Int.J.Curr.Microbiol.App.Sci (2020) *9*(9): 3540-3551

EXCELLENT PUBLISHERS

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 9 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.909.439

Detection of Aflatoxins in Milk and Feed from Cases of Reproductive Tract Disorders in Cattle and Sheep

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Keywords

Brucella, Aflatoxin, PCR, Milk, Feed

Article Info

Accepted:
26 August 2020
Available Online:
10 September 2020

ABSTRACT

In this study, a total number of 135 samples were collected and out of them 80 samples were from reproductive tract, 25 feed samples and 30 milk samples from cattle and sheep. Samples collected from reproductive tract of cattle and sheep were subjected to bacteriological and fungal examination for isolation and identification. A total of 105 bacterial isolates have been found from 68 primary bacterial cultures out of which 50 isolates (47.61%) were found to be Gram-positive and 55 isolates (50.47%) were Gram-negative. Out of mixture of gram positive isolates, 31 isolates (62%) were confirmed to be *Bacillus* spp., 14 isolates (28%) *Staphylococcus* spp., 3 isolates (6%) *Streptococcus* spp. and 2 isolates (4%) were *Arcanobacterium pyogenes*. Among all Gram-negative isolates, 3 isolates were found to positive for *Brucella abortus*, 30 for *E. coli*, 13 for *Klebsiella pneumoniae*, 7 for *Proteus* spp. and 2 for *Pseudomonas aeruginosa*. Amid abortion specific bacteria, 3 isolates have been confirmed to be positive for *Brucella* sps. by polymerase chain reaction using genus specific primers. A total of 34 fungal isolates were obtained from 68 fungal cultures, out of which a total of 8 fungal species from 6 genera have been identified. Major proportion of these isolates belong to *Aspergillus* spp. (41.17%), followed by *Alternaria* spp. (26.47%), *Penicillium* spp. (8%), *Pithomyces* spp. (8%) and *Rhizopus* spp. (8%) and *Mucor* spp. (5.8%). and are confirmed by molecular method using genus and species specific primers. Collected 25 feed and 30 milk samples were examined for aflatoxin contamination. A total of four feed samples were found to have aflatoxin contamination above the permissible limit i.e. 30ppb according to Indian standards. The level of aflatoxin contamination in feed samples was in between of 4 ppb and 52 ppb. All 30 collected milk samples were tested negative for aflatoxin contamination.

Introduction

Reproductive potency is one of major factors that build the livestock industry financially and animal health-wise well built. The elements effecting reproduction potency of domestic animals have serious influence on the economy of dairy industry producers. The sources of reproductive tract infections like repeat breeding and abortion are many and extent from simple management defects to various diseases because of some various

factors like physical, chemical, biological and environment factors (Yoo 2010). Reproduction failure occurs due to many causes but infectious causes are the most regularly identified. Infections may occur due to a wide variety of infectious agents like bacteria, virus, fungi and protozoa have direct effect on reproductive tract of cattle and sheep (Wolf-Jäckel *et al.*, 2020). These infectious agents have not only remarkable effects on dairy industry but also affect the human health due to zoonotic potential (Staric *et al.*, 2020). Genital tract infections can occur at any stage but these infections are common after parturition because of opening of cervix and immune compromised condition of uterus (Sheldon *et al.*, 2020). After parturition uterus epithelium is damaged and has debris which supports microbial growth (Konigsson *et al.*, 2002). The damaged physical barrier of cervix, vagina and vulva enhance the entry microbes into the reproductive tract of animal from the outside (Sheldon and Dobson 2004). Infection requires the first step to be established i.e. attachment of the organism on the mucosa followed by penetration of surface epithelium and liberation of toxins that ultimately leads to uterine diseases (Sheldon *et al.,* 2006). Majority of reproductive tract infections caused by bacteria such as *Brucella abortus, Brucella melitensis, Brucella ovis, Campylobacter jejuni, C. fetus* subsp. *fetus, Leptospirainterrogans* and *Listeria monocytogenes*. Some non-specific bacteria like *Salmonella* subspecies Dublin and others like *E. coli, Arcanobacterium pyogenes, Staphylococcus* spp.*, Streptococcus* spp.*, Proteus* and *Pseudomonas aeruginosa* also cause reproductive tract infections (Gani *et al.,* 2008).

