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Isolation, cultural and morphological characterization of *Salmonella Spp*. from diarrhoeic cases of Pigs

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Abstract
The present study was designed to study the cultural and morphological characterization of the *Salmonella spp.* from pigs. A total of 175 feacal samples collected from different pig farms which are located in Bidar, Hassan and Tumakuru districts of Karnataka out of which 24feacal samples were found to be positive for *Salmonella spp.* Based on the morphological and cultural characters. Out of 175 suspected diarrhoeic cases total number of isolates were 24 with 13.71 percent prevalence rate. The colony characters showed on different selective media are Hichrome *Salmonella* agar showed transparent colonies with black centres due to production of H_2S, pink colonies with black centre on XLD agar due to production of H_2S, colourless or red to pinkish white colonies often surrounded by pink or red zone on BGA agar because the bacterium does not ferment lactose or sucrose and Non-lactose fermenter *Salmonella spp.* colonies appear colourless and transparent on MacConkey agar plates and typically did not alter appearance of the medium and colourless with transparent colonies on nutrient agar. On Gram's staining, the morphologically isolated bacteria was small rod shaped, Gram negative, single or paired in arrangement.

Keywords: gram's staining, brilliant green agar, mac conkey agar, hichrome *salmonella* agar

1. Introduction
Pig rearing is one of the traditional activities in India carried out by rural folk. Among various livestock activities, piggery is most efficient way of meat production utilizing kitchen waste, vegetable waste, etc. Though initially local breeds have been raised, nowadays exotic pig breeding is popular and pork from such animal is having wide acceptance. Further, pig farming requires small investment on building and equipments.

A systematic of production pork involves food safety and food standard issues. The pork produced should be safe and wholesome. Food safety hazards caused by food-borne pathogens such as *Salmonella spp.* or serotypes remain a major problem for the food industry. Salmonellosis is an important health problem and a major challenge worldwide having greater significance in developing countries (Wang *et al*., 2008) [26]. Pork and pork products are recognized as an important source for human Salmonellosis (Smith *et al*., 2010) [22]. *Salmonella spp.*is an important cause of food-borne (alimentary) health problems in humans (Pang *et al.*, 1995; De Jong Skierus, 2006; Hernandeza *et al*., 2013) [19, 8, 12]. The risk of *Salmonella spp.* might differ between the production systems, caused by components of the husbandry systems affecting disease development and pathogen shedding or differences in the level of resistance to the pathogen (Zheng *et al*., 2007) [28]. The increased consumption of pork coupled with the high prevalence of enteropathogens in the swine industry suggests a rise in food-borne illness cases which can lead to human food-borne illness and loss of product shelf-life.

The World Health Organization (WHO) reports that the incidence and severity of cases of Salmonellosis have increased significantly (WHO, 2010) [27]. Strains resistant to a wide range of antimicrobials emerged in the 1990s and constitute a serious additional concern for public health (WHO, 2010) [27]. The economic impact of this zoonosis in commercial food production is also substantial and control of *Salmonella spp.* is becoming more challenging with the trend towards cheaper and faster food.

2. Materials and Methods
The material and methods used in the present study are presented in this chapter.

2.1 General considerations
The glassware used in this study were of neutral glass of Corning or Borosil India Ltd. make the culture media and buffers were prepared in MilliQ water (Millipore). Plasticware including microcentrifuge tubes, micropipette tips, cryovials, petri dishes and autoclave bags were procured from M/s. Tarson Products Pvt. Ltd., Kolkata.

2.2 Preparation of glassware
The glassware used in the study wereprepared by soaking them in detergent solution overnight. The following day, they were washed thoroughly in running tap water, followed by rinse in deionised/distilled water (DW). Then oven dried glassware were packed and sterilized in hot air oven at 160 ºC for 1 hr

2.2.1 Preparation of plastic ware
The new plastic ware including microcentrifuge tubes and micropipette tips were sterilized by autoclaving at 121 ºC for 15 min at 15 psi.

2.3 Media, reagents and stains used
MacConkey agar, Brilliant green agar, Hichrome *Salmonella* agar, Xylose liysine Deoxycholate agar, Nutrient agar, Triple sugar iron agar, Nutrient broth, Buffered peptone water, Selenite broth, Tetrathionate broth, Tryptone water broth, Peptone water, Glucose phosphate peptone water, and gram's staining kits were obtained from M/s Hi media Laboratories Ltd., Mumbai. Media and reagents were prepared as recommended by the manufacturer instructions and sterilized by autoclaving.

2.3.1 HiChrom *Salmonella* agar
HiCrome *Salmonella* Agar 27.9 gm
Distilled water 1000 ml
Heated to boiling with frequent agitation to dissolve the medium completely. Did not autoclave or overheat to avoid destruction of selectivity of the medium. Cooled to about 50º C and mixed well and poured into sterile petri plates.

