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Isolation and Molecular Detection of Antimicrobial Resistance Genes in *Escherichia coli* and *Staphylococcus* Species from Joint Ill Cases of Calves in Puducherry

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ABSTRACT

Background: Joint ill is a septicemic polyarthritis condition due to the localization of pathogenic bacteria within the joints of young calves. The emergence of antibiotic resistant pathogen in such cases may lead to further complication in treatment. Thus, the present study was aimed to isolate and identify the bacterial pathogens and its antimicrobial resistant genes from joint ill cases in calves.

Methods: A total of 31 pus swabs were collected from joint ill cases from calves and subjected for bacterial isolation and identification by phenotypic and genotypic method followed by determination of its antimicrobial resistant genes (*tet* and *mec*A gene) and antibiogram.

Result: Based on colony characters, microscopic observation, biochemical tests, 17/31 (54.83%) *Escherichia coli* and 14/31 (45.16%) *Staphylococcus aureus* were isolated and identified phenotypically. All the isolates were further confirmed by polymerase chain reaction using *E. coli* and *S. aureus* species specific primers. The antimicrobial resistant genes carrying *E. coli* and *S. aureus* isolates were also detected, in which 4/17 (23.5%) isolates were positive for *tet* gene and 4/14 (28.57%) were positive for the *mec*A gene. The antibiotic susceptibility test showed that the isolates were highly sensitive to Enrofloxacin (100%) and Gentamicin (77%), but were resistant to Amoxyclav (70%) and tetracycline (50%). On conclusion, *E. coli* and *S. aureus* are the most common bacterial pathogens identified from this study. The presence of antimicrobial resistant genes (*tet* and *mecA*) and antibiogram pattern of the bacterial isolates indicating the possible treatment failure and serious public health risks in future.

Key words: Antibiogram, *E. coli,* Joint ill, MRSA, *S. aureus*, *Tet.*

INTRODUCTION

Joint ill is a septicemic polyarthritis condition, primarily affects young calves and neonates in the age group of one week to one month. It is considered to be one of the major cause of calf mortality and leads to significant economic loss to the livestock farming (Bagga *et al.,* 2009). Severe inflammation of the affected joints as a result of infection from the umbilicus and its associated structures causes critical lameness in young farm animals (Abdulla *et al.,* 2015). Prevalence of arthritis in cattle is about 8.34% in India (Rao *et al.,* 2020). Localization of bacteria within the joints to cause an infectious arthritis. Bacteria enter the bloodstream from the gut and upper respiratory tract in calves born in poor sanitary conditions with delayed or inadequate colostrums intake (Lorenz *et al.,* 2011).

Joint illness, also known as neonatal arthritis, is a result of unsanitary environmental conditions in the calving shed and improper umbilical cord asepsis (Radostitis *et al.,* 2007). The clinical signs include varying degrees of lameness, fever, inappetence, synovial effusion, lameness, hot and swollen joints with open wounds, limited range of joint motion, pain on manipulation and flexion of the affected joints and varying levels of hypersensitivity.

The most common cause of joint illness is bacteria, which includes *Escherichia coli, Corynebacterium sp, Staphylococcus aureus* and *Pseudomonas aeruginosa*. These bacteria commonly inhabits the environment, food and warm blooded animals lower gut, skin and urinary tract etc (Tamilarasu *et al.,* 2020). For successful treatment of joint ill, early diagnosis is essential. The choice of antibiotic will depend on the causative bacterial pathogen (Robson, 2003).

Antimicrobial resistance occurs when microorganisms develop resistance to antimicrobial drugs such as antibiotics over time. Antibiotic resistant bacteria (superbugs) are of emerging problem now a days both in human and veterinary medicine (Chaodhary *et al.,* 2017, Venkatvasan *et al.,* 2020, Devanathan *et al.,* 2024). The widespread use of antibiotics has resulted in the emergence and spread of resistance genes which leads to an imbalance in the system as well as in environment's

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microbiota (Thakur *et al.,* 2018, Indhuja *et al.,* 2021). Inappropriate antibiotic usage and delayed treatment results in the formation of irreversible lesions in the joint tissue of calves.

