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SV Raghavendra
Department of Veterinary Pathology, College of Veterinary Science, Proddatur, Andhra Pradesh, India

A Anand Kumar
Associate Dean, College of Veterinary Science, Proddatur, Andhra Pradesh, India

P Amaravathi
Assistant Professor, State Level Disease Diagnostic Laboratory, SVVU, Tirupati, Andhra Pradesh, India

S Somasekhar Goud
Veterinary Assistant Surgion, Adoni Mandal, Kurnool District, Andhra Pradesh, India

Madhava Rao T
Professor & Uni. Head, Department of veterinary Public Health & Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

P Sudheer
Department of Veterinary Microbiology, College of Veterinary Science, Proddatur, Andhra Pradesh, India

Correspondence
SV Raghavendra
Department of Veterinary Pathology, College of Veterinary Science, Proddatur, Andhra Pradesh, India

Cultural isolation and molecular characterization of etiological agents with reference to lamb mortality in Rayalaseema region of Andhra Pradesh, India

SV Raghavendra, A Anand Kumar, P Amaravathi, S Somasekhar Goud, Madhava Rao T and P Sudheer

Abstract
The etiology for mortality in lambs may be due to infectious agents followed by non infectious origin. A total of 53 different samples were collected from ailing lambs (diarrhoea, nasal discharges, dullness, lambs with mixed signs) and during post mortem examination (showed suppurative pneumonia and abscesses on different organs) were inoculated into nutrient broth and incubated at 37 °C for 24 hrs. A loopful of inoculum from nutrient broth was streaked on different selective medias like EMB agar plates, MSA agar plates and BGA agar plates by following all the aseptic precautions. The plates were incubated at 37 °C for 48 hrs. The plates were observed for small dark colonies with green metallic sheen on EMB for *E. Coli,* pink to white colonies surrounded by red zone on BGA for *Salmonella* sp and yellow colonies on MSA for *S. aureus*. All the isolates were subjected to Gram's staining and revealed that the isolates which were positive for *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* sp were found gram negative coccobacillary rods, gram positive purple coloured cocci in clusters and gram negative bacilli respectively. Out of 32 *E. coli* isolates from different sources one (3.125%) isolate was found to be positive for the presence of stx2 gene at about 255 base pairs. Out of 7 cultures three (42.86%) isolates were found to be positive for *Salmonella* and positive isolates for the presence of invA gene at about 389 base pairs. Out of 29 cultures 23 (79.31%) isolates were found to be positive for *S. aureus* and positive isolates for the presence of nuc gene at about 279 base pairs.

Keywords: Ailing lambs, cultural isolates, PCR, stx2, invA, nuc genes

Introduction
Sheep rearing was happened to be man's oldest profession (Mahanta, 1987) [11]. Several characteristics such as a relative lack of aggression, a manageable size, early sexual maturity, social nature and high reproduction rates that made sheep particularly suitable for domestication. Today, *Ovis aries* is an entirely domesticated animal for farmer's livelihood and it is our responsibility to take care of health and survival. Infectious origin is the major etiology for lamb mortality followed by non-infectious conditions. In the present study cultural isolation and molecular characterization of *E. coli, Staphylococcus* sp and *Salmonella* sp were carried out.

Material and Methods
Agar-agar, Blood agar base, Brilliant green agar, Egg yolk agar, Eosin methyline blue (EMB) agar, Fluid thioglycolate broth, MacConkey agar, Neutrient broth, Peptone water, Neutrient agar, Manitol salt agar, Methyl red and Voges-proskauer medium (MR-VP Medium), Simmon's citrate agar and TSI agar were used in this study.
Faecal, nasal and swabs during postmortem examination from the representative organs were collected aseptically and inoculated into nutrient broths and incubated overnight at 37 °C immediately. A loop of inoculum was streaked onto the cultural media and incubated the media at 37 °C for 24-48 hours for bacteriological growth. The inoculated plates were examined for morphological characteristics and growth of bacterial colonies after incubation period. The isolates were then identified on the basis of colony characteristics, staining characteristics (after staining with Gram's Stain), microscopic morphology, catalase test and IMViC tests (Quinn *et al.*, 2002) [18]. A cultural smear was prepared and subjected to routine Gram staining followed by examination under oil immersion objective. The cultural isolates were subjected to catalase activity, tube coagulase test, TSI test and IMVC tests by following standard protocols for biochemical confirmation.