Espinel-IngroffA (2009) observed that fungal infections cause wide spread and prevalent mycotic disease in man and animals. These infections are a significant reason of illness and death rate mainly in immune compromised animals. Abortions are caused by fungal infections but not as much as bacterial infections. The most important fungal species responsible for abortions are *Aspergillus*, *Mucor*, *Absidia* and *Rhizopus* which make up to 60-80% of total mycotic abortions. Jensen *et al.,* (1991) studied that *Aspergillus fumigates* constitute the major cause of bovinemycotic placentitis and abortion as it spreads to the placenta. Many researchers have isolated pure cultures of *Aspergillus fumigates* and *A. niger* in pure culture from cases of abortion in ewes (Siddique *et al.,* 1976).

Mycotoxins are considered to be the harmful secondary metabolites produced by the growing fungi present in processed food and feed, either in field or during storage. More than 100 species of fungi are known to produce mycotoxins and about 400 secondary metabolites with toxigenic activity are produced by these fungi. These fungi belong to the different genera like *Penicillium*, *Aspergillus*, *Fusarium* and *Claviceps* (Quinn *et al.,* 1994). About 5-10% of agricultural products all over the globe are destroyed by fungal which is not suitable for humans and animals consumption (Ephrem Guchi, 2015).Because of inferior drying, storage and post-harvest waste are quite significant in developing countries like India. Fungi are saprophytic in nature present in environment abundantly and germinate on food material and produce mycotoxins under favourable conditions (Brera *et al.,* 1998).

Different types of disease causing mycotoxins include aflatoxin ochratoxins, citrinin, sterigmatocystin, alkaloids, zeralenone etc. out of which aflatoxin is commonly present as contaminants in animal feed. Effects caused by mycotoxins may not be instantly found in ruminants but incidence of decreased fertility or high rate of abortions may happen (Anonymous 2003).

Aflatoxins are very toxic, carcinogenic as well as mutation causing and teratogenic immunosuppressive metabolites found in fungal spp. like *Aspergillus flavus* and *A. parasiticus* and *A. nomius* derivatives of difuranocoumarin. B_1, B_2, G_1, G_2 these four aflatoxins are considered to be naturally produced aflatoxins and the B and G nomenclature are specific based on their expressionof blue and green fluorescent colours when viewed under UV light on thin layer chromatography plates (Edwards *et al* 2002). Out of four aflatoxins, aflatoxin B_1 is most potent carcinogenic and is excreted in milk of lactating animal in the form of aflatoxin M_1 that have consumed the AFB_1contaminated feed. Aflatoxin contaminated feed taken by animal for a long time in low doses, they produces slow and continuous damage to the body of animal. The Food and Drug Administration (FDA) limits AFB not more than 20 ppb in lactating dairy feeds and to 0.5ppb in milk (Regis *et al.*, 2019). Animal taking feed having 30ppb aflatoxin produce milk containing AFB residues above the FDA action level of 0.5 ppb. Aflatoxicosis results in reduced feed efficiency, lactation yield, diminish the disease resistance and also can interfere with acquired immunity due to vaccination in livestock and thus making them more prone to various diseases (Diekman and Green, 1992). The purpose of this study was to observe the post effect of aflatoxin contamination in feed given to the animals suffering from reproductive tract disorders.