Antibiotics commonly used for treating joint infections include penicillin and tetracycline. The mechanism of antimicrobial resistance (AMR) in Staphylococcus aureus is particularly notable, as it has become a prominent superbug resistant to β-lactam antibiotics, including methicillin, resulting in methicillin-resistant Staphylococcus aureus (MRSA) (Dookie *et al.,* 2016). The resistance to methicillin is attributed to the presence of Penicillin-binding protein 2a (PBP 2a), which has a low affinity for β-lactam antibiotics and is encoded by the mecA gene (Peacock and Paterson, 2015).

Tetracycline resistance in various bacterial species arises from multiple factors, including the acquisition of mobile genetic elements, mutations in ribosomal binding sites and chromosomal mutations that enhance the expression of intrinsic resistance mechanisms. Increased antibiotic resistance in animals may have great impact on human health especially in an environment where animals and humans share the same ecosystem. (Pavelquesi *et al.,* 2021).

Although there are various studies on detection of bacteria causing joint illness in calves (Naik *et al.,* 2011, Goodarzi *et al.,* 2015, Jalal *et al.,* 2016), there is no reports on antibiotic resistant determinants among the bacterial pathogens causing joint ill condition of calves in India. Hence, the present study was aimed to isolate and identify the bacterial pathogens from a joint ill cases in calves at Union Territory of Puducherry (India) and to detect the presence of antibiotic resistant genes in them.

MATERIALS AND METHODS

Source of samples

A total of 31 pus swabs were collected aseptically from joint ill cases presented to the Veterinary Clinical Complex (VCC), Rajiv Gandhi Institute of Veterinary Education and Research (RIVER), Puducherry, India, with the history of painful and swollen joints with pus accumulation and the samples were collected during the period of January 2023 to August 2023 (8 months). The samples were transported to the Department of Veterinary Microbiology, RIVER, Puducherry under cold chain for the bacterial isolation and further microbiological analysis.

Isolation and identification of the bacteria

The samples were inoculated onto Luria broth and incubated at 37°C for 16 to 18 hours. The inoculum were streaked onto Muller Hinton agar. Further isolation was done by sub culturing onto MacConkey's agar, Eosin-methylene blue agar and Mannitol salt agar. The isolated colonies in the culture medium were subjected to Gram's staining and conventional biochemical methods includes catalase, oxidase, IMViC (Indole, Methyl red, Voges-Proskauer and Citrate), urease and sugar fermentation tests for species-level identification as per Cowan and Steel (1974). The biochemically confirmed *E. coli* and *S. aureus* isolates were further subjected to molecular detection using polymerase chain reaction (PCR).

Molecular detection of *E. coli* and *S. aureus*

The template DNA was extracted from colonies by boiling and snap chilling method as described by Zhang *et al.,* (2015). The PCR was carried out with the primers targeting *alr* genes specific for *E. coli* with the product size of 366 bp (Yokoigawa *et al* 1999) and *nuc gene* specific for *Staphylococcus aureus* with the product size of 270 bp (Brakstad *et al.,* 1992) as described in Table 1. For both the genes, PCR assay was optimized with 12.5 µl reaction mixture containing 2.5 µl of DNA template, 6.25 µl of 2 X master mix (Taq amplicon red Mix, Emerald), 1 µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease free water for 31 reactions. The cycling conditions were as follows: initial denaturation at 95°C for 6 min; 35 cycles of 95°C for 1 min, 55°C for 45 sec and 72°C for 45 sec and a final elongation step at 72°C for 5 min. The PCR amplification was carried in an automated thermal cycler (Eppendorf Master Cycle, Germany). The PCR products were analyzed by 1.5% agarose gel electrophoresis, visualized under UV transilluminator.

Molecular detection of antimicrobial resistance gene

All the PCR positive *E. coli* and *S. aureus* isolates were further screened for the presence of AMR gene by PCR using the primers targeting *tet* and *mec A* respectively (Table 1). For both genes, the PCR assay was optimized using a 12.5 µl reaction mixture, as described earlier for

Table 1: Details of the Primers used in this study.

