedding of *S. enterica* were the use of antimicrobial-supplemented milk replacer and maintenance of a closed herd (Berge *et al*., 2006). The levels of *Salmonella* in the calves declined with age.
Calves may be infected during transport and disseminate infection to other calves either at the rearing farm or in other markets.

In the present study out of the 50 samples collected from adult animals 01 sample collected from adult non-diarrhoeic cattle was found positive for *Salmonella* spp. Huston et al. (2002) suggested that subclinical faecal *Salmonella* shedding can persist in dairy herds for up to 18 months with no measurable effects on health or production of individual cows. McEvoy et al. (2003) isolated *Salmonella* spp from 2 per cent of faecal samples of adult cattle. Murugkar et al. (2005) recovered a total of 13 (9.7%) *Salmonella* isolates from diarrhoeic cattle rectal swabs comprising *S. enteritidis*, *S. typhimurium* and *S. bareilly*. Sychanh et al. (2013) reported that the prevalence of *Salmonella* in the adult bovines was 6.69 per cent (18 of 269), with a more pronounced prevalence in buffaloes (7.11%) than in cattle (4.54%).

Although in the present study a low prevalence of *Salmonella* was determined in adult population they represent a potential source of infection for calves, humans and also for other animals passing through. Sychanh et al. (2013) have rightly pointed out that the *Salmonella* positive dairy animals constitute a potential reservoir of infection with the added risk of entering the human food chain.

Studies have indicated that on dairies *Salmonella* shedding in cows can range from 0 to 99 per cent and 0 to 67 per cent of pre-weaned calves can acquire the infection and this can continue for several years. The pre-weaned calf is exposed to *Salmonella* through calving, cow’s milk and environment. Thus, *Salmonella* excretion in faeces and milk samples of adult animals may be continuous source of infection to calves their by contributing to increased calf mortality.

All the isolates of *Salmonella* obtained in the present study were subjected to biochemical characterization. Biochemical profiling is a fast and accurate method for the identification of bacteria when it is performed with a set of reactions, but it is commonly disregarded as a means of grouping *Salmonella* isolates because most serovars within a given subgroup display a
very similar biochemical reaction profile. Results of our study revealed that all the isolates were Indole and VP negative and positive for citrate utilization. Similar to our findings Nesa et al. (2011) in their study observed that, all the salomonella isolates were negative to indole tests positive to MR and negative to VP. Variable results for fermentation of sugar by different isolates were obtained in our study These results are strongly correlated with the observations of Kwon et al. (2010) and Sujatha et al. (2003) and we agree with the observations made by earlier workers that identification of serovars on the basis of sugar fermentation reactions is difficult (Han et al., 2011) The differences among the biochemical reactions amongst the isolates may plausibly be due to the species differentiation, hygienic, environmental and geographic variation and technical limitations of the laboratory of the study.

In motility test, 10 isolates (71.42%) were motile whereas 04 were found to be non motile. This collaborates with the findings of earlier workers who have also reported maximal isolates of Salmonella recovered from cattle to be motile (Nesa et al., 2011). Motility test is fundamental basis for the detection of motile and non-motile Salmonella organisms.

The 14 isolates of presumptive colonies of Salmonella spp. were further confirmed by using Latex Agglutination test to identify S. typhimurium , S. enteritidis, S. typhi, and S. cholerasuis. Surprisingly, S. choleraesuis was identified in 01 isolate whereas the remaining 13 isolates were not found positive for any of the four species. S. choleraesuis is known to be host adapted for pigs and the only reason assigned to the present result could be a possible contamination of the sample.

In India, Murugkar et al. (2005) in their study recovered a total of 13 (9.7%) Salmonella isolates from cattle diarrhoeic rectal swabs comprising S. enteritidis, S. typhimurium and S. bareilly. Kaushik et al. (2014) isolated S. typhimurium and S. newport from milk samples of cattle. Since the latex agglutination test is able to identify only four species of Salmonella and literature reveals presence of species other than these four also in Indian cattle, it is concluded that probably the isolates of Salmonella recovered in the present work belonged to some other species.
5.2 Antimicrobial resistance of *Salmonella* isolates

The development and use of antibiotic has been one of the most important steps towards controlling of infectious bacterial diseases in 21st century. However, the subsequent appearance and spread of antibiotic resistance in pathogenic organisms have made many currently available antibiotics ineffective (Kam *et al.*, 2007) To successfully fight the increasing number of drug resistant and multi drug resistant bacteria, extensive knowledge of the molecular mechanisms of acquiring antibiotic resistance and updated information is required.