Materials and Methods

Sampling

Reproductive tract samples

A total 80 samples of reproductive tract disorders consisting of vaginal mucus, cervical mucus, uterine discharges, aborted foetus stomach contents, placenta were taken from cattle and sheep having reproductive tract disorders such as abortion, retained placenta, repeat breeding, anoestrus, pyometra, metritis and endometritis. Based on clinical signs and history of animals, samples were collected in a sterile sample container from different dairy farms in Ludhiana, Punjab as well as the reproductive tract cases of cattle coming in GADVASU clinical complex and adjoining areas of Punjab. Samples were processed for bacterial and fungal isolation by inoculating on suitable media and studying their cultural/colonial characteristics. Samples were stored at 4˚C until processed, if processing had to be delayed.

Feed and milk samples

The feed and milk samples collected from same farm where the clinical samples of reproductive tract disorders were collected. These samples were collected in and adjoining areas of Punjab. The total of 25 feed samples were processed for presence of aflatoxin by Pressure Mini Column method which was detected by visual method of measuring the intensity of fluorescence under UV chamber at a wavelength of 365nm. The feed samples were further processed for quantification of aflatoxin using an improved aflatoxin detection kit devised by charms sciences USA, provided by JC Bioage, Hyderabad. Total 30 milk samples were processed for presence of aflatoxins by qualitative method using Charms SL Aflatoxin M_1 Test.

Molecular detection of the isolates

Genus specific Polymerase Chain Reaction (PCR) for confirmation of *Brucella* isolates from clinical samples

Brucella isolates were confirmed by using genus specific PCR primersB4/B5 (Bailey *et*

al., 1992) (Table 1 and 2). Colonies from inoculated clinical samples were taken and used for DNA isolation. These colonies were earlier subjected to various biochemical tests for confirmation of *Brucella*.

Extraction of genomic DNA

Genomic DNA from *B. abortus* strain19 and clinical isolates were extracted by hot-cold lysis method (Dashti *et al.,* 2009). 500 µl of normal saline solution was taken in an Eppendorf tube. Suspected colonies were taken and suspended in tube and mixed properly with normal saline solution. Tube was closed and subjected to 100˚C in a dry bath apparatus for 10 minutes. After this incubation, tube was transferred immediately to -20˚C to give a temperature shock. Tubes were kept in cold temperature for 30 minutes. The contents of tube were then mixed properly by pipetting the contents of tube in and out. The DNA obtained was used in further molecular work.

Standard/Reference strains

*B. abortus*S19 and *B. abortus* S99 available in Department of Veterinary Microbiology, GADVASU, Ludhiana, and (Punjab) were used as reference strains for molecular work. These isolates were preserved by regular sub-culturing after every 15 days on BSM plates.

PCR protocol (B4/B5)

PCR reaction mixture was prepared for one reaction in a volume of 25µl containing 12.5µl master mix, 1µl forward primer, 1µl reverse primer, 5.5µl nuclease water, 5µl of template DNA. PCR cycling conditions are depicted in Table 3 and consisted of initial denaturation at 94˚C for 5 min, followed by 35 cycles each of 94˚C for 1 min (denaturation), 65˚C for 1 min (annealing) and 72˚C for 1 min (extension) and a final extension of 72˚C for 10 min.

Analysis of PCR product

The PCR product were evaluated by gel electrophoresis using 1% agarose gel in 1X TBE buffer containing ethidium bromide at 90 V for 1 hour and visualized under Alpha Imager 3400HP Gel Documentation System and photographed Fig. 1.

Molecular detection of *Aspergillus* spp.

Polymerase chain reaction (PCR) for confirmation of *Aspergillus* isolates from clinical samples