Primer	Sequence	Size (bp)
alr (Yokoigawa *et al*., 1999)	CTGGAAGAGGCTAGCCTGGACGAG	366
	AAAATCGGCACCGGTGGAGCGATC	
nuc (Brakstad *et al*., 1992)	GCGATTGATGGTGATACGGT	270
	AGCCAAGCCTTGACGAACTAAAGC	
tet (Olobatoke and Mulugeta, 2015)	GCACTTGTCTCCTGTTTACTCCCC	700
	CCTTGTGGTTATGTTTTGGTTCCG	
mecA (Oliveira and de Lencastre, 2002)	TCCAGATTACAACTTCACCAGG	162
	CCACTTCATATCTTGTAACG	

PCR reaction mixture preparation for *E. coli* and *S. aureus*. The amplification process with the annealing temperature of 53°C for 1 min for *tet* (Olobatoke and Mulugeta, 2015) and 50°C for 1 min for *mec A* (Oliveira and de Lencastre, 2002) was followed. The PCR amplification was carried in an automated thermal cycler (Eppendorf Master Cycle, Germany). The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized under UV transilluminator. The PCR products which yielded 700 bp and 162 bp was considered positive for *tet* and *mec A* gene of *E. coli* and *S. aureus* respectively.

Antibiotic sensitivity test (ABST)

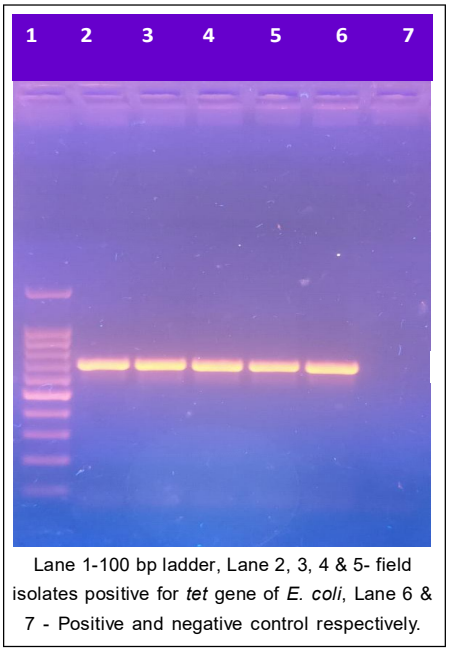
All the isolates were subjected to antibiotic sensitivity tests using 8 antibiotic agents by the disc diffusion method (Bauer *et al*., 1966). The inoculum was prepared from a single bacterial colony picked up with sterile loop from at least 4-5 well isolated similar type colonies and inoculated into 5 ml sterile Luria broth and then the inoculated broth was incubated at 37°C until a slight visible turbidity appeared usually within 2-4 hours, so as to correspond with McFarland tubes. The incubated broth was swabbed and lawn cultured over the entire surface of the MH agar plate three times, with the plate rotated approximately 60° each time to ensure even distribution of the inoculum. A final sweep of the swab was made around the agar rim. The antimicrobial agents used were Enrofloxacin (EX, 5 µg), Tetracycline (TE, 30 µg) Cefotaxime (30 µg), Co-Trimoxazole (COT, 25 µg), Amoxyclav (AMX, 5 µg), Gentamicin (GEN, 10 µg), Pencillin-G (10 µg) and Ceftriaxone (CTR, 30 µg) were used in this study. The inoculated plates were incubated at 37°C for 24 hrs. The interpretation of zone of inhibition was read as per Clinical Laboratory Standard Institute guidelines (CLSI, 2022. The reference strain *S. aureus* MTCC 87 and *E. coli* ATCC 25922 maintained in the Department of Veterinary Microbiology, RIVER, was used as positive control for genus and species specific PCR assay.