A slight variation was noticed in the results of the sensitivity of isolates against 15 different antibiotics used. Antibiotic sensitivity assay in the present study revealed that all the isolates were highly sensitive (100%) for cephalexin, ciprofloxacin, norfloxacin, enrofloxacin, chloramphenicol, nitrofurantoin and gentamicin followed by tetracycline (83.3%), amoxyacilin, and trimethoprim (50%). All the *Salmonella* isolates were found resistant to ampicillin.

McEvoy *et al.* (2003) observed that *S Typhimurium* isolates were resistant to ampicillin, chloramphenicol, streptomycin, sulphafurazole and tetracycline which was not in accordance to our observations as in our study 100 per cent sensitivity to chloramphenicol was achieved.

In accordance with our findings Berge *et al.* (2006) found that many *Salmonella* isolates (33%) were resistant to multiple antimicrobials. Bouchrif *et al.* (2009) studied the prevalence and antibiotic-resistance of *Salmonella* isolated from food and found resistance to tetracycline. Singh *et al.* (2010) observed that multiple drug resistance was common among *Salmonella* isolates of buffalo origin, particularly against aminoglycosides, oxytetracyclin, ampicillin and cephalaxin.

In agreement to our findings Nagal *et al.* (2006) Jadidi *et al.* (2012) reported that *Salmonella* isolates showed the highest sensitivity to ceftriaxone and enrofloxacin but were resistant to ampicillin. Much recently, Abdullah *et al.* (2013) observed that most of the, *Salmonella* spp. were susceptible to azithromycin, gentamicin and ciprofloxacin. Ansari *et al.* (2014)
also reported a high sensitivity of *Salmonella* towards ciprofloxacin, levofloxacin, azithromycin and cefotaxime.

In the last two decades, the emergence of antibiotic-resistant *Salmonella* has become a serious health hazard worldwide. The widespread use of antimicrobial agents in food animal production and the routine practice of giving antimicrobial agents to domestic livestock as a means of preventing and treating diseases has contributed to the occurrence of *Salmonella* with decreased susceptibility to drugs. However, fortunately none of the isolates in our study showed multiple drug resistance and were susceptible to routinely used antimicrobials. The variation in the sensitivity of antibiotics of the faecal isolates may be due to the outcome of choice and also the indiscriminate use of antibiotic in different disease stage to various species of animals. The results of study will provide guidelines to the veterinarian to select the appropriate antibiotics to reduce the economic losses by selecting the sensitive antibiotics.

### 5.3 Seroprevalence of *Salmonella* in bovines

Slide micro agglutination test (SAT) was done to assess the sero-prevalence of *Salmonella* agglutinins in different animals. The agglutination was observed in 14.5 per cent diarrhoeic cases comprising 02, 03, 01 and 01 cases from cattle calf, buffalo calf, adult cattle and adult buffalo, respectively. Maximal sero-prevalence was detected in diarrhoeic adult buffalo (50%).

Singh *et al.* (2009) found that 48.3 per cent cows with pyrexia of unknown origin (PUO), 47.4 per cent cows which aborted within previous 15 days of serum collection, 6.2 per cent healthy breeding cows, 10.7 per cent breeding buffaloes and 8.3 per cent buffaloes slaughtered for meat were detected positive for *Salmonella* antibodies. Sachan *et al.* (2013) reported 11 per cent sero-prevalence of *Salmonella* agglutinins in cattle.

In our study a high sero-prevalence was observed in adult animals. In agreement to our findings, Verma *et al.* (2008) have also reported high sero-prevalence of *Salmonella* agglutinins in dogs with the increasing age, which might be either due to increase in susceptibility of dogs with age
as in humans or repeated subclinical infection increased the antibodies detected by the test. Detection of *Salmonella* antibodies in large number of healthy and sick animals is not surprising because salmonellosis is hyperendemic in India in human beings (John, 1996) however, little is known about its prevalence in livestock and pet animals.