Extraction of fungal DNA from fungal cultures

The DNA was extracted from fungal cultures suspected to be *Aspergillus* as well as from standard cultures by Chloroform Isoamyl alcohol method (Moller *et al* 1992) using liquid nitrogen. A volume of 30-60 mg of mycelia was ground quickly in 50 ml liquid nitrogen in sterilized pestle and mortar for extraction of DNA. The powdered mycelia were rinsed with a volume of 500 µl of TES buffer and collected in an Eppendorf tube. A volume of 3.5 µl of Proteinase K was added and tube was kept at 60˚C for 1 h with gentle mixing. A volume of 140 µl of NaCl solution along with 65 µl of CTAB was added to it and incubates at 65˚C for 10 minutes. 700 µl of Chloroform-Isoamyl alcohol solution was added and mixed gently. Then tube was incubated at 0˚C for 30 minutes and was centrifuged at 14000g for 10 minutes at 4˚C. The supernatant was transferred to a fresh Eppendorf tube to which 225 µl of 5M ammonium acetate was added. It was mixed gently and place on ice for 30 minutes. The tube was centrifuged at 14000g for 10 minutes at 4˚C. 510 µl of ice cold isopropanol alcohol was added to an Eppendorf tube and supernatant was also transferred to this tube for precipitating DNA. Then tube was

centrifuged at 14000g for 5 minutes at 4˚C. DNA lumps become visible as pellet and supernatant was drained off.

The pellet was washed twice with chilled 70% ethanol and was made air dried. The pellet was reconstituted in 50 µl TE buffer. 1 µl RNase stock solution was added and tube was incubated at 37˚C for 45 minutes before storing it at -20˚C.

Primers Used

Analysis of PCR product

The amplified product were analyzed by gel electrophoresis at 80 V for 1 hour in 1% agarose gel in 1X TBE buffer containing ethidium bromide and visualized under Alpha Imager 3400 HP Gel Documentation System and photographed figure 2, 3 and 4.

Detection of aflatoxin in feed and milk

Feed analysis through PMC

Each feed sample (10g) was taken in a conical flask and 50 ml acetone water was added to it. Then it was shaked on orbital shaker for 30 minutes. The solution was filtered using WFP No. 1.filterate was taken in large test tube and equal volume of 20% lead acetate solution was added to it and it was mixed thoroughly for 3 min. it was again filtered through WFP No. 1.

Then 2 ml benzene was added to the filtrate and mixed thoroughly and was allowed to stand for 2 min. upper layer was collected in another tube containing 400 mg of natural alumina, mixed and allowed to stand for 2 min. The detected aflatoxins were quantitated by aflatoxin quantitation kit using a strip method base on lateral flow assay principle provided by Charm Sciences, USA which could detect total aflatoxin present in feed. This kit has detection range of 0- 150ppb.

Qualitative detection of aflatoxin in milk

The Charm SLAflatoxin M1 Test (SLAFM) provides quick and exact results using ROSA® (Rapid One Step Assay) lateral flow technology. SLAFM facilitates dairies and food manufacturers to observe the level Aflatoxin M1 at 350 ppt in raw, mixed cow milk. This qualitative test provides a useful method for customers where a fast screening test is needed and Achieves results in 3 minutes.

Results and Discussion

A total of 80 samples of reproductive tract of cattle and sheep were collected out of which 68 yielded 105 isolates of bacteria and 30 isolates of fungi. The bacteria isolated from samples were *Brucella abortus, E. coli, Arcanobacterium pyogenes, Bacillus spp., Klebsiella pneumoniae, Staphylococcus spp., Streptococcus spp., Pseudomonas aeruginosa* with incidence of 2.80%, 28.57%, 1.90%, 29.52%, 12.38%, 13.34%, 2.8%, 1.90% respectively (Table 4 and 5). Out of 80 samples only 30 samples yielded 34 isolates of fungi of which 10 were *Alternaria* spp., 14 *Aspergillus* spp., 2 *Mucor* spp., 3 *Rhizopus* spp., 3 *Penicillium*spp., 3 *Pithomyces* spp. (Table 6).

A total 30 milk samples were tested for detection of aflatoxin. All of 30 samples were found negative for aflatoxin.

Reproductive tract infections constitute the most important problem causes great economic implications in terms of milk yield, meat production, wool production and fertility of animals.