RESULTS AND DISCUSSION

Joint ill usually occurs less than 1 year of age in calves, as a result of inflammation due to infection of the tissue of the umbilicus after parturition in dirty environment (Blowey and Weaver, 2011). The tissue of umbilicus get infected by bacterial contamination soon after parturition in unhygienic areas. Among 31 samples, 17 lactose fermenting colonies in MacConkey's agar and 14 mannitol fermenting colonies in mannitol salt agar (Fig 1 and 2) were obtained. The isolated organisms were identified as *E. coli* (54.84%) and *S. aureus* (45.16%) respectively by conventional biochemical tests. The results of morphological, cultural and biochemical tests were in agreement with the reports of Nuss (2012), Goodarzi *et al.* (2015), Waseem *et al.* (2016), Ruban *et al*. (2018) and Debbarma *et al.* (2020).

Fig 1: Lactose fermenting colonies in Mac conkey's agar.

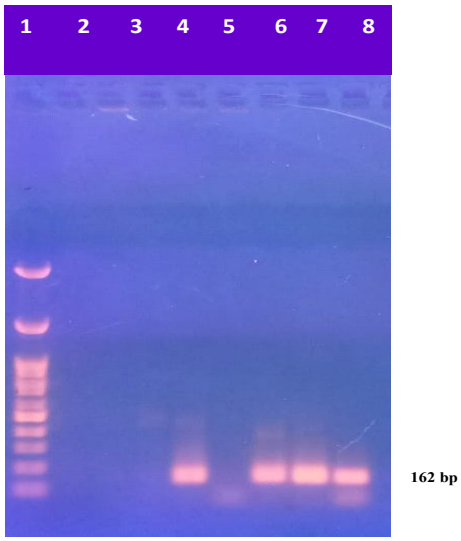
Fig 2: Mannitol fermenting colonies in Mannitol salt agar.



Lane 1-100 bp ladder, Lane 2, 3, 4 & 5- field isolates positive for *tet* gene of *E. coli*, Lane 6 & 7 - Positive and negative control respectively.

Fig 3: Agarose gel electrophoresis showing the results of PCR amplified product of *tet* gene with the product size 700 bp.

All the 17 (54.84%) isolates were further confirmed as *E. coli* using PCR assay by targeting *alr* gene with the amplicon size of 366 bp and 14 (45.16%) isolates were confirmed as *S. aureus* by amplifying the *nuc* gene with the product size of 270 bp. The study conducted by Waseem *et al.,* (2016) reported 9/20 (45%) *S. aureus* isolates from synovial fluid samples from kids in Uttarpradesh. Lower incidence of *E. coli* (2, 5%) and *S. aureus* (12, 30%) isolates were detected from the synovial fluid of calf in Iran were reported by Goodarzi *et al.,* (2015). Jalal *et al.* (2016) found the 2.47% prevalence rate of joint ill in calves at Bangladesh and Wudu *et al.* (2008) detected



Lane 1-100 bp ladder, Lane 2, 3 –Negative field isolates, 4 and 5 Positive and Negative control, Lane 6, 7 & 8 – field isolates positive for *mec A* gene of *S. aureus* isolate.

Fig 4: Agarose gel electrophoresis showing the results of PCR amplified product of *mec* A gene with the product size 162 bp.

6% prevalence rate in Ethiopia which were lower than the report in our study. It might be due to differences in the geographical area. Anderson and Rings (2008) reported that most umbilical infections are caused by *Actinomyces pyogenes* and *Escherichia coli* being the second most commonly isolated bacteria and is believed to be the most likely cause of systemic infection and septic polyarthritis.

According to this study, the occurrence of joint ill was higher in crossbred female calves (57.14%) than male calves (42.86%) which are supported by the research findings of Ramanathan (2007), Goodarzi *et al.*, (2015), Jalal *et al.* (2016), Dogan *et al.*, (2016) and Ibrahim (2019). Septic arthritis was frequently reported as neonatal polyarthritis in young calves (Blowey and Weaver (2011). An abnormal birth circumstance was also may be a reason for increased susceptibility to neonates to septic arthritis in calves (Firth, 1983). In our study, 24 animals were less than 1 year of age (77.42%) and 7 animals were more than 1 year of age (22.58%). Similar findings were also reported by Jackson (1999) in first 8 weeks of age. Rohde *et al.* (2000) observed higher incidence in calves of below 1 year age (70.49%) than adults (29.51%). Whereas Merkens *et al.* (1984) reported that the incidence was not age dependent. The failure of passive transfer of immunity lead to high risk of developing calf hood infections in neonatal calves (Wittum *et al.*, 1985, Blowey and Weaver, 2011).