Polymerase Chain Reaction
Oligonucleotide Primers
The primers used in the study for detection of nuc gene in *S. aureus*, invA gene in *Salmonella* and stx2 gene in *E. coli* (Table 1).

Pathogen	Target gene	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
E. coli	Stx2	Stx2:F	GGCACTGTCTGAAACTGCTCC	255	Paton and Paton (1998) [17]
		Stx2:R	TCGCCAGTTATCTTGACATTCTG		
Salmonella	invA	Salm-3	GCT GCG CGC GAA CGG CGA AG	389	Malorny *et al.* (2003) [13]
		Salm-4	TCC CGG CAG AGT TCC CAT T		
S. aureus	nuc gene	nuc-F	GCG ATT GAT GGT GAT ACG GTT	279	Jung *et al.* (2015) [10]
		nuc-R	AGC CAA GCC TTG ACG AAC TAA AGC		

Template DNA preparation by boiling and snap chilling method
Preparation of template DNA from *Escherichia coli* strains was carried out as per Lee *et al.* (2003) with slight modifications. About 2 ml of overnight grown culture was taken in micro centrifuge tube and centrifuged at 12,000 rpm for 10 minutes. The pellet was suspended in 200 µl of nuclease free water and boiled for 15 min in a boiling water bath. The micro centrifuge tubes were transferred immediately on to ice. After 20 min, the tubes were centrifuged at 12,000 rpm for 10 min at 4 ^{0}C and the supernatant was used as template for multiplex PCR assay.

Amplification of stx2 gene, invA gene and nuc gene
PCR for amplification of stx2 gene, invA gene and nuc gene was set up in 25 µl reaction separately. Following initial trails varying with concentrations of components, the reaction mixture was optimized as below. (Table 2)

S.No.	Name of the reagent	Quantity (µl)
1.	10X PCR buffer with 15mM $MgCl_2$	2.5
2.	dNTP mix	0.2
3.	Primer-F	2.5
4.	Primer-R	2.5
5.	Taq polymerase	0.3
6.	Template	5.0
7.	Nuclease free water	12.0

PCR tube containing the reaction mixture was flash spun in a micro centrifuge tube to settle the reactants at the bottom. Thermal cycling for stx2 gene was performed in a 96-well Eppendorf gradient Thermo cycler (Germany) with heated lid. It consisted of an initial denaturation at 95 ^{0}C for 5 min, followed by 35 cycles of denaturation at 9 ^{0}C for 1 min, 1 min of annealing at 55 ^{0}C and 1 min of elongation at 72 ^{0}C, and final cycle elongation at 72 ^{0}C for 7 min. PCR product were stored at 4 °C until further confirmation by 1% agarose gel electrophoresis containing ethidium bromide.
PCR tube containing the reaction mixture was flash spun in a micro centrifuge tube to settle the reactants at the bottom. Thermal cycling for invA gene was performed in a 96-well Eppendorf gradient Thermocycler (Germany) with heated lid. It consisted of an initial denaturation at 95 ^{0}C for 5 min, followed by 35 cycles of denaturation at 95 ^{0}C for 1 min, 80 min of annealing at 58 ^{0}C and 1 min of elongation at 72 ^{0}C, and final cycle elongation at 72 ^{0}C for 7 min. PCR product were stored at 4 °C until further confirmation by 1% agarose gel electrophoresis containing ethidium bromide.
PCR tube containing the reaction mixture was flash spun in a micro centrifuge tube to settle the reactants at the bottom. Thermal cycling for nuc gene was performed in a 96-well eppendorf gradient Thermocycler with heated lid. It consisted of an initial denaturation at 95 ^{0}C for 5 min, followed by 30 cycles of denaturation at 94 ^{0}C for 1 min, 2 min of annealing at 55 ^{0}C and 1 min of elongation at 72 ^{0}C, and final cycle elongation at 72 ^{0}C for 10 min. PCR product were stored at 4 °C until further confirmation by 1% agarose gel electrophoresis containing ethidium bromide.