Appearance: Cream to yellow homogeneous free flowing powder
Gelling: Firm, comparable with 1.3 per cent Agar gel
Colour and Clarity of prepared medium:-Light amber coloured, slightly opalescent gel forms in Petri plates

2.3.2 Xylose-Lysine Deoxycholate Agar (XLD Agar)
XLD Agar 56.68 gm
Distilled water 1000 ml
Heated with frequent agitation until the medium boils. Did not autoclave or overheat. Transfer immediately to a water bath at 50 °C. After cooling, poured into sterile Petri plates. Here not to prepare large volumes that require prolonged heating, thereby producing precipitate.

Appearance:-Light yellow to pink homogeneous free flowing powder
Gelling: Firm, comparable with 1.5% Agar gel
Colour and Clarity of prepared medium: Red coloured clear to slightly opalescent gel forms in Petri plates.

2.3.3 Brilliant Green Agar Medium (BGA)
BGA Agar 58.09 gm
Distilled water 1000 ml
Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (115 °C) for 15 min. Precaution is taken avoid overheating. Cooled to 45-50 °C. Mixed well and poured into sterile Petri plates.

Appearance: Light yellow to light pink homogeneous free flowing powder
Gelling: Firm, comparable with 2.0 per cent agar gel.
Colour and Clarity of prepared medium: Greenish brown clear to slightly opalescent gel forms in Petri plates.

2.3.4 MacConkey Agar
MacConkey Agar 49.53 gm
Distilled water 1000 ml

Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (115 °C) for 15 mi. Precaution should be taken avoid overheating. Cooled to 45-50 °C. Mixed well before poured into sterile Petri plates. The surface of the medium is dried when inoculating.

Appearance:-Light yellow to pink homogeneous free flowing powder

Gelling: Firm comparable with 1.35% Agar gel

Colour and Clarity of prepared medium: Red with purplish tinge coloured clear to slightly opalescent gel forms in Petri plates.

2.3.5 Nutrient Agar
Nutrient Agar 28 gm
Distilled water 1000 ml
Heated to boiling to dissolve the medium completely. Dispense as desired and sterilized by autoclaving at 15 lbs pressure (115 °C) for 15 min. Mixed well before poured into the petri plates

Appearance: Cream to yellow homogeneous free flowing powder
Gelling: Firm, comparable with 1.5 per cent Agar gel
Colour and Clarity of prepared medium: Light yellow coloured clear to slightly opalescent gel forms in Petri plates.

2.3.6 Nutrient broth
Nutrient broth 13 gm
Distilled water 1000 ml

2.3.7 Nutrient- glycerol broth
Nutrient broth (Sterile) 80 ml
Sterile glycerol 20 ml
Nutrient and glycerol broth were prepared for preservation of isolated samples. Glycerol was sterilized in hot air oven at 160 ºC for one hr and added in to nutrient broth sterilized by autoclaving mixed well and aliquot in to sterile tubes in 5 ml quantities and store at 4 ºC.

2.4 Source and collection of samples
A total 175 feacal samples collected from different pig farms which are present in Bidar, Hassan and tumukurudistricts of Karnataka. The feacal samples were collected in sterilized transport media swabs (Hi Media) were collected aseptically

collected from rectal feacal swabs from the suspecteddiarrhoeal cases were all collected samples transported to department of veterinary microbiology in thermocol box containing icepacks. All the samples were subjected to bacteriological isolation was carried out in Department of Veterinary Microbiology, Veterinary College, Bidar.

2.5 Isolation of *Salmonella spp.*

The study was conducted utilizing the conventional methods for the detection of *Salmonella spp.* following the standard guide lines from ISO 6579: 2002. This isolation andidentification procedure involved four principal stages: pre-enrichment, selective enrichment, selective plating and confirmation.

2.5.1 Non-selective pre-enrichment

2.5.1.1 Buffered Peptone Water

Buffered peptone water 20 gm
Distilled water 1000 ml

Dispensed in 50 ml amounts in sterilizaed test tubes. Sterilize by autoclaving at 15 lbs pressure (115 °C) for 15 min. Here feacal samples collected swabs directly inoculated aseptically added to 45 ml of buffered peptone water (BPW, Oxoid), and homogenized by shaking the sample mixture for about 2 min. After homogenization, all the samples were incubated at 37 ºC for 18 hrs.

2.5.2 Selective enrichment

3.5.2.1 Selenite Broth (Selenite F Broth) (Twin Pack)

Part A 19 gm
Part B 4 gm
Distilled water 1000 ml

Mixed and Warmed to dissolve the medium completely. Distributed into sterile test tubes. Sterilized in a boiling water bath or free flowing steam for 10 min. Did not autoclave. Precaution to taken excessive heating is detrimental. Discard the prepared medium if large amount of selenite is reduced (indicated by red precipitate at the bottom of tube/bottle).