Table.1 Sequence of primers used for detection of genus *Brucella*

Name of primers	Gene	Sequence (5'-3')	Size of amplified product	Reference
B4	*bcsp*31	TGG CTC GGT TGC CAA TAT CAA	**223 bp**	Bailey *et al* (1992)
B5		CGC GCT TGC CTT TCA GGT CTG		

Table.2 Sequence of primers used in nested PCR of 18 rRNA gene in *Aspergillus* spp.

S. no.	Primers	Sequence (5'-3')	Size of amplified product	Reference
1.	ASAP1	CAG CGA GTA CAT CAC CTT GG	521 bp	Sugita, *et al* (2004)
	ASAP2	CCA TTG TTG AAA GTT TTA ACT GAT T		
2.	ASPU	ACT ACC GAT TGA ATG GCT CG	190bp	
	F12r	TTC ACT AGA TCA GAC AGA GT		
3.	ASPU	ACT ACC GAT TGA ATG GCT CG	300bp	
	Af3r	CAT ACT TTC AGA ACA GCG TTC A		

Table.3 Cycling reaction for first step PCR reactions

Stage	Step	Temperature(˚C)	Duration	No. of cycles
1.	Initial Denaturation	94	4 min	1
2.	Denaturation	94	1min	30
	Annealing	55	2 min	
3.	Extension	72	1 min	
	Final Extension	72	10 min	1

Table.4 Cycling reactions for nested PCR reactions

Stage	Step	Temperature(˚C)	Duration	No. of cycles
1.	Initial Denaturation	94	4 min	1
2.	Denaturation	94	1min	25
	Annealing	60	1 min	
3.	Extension	72	15 min	
	Final Extension	72	10 min	1

Table.5 Bacteria isolated from reproductive tract of cattle and sheep

S. No.	Bacteria isolated	Number			
1.	*Arcanobacterium pyogenes*	2 (from mixed cultures)			
2.	*Bacillus*	31	Pure	15	*B. licheniformis* (3)
					B. cereus (2)
					Other spp. (12)
			Mixed	16	
3.	*Brucella*	3 (all pure growths)			
4.	*E. coli*	30	Pure	5	
			Mixed	25	
5.	*Klebsiella pneumonia*	13	Pure	2	
			Mixed	11	
6.	*Proteus* spp.	7 (all from mixed growths)			
7.	*Pseudomonas aeruginosa*	2	Mixed	1	
			Pure	1	
8.	*Staphylococcus* spp.	14 (all from mixed growths)			
9.	*Streptococcus* spp.	3	Pure	1	
			Mixed	2	

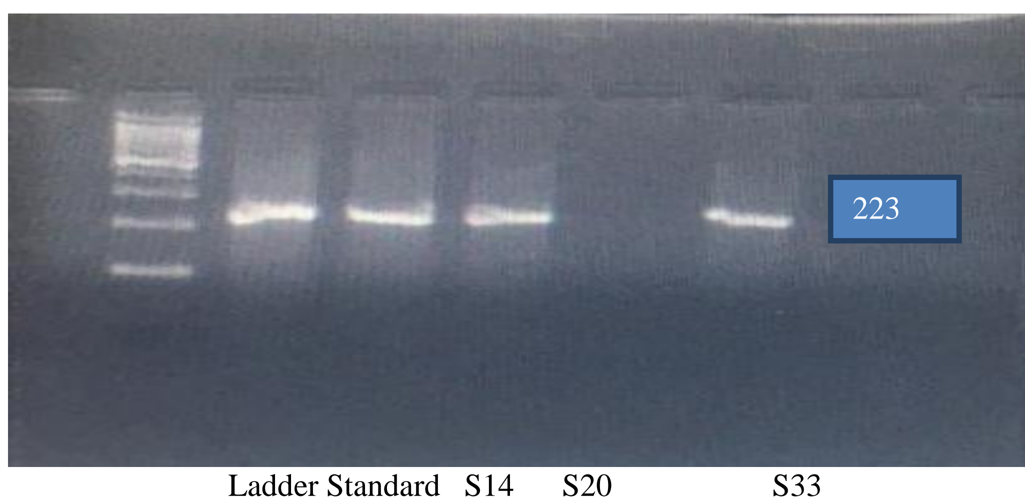
Table.6 Fungi isolated from reproductive tract of cattle and sheep

