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Rapid diagnosis of bovine brucellosis by peptide nucleic acid-fluorescence *in situ* hybridization assay

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Brucellosis is one of the major zoonotic, reproductive and highly contagious bacterial disease caused by *Brucella* spp. affecting various livestock species. It causes major economic losses to famers through abortions (Birley and Lock 1999). A 20% drop in milk yield is reported in a sizable proportion of animals that are in carrier state (Aiello and Mays 1998). *Brucella* are small, Gram-negative, non-spore forming, non-motile, facultative intracellular coccobacilli. The isolation of *Brucella* from host tissues, milk, vaginal exudates, etc. continues to be 'gold standard', followed by biochemical identification. The drawback of isolation is that it is time-consuming, has low sensitivity because *Brucella* can be overgrown easily by other contaminating bacteria due to their fastidious nature and isolation is a less sensitive technique in chronic infections. More importantly, *Brucella* organisms are class III pathogens that need to be handled with the utmost care, and the isolation must be performed in an authorized laboratory by highly skilled persons. To address these adversities, assays for detection of nucleic acid have been carried out for quick diagnosis. Fluorescence *in situ* hybridization (FISH) technique is done by targeting 16S rRNA with a probe to detect specific organism from cultures. Peptide nucleic acid-Fluorescence *in situ* hybridization (PNA-FISH) is a unique diagnostic technique that provides a quick and accurate diagnosis of various infectious diseases. Peptide nucleic acid probes have better hybridization capability when compared to traditional DNA probes as they possess an uncharged backbone (Perry *et al.* 2001). Therefore, the main objective of the current study was to test the efficacy of PNA-FISH on *Brucella* isolates and directly on the clinical samples for comparison of both the techniques and to evaluate PNA-FISH as a rapid diagnostic test.

An aggregate of 50 samples comprising fetal stomach contents, vaginal mucus, uterine discharges, and placenta were collected from cattle and buffaloes suffering from abortions and other reproductive disorders from areas in and around Ludhiana, Punjab. The samples collected were inoculated on BSM (*Brucella* selective medium) and incubated at 37°C under 5-10% CO_2 for 3-5 days. The isolates were identified based on morphology, cultural characteristics, various biochemical tests viz. urease, indole, oxidase, catalase, nitrate reduction, H_2S production, and growth in the presence of dyes (i.e. thionin and basic fuchsin). The isolates obtained were subjected to hot cold lysis method for DNA extraction and confirmed by PCR using genus-specific primers B4/B5 (Baily *et al.*1992) (Table 1). The reaction mixture and conditions of PCR are mentioned in Tables 2, 3 respectively.

In the present study, PNA-FISH was performed on *B. abortus* strain S99, *Brucella* isolates obtained from the clinical samples and directly on the clinical samples. The fluorescently labeled probe sequence was 5'-Flu-OO-labeled (Flu = 5,6 carboxyfluorescein and O = 8-amino-3,6-dioxaoctanoic acid) 16S rRNA of *B. abortus* (Flu-OO-gcc gct cac cct tgc; Fernandez *et al.* 2000) procured from PNA Bios (PNA Inc., USA). The smear of *B. abortus* S99 and *Brucella* isolates was prepared by standard smear preparation technique as per Perry (2001). For performing the PNA-FISH assay, the isolates obtained and standard strain of *Brucella* S99 were resuspended in PBS with 1% Triton X-100 in well of a coated microscopic slide (Tekdon Inc, USA). The slide was incubated at 55°C for 20 min, treated by 96% ethanol for 10 min and they were air-dried and fixed. PNA-FISH method was performed as per the method of Rigby *et al*. (2002). The smears were treated with hybridization solution (20 µl) comprising

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

Name of primer	Gene	Sequence (5′-3′)	Size of product	Reference
B4 (F)	*bcsp*31	TGG CTC GGT TGC CAA TAT CAA	223 bp	Baily *et al.* (1992)
B5 (R)		CGC GCT TGC CTT TCA GGT CTG		

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Table 2. *Brucella* PCR reaction mixture for B4/B5 primer pair

PCR component	Required concentration	Amount (µl)
H_2O (PCR grade)	Up to 25 µl	14.3
PCR buffer (10×)	1×	2.5
$MgCl_2$ (25 mM)	1.5 mM	1.5
dNTPs (10 mM)	200 µM	0.5
Primers (40pmol/µl each)	20 pmol each	0.5 + 0.5
Taq (5U/µl)	1 U	0.2
DNA template	~100 ng	5.0
Total volume		25