The pre-enrichment broth after incubation was mixed and 1 ml of the broth was transferred into a tube containing 9 ml Selenite broth (Himedia) and the inoculated Selenite broth were incubated at 37 ºC for 48 hr.

2.5.3 Plating out and identification of *Salmonella* spp.

After incubation for 18-24 hrs., a loop-full of material from the Selenite broth was transferred and streaked separately onto the surface of Brilliant green agar, HiChrome *Salmonella spp.* agar, Xylose lysine deoxycholate agar, Mac Conkey agar, and plates separately. The plates were incubated at 37 ºC for 18-24 hrs. After incubation, the plates were checked for growth of typical *Salmonella spp.* colonies. Non lactose fermenter *Salmonella spp.* colonies appear colourless and transparent on Mac Conkey agar plates and typically did not alter appearance of the medium. Transparent colonies with black centres on Hi Chrome *Salmonella spp.* agar, pink colonies with or without black centre on XLD agar and colourless or red to pinkish or opaque-white colonies aften surrounded by pink or red zone on BGA agar because the bacterium does not ferment lactose or sucrose. The pure cultures were sreaked on triple sugar iron (TSI) agar slant and incubated at 37 ºC for 24-48 hrs. Those producing typical reaction on TSI (pinkslant and black butt with H_2S production-blackening of agar). The suspected colonies were confirmed by staining characters and biochemical tests (BAM, 2007)

3.5.4 Triple sugar iron agar

Triple Sugar Iron Agar 64.62 gm
Distilled water 1000 gm

Heated to boil to dissolve the medium completely. Mixed well and distributed into test tubes. Sterilized by autoclaving at 15 lbs pressure (115 °C) for 15 min. Allow the medium to set in sloped form with a butt of depth about 2.5 cm-5 cm

3.5.5 Morphological characterization by Gram's staining method

The representative *Salmonella spp.* colonies were characterized microscopically using Gram's stain (Himedia) done as per the standard methods.

3. Results

The present study was carried out with an objective of isolation, cultural and morphological characterization of the *Salmonella spp.* from suspected diarrhoeic cases in pigs from different pig farms in Bidar, Hassan and Tumkuru districts of Karnataka. The result obtained during the programme of research work was documented as follows.

3.1 Collection of samples

A total 175 samples were collected from different pig farms of Karnataka (Table 1, Plate 1). The samples were processed in the Dept. of Veterinary Microbiology, Veterinary College, Bidar by standard protocols.

3.2 Isolation of *Salmonella spp.*

All the samples were subjected to bacteriological isolation, a total of 24 (13.71%) *Salmonella spp.* isolates were obtained in the present study (Table 1).

3.3 Cultural and morphological characterization

Samples were positive for *Salmonella spp* organisms was identified by cultural and morphological characterization. The colony characters showed on different selective media are Hichrome *Salmonella* agar showed transparent colonies with black centres due to production of H_2S (Plate 3), pink colonies with black centre on XLD agar due to production of H_2S (Plate 4), colourless or red to pinkish white colonies aften surrounded by pink or red zone on BGA agar because the bacterium does not ferment lactose or sucrose (Plate 5) and Non lactose fermenter *Salmonella spp.* colonies appear colourless and transparent on MacConkey agar plates and typically did not alter appearance of the medium (Plate 6) and colourless with transparent colonies on nutrient agar (Plate 6).

On Gram's staining, the morphologically isolated bacteria was small rod shaped, Gram negative, single or paired in arrangement (Plate 2).

Table 1: Collection of feacal samples from different farms of Karnataka

Sl. No.	Details of Pig farm in Karnataka	Suspected diarhhoeic cases	Total no. of isolates	Isolates (%)	Overall prevalence %
1.	Krishna farm Shivanagar, Bidar	41	5	12.2	13.71
2.	Slaughter house Bidar	34	5	14.7	
3.	Kumar Naik, Hassan (D)	15	2	13.3	
4.	Konehalli farm, Tumukur (D)	10	1	10	
5.	KVK, Hassan.	17	2	11.7	
6.	ILFC, HVC, Hassan	58	9	15.5	
Total		175	24	13.71	

Plate 1: Sample collection in pigs from rectum by using transport media swab method