As per OIE 2010, the SAT is relatively insensitive, and many older animals have low levels of agglutinins in their sera caused by enterobacteria other than *Salmonella*. Single samples are of little diagnostic value except for initial screening on a herd basis. Paired samples are needed as the minimum requirement for confirmation of active infection.

### 5.4 Enteric Pathology in bovine salmonellosis.

Gross and histopathological studies were done in total 10 cases from which *Salmonella* spp. was isolated. These included 04 cattle calves and 06 buffalo calves. In maximum cases small intestine was congested and mild erosions were present on mucosa. Microscopic examination of duodenum revealed catarrhal enteritis with infiltration of polymorhonuclear cells in the lamina propria. Ileum showed consistent mucosal thickening with increased exudates and necrosis of villi. Caecum and colon revealed villi atrophy with proliferative reaction in the crypts.

A large number of researchers have created experimental bovine models of salmonellosis using *Salmonella* dublin delivered to calves through an oral route (Forbes *et al.*, 1977; Masalski *et al.*, 1987., Frizzo *et al.*, 2012). In general, the oral entry of *Salmonella*, proliferation of this microorganism in the small intestine, and its rapid penetration into the lamina propria cause edema, macrophage and lymphocyte proliferation, and polymorphonuclear (PMN) recruitment. This is accompanied by expansion of the central lacteals, and generates a sharp decline in apical enterocytes while provoking a proliferative reaction in the bottom of the crypts (enteritis regenerative). This process is rapidly decompensate, causing atrophy and fusion of the villi. Salmonellosis caused by low virulence bacterial strains usually generate haemorrhagic type lesions in the course of several days (Gelberg, 2001)
In the present study, a consistent feature observed in all cases was degenerated villi, oedema of lamina propria and microhaemorrhages in the small intestine. This is similar to observations of Zhang et al. (2003) that in *S. enterica* infection the resulting acute inflammatory response is associated with an increase in vascular permeability resulting in mucosal edema. Frizzo et al. (2012) have opined that circulatory disorders lead to irreversible damage of the villi with apical necrosis, hemorrhage, and fibrin exudation (fibrinous necrotic enteritis).

Gelberg (2001) have expressed that in clinical cases of salmonellosis observed on farms, ileum lesions usually involve fibrinous necrotic enteritis and the possible presence of polymorphonuclear cells. Zhang et al. (2003) also described an influx of neutrophils associated with necrosis of the uppermost ileal mucosa in calves with salmonellosis. In accordance with their observations in our study also, the lumen of ileum contained necrotic debris with degenerated neutrophils.

Bacteria spread through blood and the lymphatic system cause septicemia with involvement of the mesenteric lymph nodes, liver, and spleen (Tizard, 1996) The bacteria invade regional lymph nodes, leading to macrophage and PMN recruitment. In our study Mesentric lymph nodes showed acute hyperplastic lymphadenitis with cortical follicular hyperplasia and increased macrophages and neutrophils. Aggressiveness of the pathogenic bacteria strain and the infection time course have a marked influence on the type of lesions found in the lymph nodes in cases seen on farms. Frizzo et al. (2012) have stated that when the strain is highly virulent and the infection process is hyperacute, lymph nodes are often the only organs in which PMN are found (being absent in the liver and spleen). In these cases, nodules are found in the subcapsular sinus as a relatively continuous mantle of necrotic cells with pyogenic PMN in a mesh of fibrin.

Microscopic section of liver in our study revealed degenerative changes with vacuolations and foci of hepatitis. Barker and Van Dreumel (1985) observed that in the liver of animals with salmonellosis, severe
cholangiohepatitis with strong infiltration in the portal space is typically observed, particularly in bile canaliculi and in calves with salmonellosis, polymorphonuclear cells are seen infiltrated in between the sinusoids. This is in agreement with our observations.