10% dextran sulfate, 50M Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM disodium EDTA, 30% formamide, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 1% Triton X-100, 0.1% sodium pyrophosphate, PNA probe. With the coverslips, the slides were incubated for 90 min at 55°C. Removing the coverslips, slides were submerged in prewarmed washing buffer containing 5 nM Tris, 15 mM NaCl, and 0.1% Triton X-100 at 55°C for 30 min. Finally, the smears were mounted with imagen mounting fluid (Dako) and observed for fluorescence under 40× and 100× power of a fluorescent microscope.

Clinical samples like fetal stomach contents and uterine discharges were centrifuged initially at a low speed to remove the larger debris. The supernatant was then centrifuged at 5,000 rpm for 5 min, the pellet obtained was washed four times with Phosphate buffer saline (PBS; pH 7.4) and resuspended and further processed for PNA-FISH by similar method as described earlier for cultures.

From the samples subjected to *Brucella* isolation, four samples yielded positive isolates. All the isolates were confirmed based on the biochemical and morphological characteristics. Upon incubation for 3-5 days, smooth, pinpoint, bluish, glistening and translucent colonies were observed which upon ageing turned opaque. Gram's staining of isolates revealed Gram-negative, coccobacilli, or rods, but in Modified Ziehl-Nielsen stain, red coccobacilli with a blue background were seen.

The isolates were non-motile and did not show any growth on McConkey's lactose agar (MLA) and were positive for catalase, oxidase, urease, H_2S production, and nitrate reduction test, whereas they tested negative for indole test. PCR performed on the extracted DNA by using primer pair B4/B5 that is specific for genus *Brucella* (Baily *et al.* 1992) and it amplifies *bcsp31* gene of *Brucella* yielded an amplicon of 223 bp in positive control as well as in the isolates.

Table 3. *Brucella* PCR program by using B4/B5 primer pair

Stage	Step	Temperature (°C)	Duration	No. of cycles
1	Initial denaturation	94	5 min	1
2	Denaturation	94	60 sec	35
	Annealing	65	60 sec	
	Extension	72	60 sec	
3	Final extension	72	10 min	1

The four *Brucella* isolates that were obtained by isolation along with the standard strain were subjected to PNA-FISH in which they were detected positive at a probe concentration of 500nm/20µl. The PNA probe was added in the hybridization buffer in various concentrations (50nm/20µl, 200nm/20µl and 500nm/20µl). All the concentrations were evaluated for hybridization with *B. abortus* S99, and ideal results were observed at a probe concentration of 500nm/20µl. Coccobacilli with yellow-green fluorescence were identified indicating that hybridization reaction occurred between the PNA probe and *Brucella* (Fig. 1). Isolation is the gold standard test for *Brucella*, hence, positive fluorescence by all the isolates and also by the standard reference strain in PNA-FISH signifies that the probe can detect the organism from the cultures.

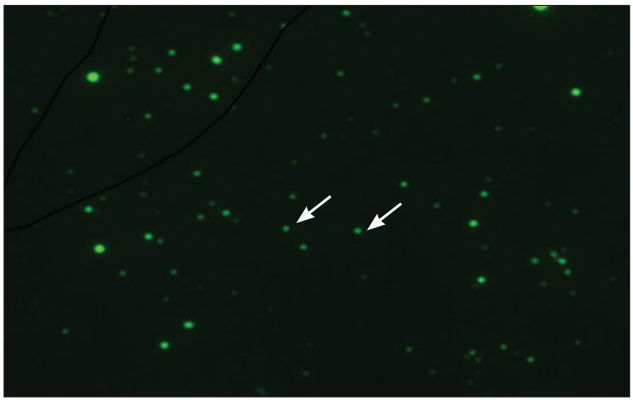

Fig. 1. Brucella isolates in PNA-FISH (100×).