Analytical Agar Gel Electrophoresis:
The product (DNA) amplified by PCR was subjected to 1% agarose gel electrophoresis as described by Sambrook and Russel (2001). Agarose gel (1%) was prepared by boiling agarose in an appropriate volume of 1 X TBE buffer and allowed to cool to 50 ^{0}C. After cooling for about 3 minutes, ethidium bromide (10 mg/ml) stock was added to the agarose solution to a final concentration of 0.5 µl / ml and mixed carefully. The molten agarose was poured in to a gel casting tray fitted with acrylic comb and allowed to solidify. Once the gel was solidified a few ml of 1X TBE buffer was added, comb was removed carefully and the tray containing the gel was then placed in a submarine horizontal electrophoresis unit filled with 1X TBE buffer upto a level of 1mm above the gel surface.
The wells were loaded with 5 µl of each PCR product was mixed with 1µl of 6X loading dye. Electrophoresis was carried out at the rate of 5-6 V/cm and the motility was monitored by the migration of the dye. After sufficient migration, the gel was observed under UV transillumination using Syngene Gel Documentation system to visualize the bands and image was captured. The PCR product size was determined by comparing with a standard molecular weight marker.

Results
Isolation of bacterial pathogens
A total of 53 different samples collected from ailing lambs (diarrhoea, nasal discharges, dullness, lambs with mixed signs and post mortem examination showed suppurative pneumonia and abscesses on different organs) were inoculated into nutrient broth and incubated at 37 °C for 24 hrs. A loopful of inoculum from nutrient broth was streaked on different selective medias like EMB agar plates, MSA agar plates and BGA agar plates by following all the aseptic precautions. The plates were incubated at 37 °C for 48 hrs. The plates were observed for small dark colonies with green metallic sheen on EMB for *E. coli,* pink to white colonies surrounded by red zone on BGA for *Salmonella* sp and yellow colonies on MSA for *S. aureus*. All the isolates were subjected to Gram's staining and revealed that the isolates which were positive for *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* sp were found gram negative coccobacillary rods, gram positive purple coloured cocci in clusers and gram negative bacilli respectively. Bio chemical tests were tabulated in table number 3.

Out of 53 samples 32 *E. coli, 7 Salmonella* sp and 29 *S. aureus* isolates were obtained. The PCR assay for the detection of *E. coli, Salmonella* sp and *S. aureus* from different samples collected from lambs was standardized by using primers targeting genes stx2, invA and nuc genes respectively. Genomic DNA prepared from isolates was used as template. In the present study 32 culture positive *E. coli* isolates from different sources were screened for the presence of stx2 gene by PCR. Of which one (3.125%) isolate was found to be positive for the presence of stx2 gene at about 255 base pairs. In 7 culture positive Salmonella isolates from different sources three (42.86%) isolates were found to be positive for the presence of invA gene at about 389 base pairs in PCR assay. In 29 culture positive *S. aureus* isolates from different sources 23 (79.31%) isolate was found to be positive for the presence of nuc gene at about 279 base pairs in the PCR assay. (Figures: 1-10)

Table 3: Biochemical tests of cultural isolates – *E. coli, Salmonella* sp. and *S. aureus*

Name of the test	*E. coli*	*Salmonella* sp.	*S. aureus*
Indole	+ve	-ve	-ve
Methyl red	+ve	+ve	+ve
Voges- Proskeur	-ve	-ve	+ve
Citrate	-ve	+ve	+ve
TSI (Butt/slant/H_2S)	Y/Y/-ve	Y/R/+ve	----------
Coagulase	--------	-------	+ve
Catalase	--------	-------	+ve

Discussion

In the present investigation *E. coli* isolates were identified from different samples by culture and biochemical characterization and one isolate was positive for stx2 gene. Almost similar results were reported by Novotna *et al.* (2005) [16], Ahmed *et al.* (2010) [1], Bkheet *et al.* (2010) [4], Aklilu *et al.* (2013) [2], Eldin *et al.* (2013) [6] and Turkyilmaz (2013) [21] by culture method. The incidence in the present study by PCR assay for stx2 gene was reported by Turkyilmaz (2013) [21] and Virpari *et al.* (2013) [22].

Three isolates were carried invA gene out of seven culture positive isolates. Almost similar results were reported by Ahmed *et al.* (2010) [1] and Eldin *et al.* (2013) [6] respectively correlated with present study (13.21%). The incidence of *Salmonella* sp by cultural method was also reported by Duffy *et al.* (1999) [5], Scanga *et al.* (1999) [20], Heredia *et al.* (2001) [8], Malkawi and Gharaibeh (2004) [12], Jamshidi *et al.* (2009) and Aklilu *et al.* (2013) [2].