Taken together, *Salmonella* species are invasive pathogens which effect the intestine leading to enteritis and serious economic losses in animal industry.
6. SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

6.1 Summary

Diarrhoea is one of the major causes of neonatal calf mortality. The infectious agents capable of causing diarrhoea in the neonatal calf include rotavirus, coronavirus, enteropathogenic *E. coli*, *Salmonella spp.* and Cryptosporidium.

Work was conducted to investigate the infection of calves with *Salmonella spp.* in organized dairy farms; to investigate the infection of adult diarrhoeic bovines with *Salmonella* in above farms and to study the enteric pathology in calves with salmonellosis. The study was conducted for a period of seven months from September 2013 to March 2014. For the study rectal faecal swabs were collected from adult cattle and buffaloes as well as cattle and buffalo calves of either sex from organized dairy farms in and around Jabalpur district. Sterile intestinal swabs and intestinal tissues were also collected from necropsy cases having enteritis. Impression smears from faeces and intestinal contents were prepared and stained by Gram’s stain for direct examination. The samples were processed for microbiological examination. Ready to use Nutrient agar, Xylose-lysine deoxycholate agar and Mueller Hinton agar plates, were used for culture of organisms and antibiogram test. Brilliant green agar was prepared and used as a selective medium for primary isolation of bacteria.

During the study period faecal swabs were collected from 82 calves, both cattle as well as buffalo from 05 organized dairy farms. Out of the 82 samples collected from calves *Salmonella spp.* was isolated in 03 samples (3.6%). Of these 02 samples (2.4%) were from diarrhoeic cow calves and 01 (1.2%) was from diarrhoeic buffalo calves. 50 faecal swabs were also collected from adult animals. Amongst these 01 sample (2%) from adult non-diarrhoeic cattle was found positive for enteric *Salmonella*. In the study 41 intestinal swabs, collected from bovine post mortem cases with enteritis, 09 cattle calves and 32 buffalo calves, were processed for isolation of *Salmonella spp.* Out of 09 intestinal swabs from cattle calves, 04 (44.44%)...
were positive for *Salmonella spp.* whereas from 32 samples collected from buffalo calves, 06 (18.75%) were found positive for *Salmonella spp.*

Total incidence of *Salmonella spp.* was determined as 8.09% (14/173) in the bovines at organized dairy farms. The total 14 isolates recovered from animals were further characterized by biochemical tests.

The 14 isolates of presumptive colonies of *Salmonella sp* were subjected to Latex Agglutination test designed to identify *S. typhimurium*, *S. enteritidis*, *S. typhi*, and *S. cholerasuis*. Latex agglutination test identified *S cholerasuis* in 01 isolate only. The remaining 13 isolates were not found positive for any of the four species.

Antibiotic sensitivity test conducted for six isolates of *Salmonella spp*. revealed that isolates were highly sensitive (100%) for cephalexin, ciprofloxacin, norfloxacin, enrofloxacin, chloramphenicol, nitrofurantoin and gentamicin followed by tetracycline (83.3%), amoxyacilin, and trimethoprim (50%). All the *Salmonella* isolates were found resistant to ampicillin.

To know the sero-prevalence of *Salmonella* in bovines, blood was collected from 101 animals including 48 diarrhoeic and 53 non diarrhoeic animals. Slide micro agglutination test revealed maximal sero-prevalence in diarrhoeic adult buffalo (50%). Agglutination was observed in 14.5% diarrhoeic cases comprising 02, 03, 01 and 01 cases from cattle calf, buffalo calf, adult cattle and adult buffalo, respectively. Agglutination was observed in 22.6% non-diarrhoeic apparently healthy animals comprising 06, 02, 03 and 01 cases from cattle calf, buffalo calf, adult cattle and adult buffalo, respectively.

Gross and histopathological studies were done in total 10 cases from which *Salmonella spp.* was isolated. These included 04 cattle calves and 06 buffalo calves. In maximum cases small intestine was congested and mild erosions were present on mucosa. Microscopic examination of duodenum revealed catarrhal enteritis with infiltration of polymorhonuclear cells in the lamina propria. Ileum showed consistent mucosal thickening with increased exudates and necrosis of villi. Caecum and colon revealed villi atrophy with proliferative reaction in the crypts.
6.2 Conclusions

The following conclusion can be drawn from the present investigations.