All physiological parameters were within the normal range, with the exception of pyrexia, which was present in all cases upon presentation. Following treatment, body temperature returned to normal levels. The elevated body temperature observed served as a clinical indicator of systemic involvement in the context of arthritis which is also reported by Mee, *et al.,* (2008).

Among the 17 *E. coli* isolates, 4/17 (23.53%) isolates were harboring *tet* gene and among 14 *S. aureus* isolates, 4 isolates (28.57%) found to harbour *mec*A gene by PCR (Fig 3

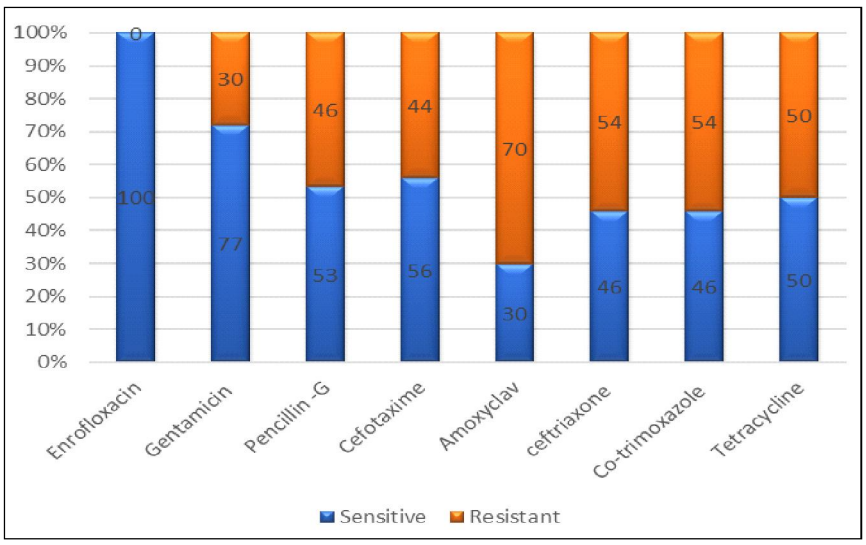


Fig 5: A bar diagram showing the antibiogram results (in percentage).

and 4). Even though only 4 isolates found to harbour *tet* gene responsible for that particular drug resistance, phenotypic disk diffusion method shows 50% of the *E. coli* isolates were resistant to tetracycline on the contrary to the study by Jalal *et al*., (2016), Paul *et al.,* (2010) and Sunder *et al.* (2021) who reported that the *E. coli* isolates were sensitive to tetracycline and doxycycline. MRSA has emerged as a significant animal health problem in veterinary medicine and resistant is mediated by *mec*A gene that encodes the production of a modified penicillin-binding protein. In the present study also, a high prevalence (28.57%) of MRSA was noticed which was in accordance with Jalal *et al.* (2016) and Nuss (2012).

Antibiogram patterns obtained from joint ill cases in this study showed resistant to most commonly used antibiotics (Fig 5). All the isolates were highly sensitive with Enrofloxacin (100%) and Gentamicin (77%), but were resistant to Amoxyclav (70%) and tetracycline (50%). Enrofloxacin and Gentamicin was detected as the most effective antibiotic which was in agreement with the previous reports of Goodarzi *et al.,* (2015).

CONCLUSION

The present study showed that *E. coli* and *S. aureus* is the frequently isolated bacteria from joint ill cases in calves in Puducherry region. Enrofloxacin and Gentamicin were found to be the most effective antibiotics and the isolates showed resistant to antibiotics such as Amoxyclav, Ceftriaxone and tetracycline. The incidence of tetracycline and methicillin-resistance genes might probably be the result of indiscriminate use of antibiotics in large animal practice in Puducherry region. Further studies on antibiotic resistance genes in *E. coli* and *S. aureus* may help to understand the prevalence of such AMR infection and to formulate control strategies against them.

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Conflict of interest

The authors declare that they have no conflict of interest.

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