In the present study total 23 isolates out of 29 were positive for carrying *S. aureus* nuc gene. These samples were processed from suspected cases of suppurative pneumonia/abscess on lungs. No reports on isolation of organism and PCR based studies are available. Coagulase positive *S. aureus* is common commensal microorganisms and opportunistic pathogens in humans and animals. However, the incidence by culture method was reported by Gundogan *et al.* (2005) [7], Normanno *et al.* (2007) [15], Alzohairy (2011) [3], Nasreen *et al.* (2012) [14] and Rahimi *et al.* (2013) [19].

Summary and Conclusion

A total of 53 different samples collected from ailing lambs showed symptoms included diarrhoea, nasal discharges, dullness, lambs with mixed signs and post mortem examination showed suppurative pneumonia, congested liver, endocardial haemorrhages, enteritis and abscesses on different organs were subjected for cultural isolation of microorganisms and molecular charecterization by using PCR assay. Total 32 *E. coli,* 7 *Salmonella* sp and 29 *S. aureus* isolates were obtained and one (3.125%) isolate of *E. coli* was found to be positive for the presence of stx2 gene, three (42.86%) isolates of *Salmonella* sp were carried the invA gene and 23 (79.31%) isolates of *S. aureus* were found to be positive for the existence of nuc gene.

Infectious origin might be due to improper managemental practices including prophylactic measures, treatment schedule and sanitation due to inadequate knowledge of the farmers. Educating the farmers, about simple improvements in the flock management both ewes and lambs before, during and after lambing will be of great use in reducing the death rate in lambs, which in turn improves the economy of farmer.

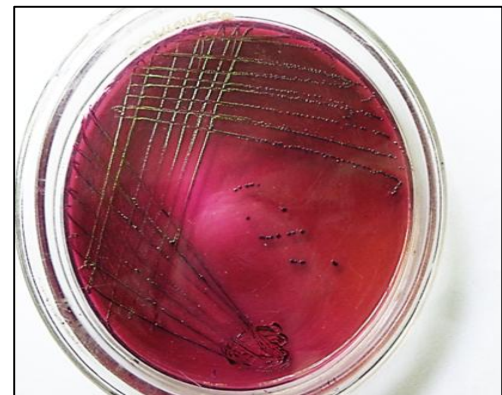

Fig 1: Plate showing green metallic sheen of *E. coli* on EMB agar

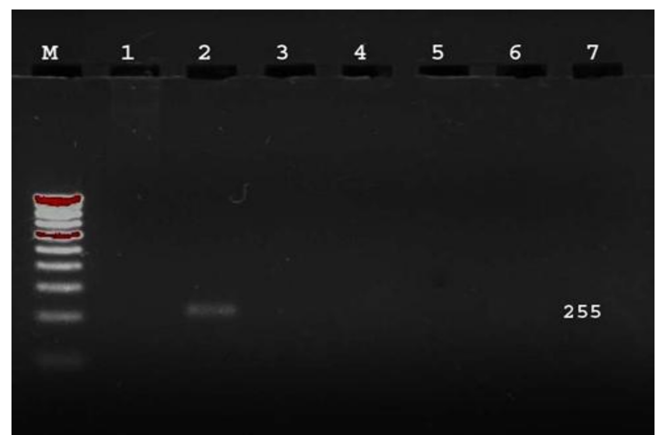

Fig 3: Detection of stx2 gene by PCR assay Lane M: 100bp DNA marker, Lane 2: Positive for stx2 gene; Lane 3, 5 and 7: Negative for stx2 gene

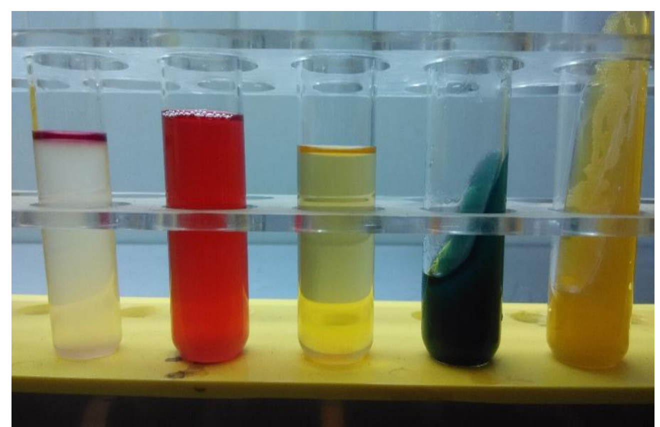

Fig 2: Tests showing results for IMViC (+, +, -, -) and TSi reaction (acid slant, acid butt & gas production) for *E. coli.*