PNA consists of a neutral polyamide backbone with a similar chemical characterization to the DNA molecules that facilitates PNA hybridization with DNA or RNA sequences that are complementary. The neutrally charged PNA diffuses efficiently through the bacterial membrane, and it has better resistance to proteases and nucleases due to its synthetic nature. As compared to DNA probes, PNA probes show better performance when used in the FISH, including greater specificity (Wilks and Keevil 2006). PNA-FISH as a reliable and rapid diagnostic method has also been applied on various other microorganisms. Rigby *et al.* (2002) reported *C. albicans* PNA-FISH method based on targeting the *Candida albicans* 26S rRNA by a fluorescein-labelled PNA probe. Similarly, Perry *et al.* (2001) formulated PNA probes targeting specific rRNA sequences of *Salmonella, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*, as well as Eubacteria and Eucarya.

From all the samples that were collected, 43 samples comprising fetal stomach contents and vaginal discharges were processed and subjected directly for PNA-FISH assay. From the samples tested, six samples yielded fluorescence, of which four were positive by isolation. Two clinical samples yielded positive results in PNA-FISH that were negative by isolation, suggesting PNA-FISH to be more sensitive assay when compared to isolation. When

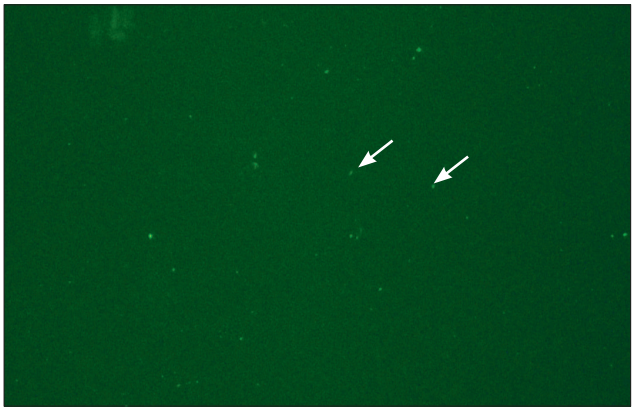

Fig. 2. PNA-FISH on clinical sample (100×).

PNA-FISH was performed directly on the clinical samples as such, fluorescent coccobacillary organisms could be detected, but they could not be visualized clearly because of the considerable amount of debris present in the neat sample. To counter this, clinical samples were washed with PBS and the debris was minimized for better visualization (Fig. 2).

Previous studies have evaluated the ability to identify pathogens in blood culture and other body fluids using similar method. PNA-FISH has been performed on other bacteria and yeasts (Stender *et al*. 1999, Olieviera *et al*. 2001, Perry *et al*. 2001) including the detection of *Staphylococcus aureus* (Oliviera *et al.* 2002) and *Klebsiella pneumoniae* (Søgaard *et al.* 2007) directly from blood culture bottles and also for detection of *Salmonella* from various samples that are artificially contaminated (Al media 2010). A similar application of PNA-FISH on the clinical sample was carried out by Prudent *et al.* (2018) on 23 *Coxiella burnetii* positive valves and thrombus samples. PNA-FISH detected more positive samples in comparison to culture and FISH. Similarly, Almeida *et al.* (2013) artificially contaminated ground beef and unpasteurized milk samples with *E. coli* O157. PNA-FISH on 60 food samples showed a 100% specificity value, a sensitivity of 97.22%, and an accuracy of 98.33%. The present study indicates PNA-FISH to be a rapid test that can be used for identification of *Brucella* directly from the clinical samples, thus eliminating the need to handle the cultures that are hazardous, time-consuming, and requires expertise. The results of the present study reveal more about the efficacy and sensitivity of PNA FISH in comparison to isolation.

SUMMARY

Brucellosis is a major economical disease of livestock and an important zoonotic disease. Rapid and accurate diagnosis is a fundamental prerequisite in the quest for control and elimination of brucellosis. The current study was conducted to evaluate a rapid molecular technique, peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) assay for the diagnosis of brucellosis. Samples, including fetal stomach contents, vaginal discharges, and placenta collected from cattle and buffaloes experiencing abortion and other reproductive disorders were processed for isolation. *Brucella* isolates obtained were confirmed by biochemical tests and polymerase chain reaction (PCR), thereby subjected to PNA-FISH which yielded positive fluorescence. The clinical samples were processed and subjected directly to PNA-FISH. On performing PNA-FISH directly on the clinical samples, this test reduces the time spent on processing the sample for isolation, avoiding the risk of handling the infectious organism while going through the process of isolation. Hence, PNA FISH can be considered as a rapid and efficient diagnostic method for Brucellosis.

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