S. no.	Fungal isolates obtained		Number		
1	*Alternaria* spp.		9	Pure	5
				Mixed	4
2	*Aspergillus* spp. (total =14)	*A. flavus*	5	Pure	3
				Mixed	2
		A. fumigatus	4 all pure growths were obtained		
		A. niger	5	Pure	2
				Mixed	3
3	*Mucor* spp.		2	Pure	1
				Mixed	1
4	*Rhizopus* spp.		3 pure cultures were obtained		
5	*Penicillium* spp.		3 pure growth culture were obtained		
6	*Pithomyces* spp.		3 pure growth culture were obtained		

Table.7 Amount of aflatoxins present in feed samples

Feed sample number	Amount of aflatoxin detected/interpretation	Clinical samples correspondence to feed
F1	48 ppb (above limit)	S1,S2
F2	20 ppb (below limit)	S7
F3	24 ppb (above limit)	S 12
F4	26 ppb (above limit)	S 13, S14
F5	22 ppb (above limit)	S 18- S 20
F6	30 ppb (above limit)	S 21
F7	15 ppb (below limit)	S 22
F8	4 ppb (below limit)	S23 , S 24
F9	15 ppb (below limit)	S25
F10	16 ppb (below limit)	S 26
F11	20 ppb(below limit)	S 27
F12	16 ppb (below limit)	S 28
F13	10 ppb (below limit)	S 29
F14	9 ppb (below limit)	S 36
F15	52 ppb (above limit)	S 37- S 43
F16	6 ppb (below limit)	S 45
F17	32 ppb (above limit)	S 47
F18	8 ppb (below limit)	S 50- S 61
F19	25 ppb (above limit)	S 64
F20	5 ppb (below limit)	S 70
F21	20 ppb (below limit)	S72