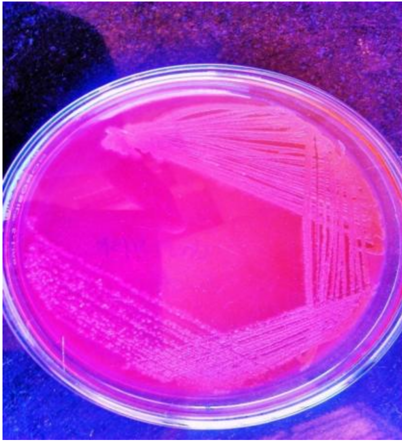

Fig 4: Plate showing *Salmonella* on BGA agar

Fig 6: Detection of invA in *Salmonella* gene. M: 100 bp DNA marker. Lane 2, 6 and 7 are positive for invA gene.

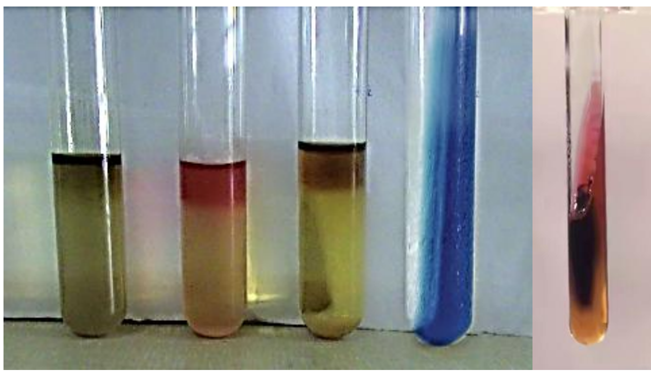

Fig 5: Tests showing results for IMViC (-, +, -, +) and TSi reaction (acid butt, alkaline slant with H_2S & gas production) for Salmonella

Fig 7: Plate showing *S. aureus* on MSA agar

Fig 8: Slide showing catalyse positivity (right side)

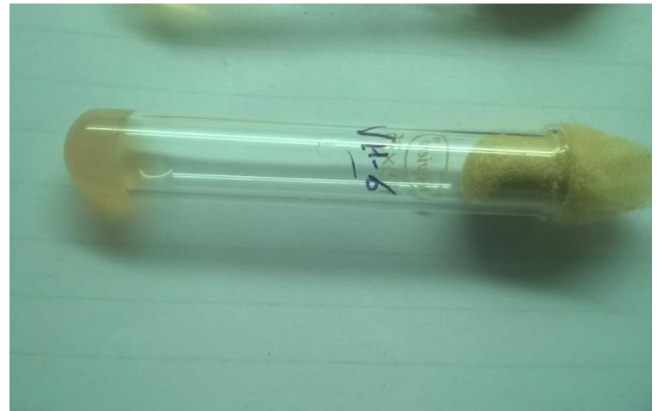

Fig 9: Tube coagulation positivity

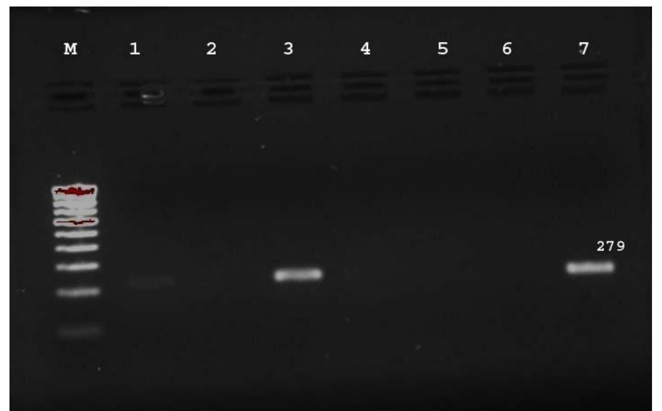

Fig 10: Detection of nuc gene in *S. aureus* Lane M: 100bp DNA marker Lane 1, 3 and 7: Positive for nuc gene Lane 6: Negative for nuc gene

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