1. Incidence of Salmonellosis in bovine calves was determined as 3.65%. The incidence of salmonellosis in diarrhoeic cow calves and diarrhoeic buffalo calves was 9.09% and 6.25%, respectively.

2. Positive percentage of *Salmonella* spp. isolated from post mortem cases of cattle calf was 44.44% and buffalo calf was 18.75%.

3. The isolates of *Salmonella* spp. were 100% sensitive for cephalexin, ciprofloxacin, norfloxacin, enrofloxacin, chloramphenicol, nitrofurantoin and gentamicin. All the *Salmonella* isolates were found resistant to ampicillin.

4. In diarrhoeic animals 14.5% and in non-diarrhoeic animals 22.6% seroprevalence for *Salmonella* was recorded by slide agglutination test.

5. The major lesions in salmonellosis include catarrhal enteritis and congestion mainly observed in small intestine.
6.3 Suggestions for further work

1. Molecular diagnostic methods like PCR, IHC etc should be attempted to know the true status of bovine Salmonellosis in Jabalpur.

2. Serotyping of *Salmonella spp.* found in cattle population of Jabalpur should be carried out so that effective vaccines can be devised.

3. The role of the virulence plasmid in pathogenesis of both the enteric and systemic phases of salmonellosis in calf needs to be determined.
REFERENCES


APPENDIX
Staining solutions

i. Weigert’ Iron hematoxilin:
   Solution A
   Hematoxilin 1.0gm
   Absolute alcohol 100.0gm
   Solution B
   29% ferric chloride 4.0ml
   Distilled water 95.0ml
   Hydrochloric acid, concentrated 1.0ml
   Working solutions
   Equal parts of solution A and solution B were mixed

ii. Boin’s solution:
   Picric acid, saturated aqueous solution 75.0ml
   Formaldehyde, 37-40% 25 ml
   Glacial acetic acid 5.0ml

iii. Biebrich Scarlet- Acid Fuchsin Solution:
   Biebrich scarlet, aqueous 1% 90ml
   Acid fuchsin, aqueous 1% 10ml
   Glacial acetic acid 1ml

iv. Phosphomolybdic – Phosphotungstic acid Solution:
   Phosphomolybdic acid 5.0gm
   Phosphotungstic acid 5.0gm
   Distilled water 200ml

v. Aniline Blue Solution:
   Aniline blue 2.5gm
   Acetic acid 2.0ml
   Distilled water 100ml

vi. 1% Acetic water
   Glacial acetic acid 1.0ml
   Distilled water 100ml
vi. Goodpasture’ stain:

   - Basic fuchsin: 0.59gm
   - Aniline: 1.0ml
   - Phenol crystals (melted): 1.0ml
   - Alcohol, 30%: 100.0ml

vii. Gram’s iodine solution:

   - Iodine: 1.0gm
   - Potassium iodine: 2.0gm
   - Distilled water: 300ml

viii. Stirling’s Gentian violet stain:

   - Gentian violet (crystal violet): 5.0 gm
   - Absolute alcohol: 10ml
   - Aniline: 2.0ml
   - Distilled water: 88.0%

ix. Saturated picric acid solution:

   - Picric acid: 1.18gm
   - Distilled water: 100.0ml

The above ingredients were mixed and dissolved in distilled water. Volume marked 100ml. Keep the stain for months prior to use.

### Chi-Square test 2 X 2 contingency table

<table>
<thead>
<tr>
<th>Agglutination Test</th>
<th>Present</th>
<th>Absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>(a)</td>
<td>(b)</td>
<td>(a+b)</td>
</tr>
<tr>
<td>Negative</td>
<td>(c)</td>
<td>(d)</td>
<td>(c+d)</td>
</tr>
<tr>
<td>Total</td>
<td>(a+c)</td>
<td>(b+d)</td>
<td>(n=a+b+c+d)</td>
</tr>
</tbody>
</table>

Chi square test $\chi^2 = \frac{(ad - bc)^2 x n}{(a+b) (a+c) (d+b) (d+c)}$