Fig.1 Electrophoresis analysis of PCR product of amplified *Brucella abortus*



Ladder Standard S14 S20 S33

Fig.2 Electrophoresis analysis of PCR product of amplified *Aspergillus* genus

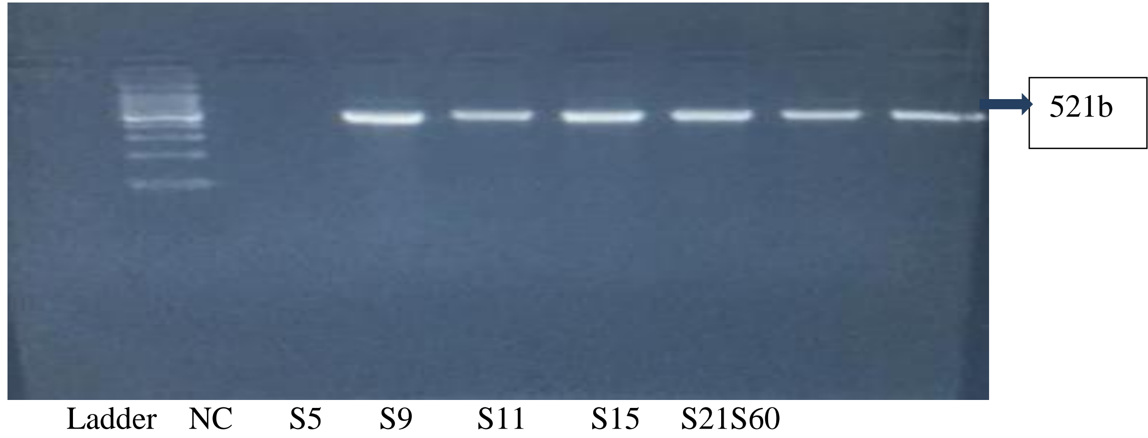


Fig.3 Electrophoresis analysis of nested PCR product of amplified *A. flavus*

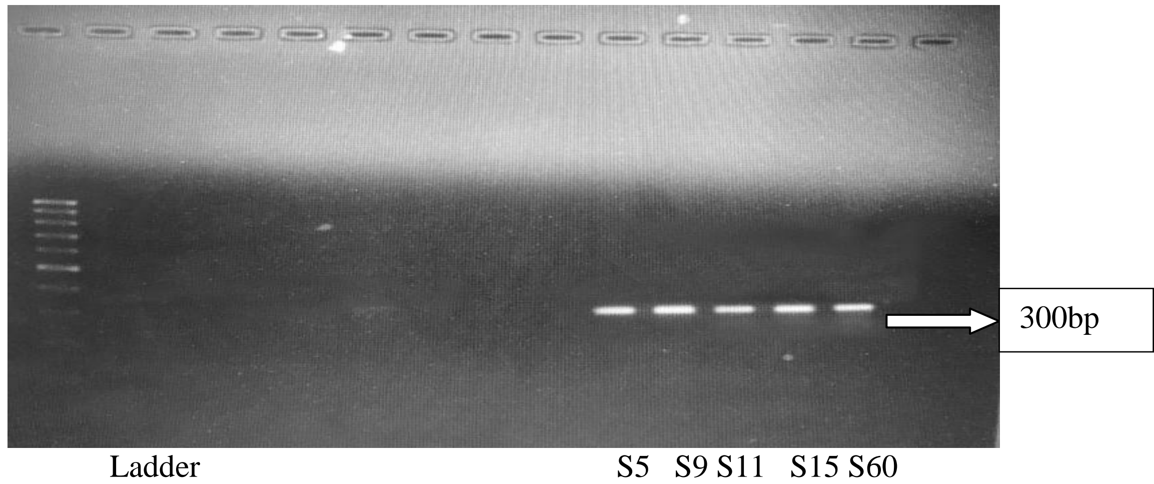
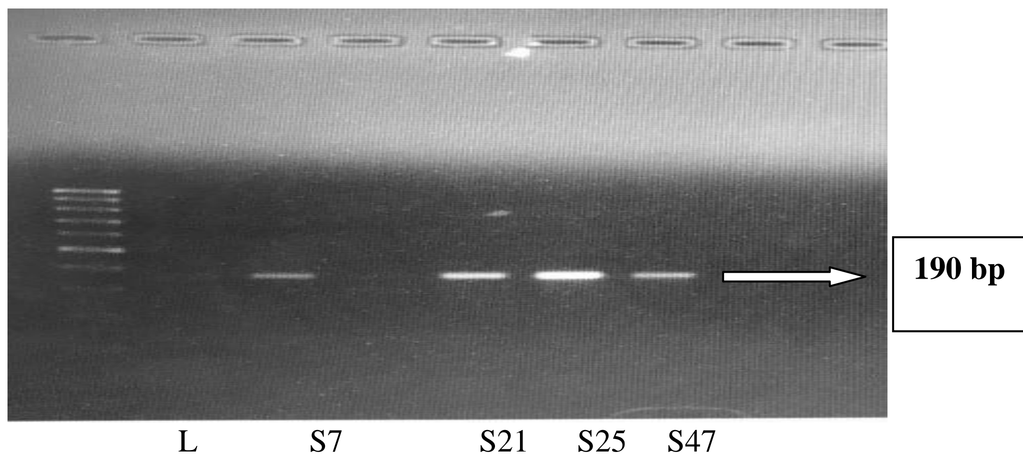