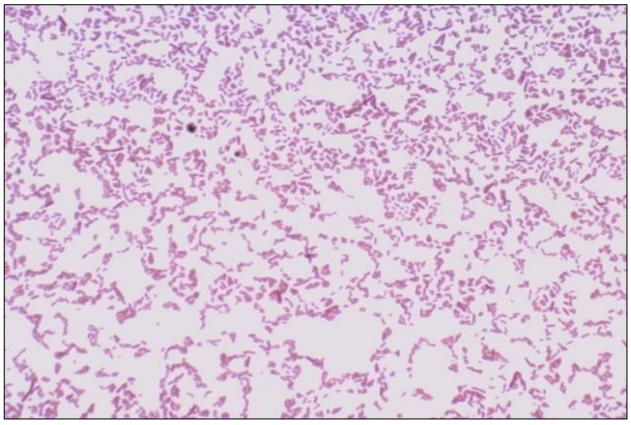

Plate 2: Gram's staining shows Small rod shaped, Gram negative, single or paired in arrangement of *Salmonella spp.*

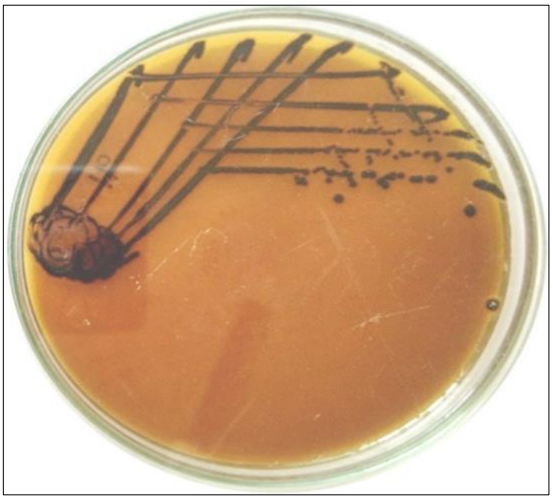

Plate 3: HiChrome *Salmonella* agar *Salmonella spp.* showing transparent colonies with black centres due to production of H_2S_4

Plate 4: Xylose lysine Decarboxylate agar *Salmonella spp.* showing pink colonies with black centre colonies due to production of H_2S

Plate 5: Brilliant green agar *Salmonella spp.* showing pink or red zone on BGA agar

Plate 6: Mac Conkey agar *Salmonella spp.* showing colourless and transparent colonies due to non-lactose fermenter

4. Discussion

Salmonellosis is one of the most important zoonotic diseases and worldwide problem. It is assuming greater significance in developing countries like India. Salmonellosis has been recognized and studied mainly in industrialized countries while occasional outbreaks of non-typhoidal Salmonellosis due to food contamination have been detected in other countries. A number of reports have indicated that the occurrence of organism in various foods *viz*. poultry, beef, pork, eggs, milk, cheese, fish, shellfish, fresh fruit and juice and vegetables have been found to be epidemiologically associated with *Salmonella spp.* infection. The presence of *Salmonella spp.* in feacal sample (rectal swab) from different pig farms with the overall incidence rates varying from 0 to 82 per cent have been reported (Venkateswaran *et al*., 1988; Ejeta *et al*., 2004; Van *et al*., 2007 and Kuhn *et al*., 2013) [25, 9, 24, 15].

The incidence of food-borne illness *Salmonella spp.* continue to be an important problem throughout the world. The economic losses associated with *Salmonella spp.* infection have attracted increasing attention in developing countries in recent year although Salmonellosis is endemic in nature and responsible for heavy economic loss in India every year. Pork products are contaminated with harmful, pathogenic and spoilage bacteria by infected stocks, cross contamination due to improper handling and during storage or improper cooking which can lead to human food-borne illness and loss of product shelf-life. Epidemiological data are needed to monitor trends over time. Food-borne Salmonellosis is a notifiable condition in many countries including US and UK (Choudhary *et al*., 2015 and Kumar *et al*., 2008) [16]

4.1 Isolation and identification of *Salmonella spp.*

The feacal samples of pigs were analyzed for the presence of *Salmonella spp.* organisms using the standard protocol of two steps pre-enrichment in buffered peptone water, enrichment in selenite broth and selective plating on Hichrome *Salmonella spp.* agar, XLD agar, BGA and MacConkey agar, the isolates obtained were having similar characters as reported by Rajeshwari. (2013) [21]; Paramesh. (2015) [20] and Choudhary *et al*. (2015) have shown isolates with similar characteristics shows in present study. Hektoen Entric agar and XLD agar were highly selective and differential media (Fagerberg and Avens, 1976) [11]

The morphology of the isolated bacteria on Gram staining showed small rod shape, Gram negative, motile single or paired in arrangement which was in agreement with standard morphological characters of *Salmonella spp*. described by several authors El-Gazzar and Marth. (1992) [10]; Monnery *et al*. (1994) [10]; Todar. (2004) [23]; Rajeswari. (2013); Kalambhe *et al*., (2013) [13]; Megha *et al*. (2015) [17] and Karthik *et al*. (2016) [14].

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