Fig.4 Electrophoresis analysis of nested PCR product of amplified *A. fumigatus*



Bacteria and fungi were usually associated with reproductive tract infections of cattle and sheep. In this study, the bacteriological and mycological examination of reproductive tract infections (80) of cattle and sheep revealed that 68 samples yielded 105 isolates and 34 isolates of fungi. From the bacteriological examination *E. coli* and *Bacillus* spp. were mostly isolated from cases of pyometra, metritis and retention of placenta in cattle and sheep with the incidence of 28.57% and 29% respectively. Other bacteria like *Staphylococcus* spp., *Klebsiella pneumoniae, Proteus* spp., *Brucella abortus Streptococcus* spp., *Arcanobacterium pyogenes, Pseudomonas aeruginosa* with the incidence of 13.3%,, 12.3%, 6.6%, 2.8%, 2.8%, 1.90%, 1.90% respectively. Another worker Paisley *et al.,* (1986) stated that toxic puerperal metritis caused by *E. coli*, *Streptococcus* spp., *Staphylococcus* spp. and *Pseudomonas* spp. and can be isolated from such cases of reproductive infections. In another study conducted by Ghanem *et al.,* (2015), *E. coli* was identified in association with *Streptococcus pyogenes* from post-partum dairy cows. Mshelia *et al.,* (2014) investigated bacterial infections of reproductive tract of ewes in tropical arid zone of Nigeria. The samples from vagina and uterus were collected and cultured using standard bacteriological techniques. The findings of the study revealed that the isolates were *Escherichia coli* (32%), *Staphylococcus* spp. (26%), *Klebsiella* spp. (16%), *Pseudomonas* (15%) and *Proteus* (11%); wherein *E. coli* and *S. aureus* were the most common bacterial isolates. The bacterial population in the vagina (64%) was significantly higher than that in the uterus (34%).

On mycological examination *Aspergillus* spp. and *Alternaria* spp. were mostly isolated from the infections of reproductive tract of cattle and sheep with incidence of 41.17% and 26.47% respectively. Other fungal species like *Mucor* spp., *Rhizopus* spp., *Penicillium* spp. and *Pithomyces spp.* with incidence of 5.8%, 8%, 8% and 8% respectively. A study conducted by Hassan *et al.,* (2012) reported isolation of a total of 20 fungal species related to 8 genera from 25 samples of vaginal swabs collected from cases of cow which suffered from abortion. The main recovered genera of fungi were *Aspergillus* spp. (80%), *Fusarium* spp. (16%), *Penicillium* spp. (32%), *Alternaria* spp. (8%) and *Candida albicans* (40%) which is similar to present study which results in isolation of 8 fungal species from 6 genera from 30 different reproductive disorders. Here, genera isolated were *Alternaria* spp. (26.47%), *Aspergillus* spp. (41.17%), *Mucor* spp. (5.8%), *Penicillium* spp. (8%), *Pithomyces* spp. (8%) and *Rhizopus* spp. (8%).

A total 25 samples tested for detection of aflatoxin, 21 feed samples were found to be contaminated with aflatoxin. Out of which four feed samples had mean concentration 40.5 ppb of aflatoxin which is higher than Indian Standards. Similar studies were done by Dhand *et al.,* 1998 in dairy cattle feed and reported that 75% of feed samples were contaminated with aflatoxins. Findings of present study are also in concurrence with other reports by Sarathchandra and Murlimanohar (2013) and Kotinagu *et al.,* (2015) where aflatoxins were found to be widely distributed in feedstuffs. A total 30 milk samples were collected from the same animals from which reproductive tract specimen and feed samples were taken and examined for detection of aflatoxin in milk. All of 30 milk samples were found negative for contamination of aflatoxin in milk of cattle and sheep.

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How to cite this article:

Bhupinder Kaur Khosa, Tejinder Singh Rai, Narinder Singh Sharma and Anil Kumar Arora. 2020. Detection of Aflatoxins in Milk and Feed from Cases of Reproductive Tract Disorders in Cattle and Sheep. *Int.J.Curr.Microbiol.App.Sci.* 9(09): 3540-3551.
doi: https://doi.org/10.20546/ijcmas.2020.909.439