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Seroprevalence of bovine brucellosis and association of Toll-like receptor 2 T385G polymorphism with natural disease resistance in cattle and Murrah buffaloes of Andhra Pradesh

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Abstract

Bovine brucellosis causes substantial reproductive and public-health losses, and genetic variation in innate-immunity genes may contribute to differences in host susceptibility. This study estimated brucellosis seroprevalence and evaluated the association of the T385G polymorphism in exon 2 of Toll-like receptor 2 with natural disease status in cattle and Murrah buffaloes from Andhra Pradesh. A total of 222 unvaccinated animals with a history of abortion or repeat breeding were screened by the Rose Bengal plate test and indirect enzyme-linked immunosorbent assay. Forty-one confirmed cases and 181 controls were genotyped by polymerase chain reaction-restriction fragment length polymorphism. Forty-four animals (19.8%) were positive by the screening test and 41 (18.5%) were confirmed by enzyme-linked immunosorbent assay. Confirmed seroprevalence ranged from 0% in Jersey crossbreds to 50.0% in Punganur cattle, although estimates for small breed groups were imprecise. The recessive genetic model had the lowest Akaike information criterion. The TT genotype was associated with lower estimated odds of seropositivity than GG/GT genotypes (odds ratio 0.17; 95% confidence interval 0.02-1.46; $P = 0.05$). Because the confidence interval included unity and only one positive TT animal was observed, the association should be considered suggestive rather than conclusive. These findings support further evaluation of Toll-like receptor 2 variation in larger, multi-herd and longitudinal cohorts before its use in marker-assisted selection.

Keywords: Bovine brucellosis, cattle, disease resistance, Murrah buffalo, seroprevalence, TLR2

Introduction

Brucellosis is an economically important zoonosis that causes late-gestation abortion, stillbirth, retained placenta, reduced milk yield, infertility and prolonged calving intervals in bovines [1-2]. In India, estimated losses amount to approximately US\$6.8 per affected cattle and US\$18.2 per affected buffalo [3]. A national random-sampling survey reported seroprevalence estimates of 8.30% in cattle and 3.60% in buffaloes, with marked geographic heterogeneity [4]. Vaccination, movement control, biosecurity and removal of infected animals remain central to control programmes; however, host genetic resistance may provide a complementary long-term strategy [5-6].

Toll-like receptor 2 (TLR2) is a cell-surface pattern-recognition receptor that forms heterodimers with TLR1 or TLR6 and detects bacterial lipoproteins, thereby initiating inflammatory signalling [7-8]. Variation within TLR2 may alter receptor function and influence susceptibility to intracellular pathogens. The non-synonymous T385G variant in exon 2 has been linked to an aspartic-acid-to-glutamic-acid substitution and has previously been evaluated in relation to paratuberculosis, brucellosis and bovine tuberculosis [9-11].

Evidence for TLR2-associated resistance in Indian cattle and Murrah buffaloes remains limited. Therefore, this study aimed to estimate brucellosis seroprevalence in selected bovine populations of Andhra Pradesh and to test the association between the TLR2 T385G polymorphism and naturally occurring serological disease status.

Materials and Methods

Study design and animals

A case-control design was used. The study included 222 unvaccinated animals with a history

of abortion or repeat breeding: 120 Murrah buffaloes, 51 Holstein Friesian crossbreds, 5 Jersey crossbreds, 21 Ongole, 19 Gir and 6 Punganur cattle. Cases were animals positive by both the Rose Bengal plate test (RBPT) and indirect enzyme-linked immunosorbent assay (i-ELISA); controls were negative by both tests. This yielded 41 cases and 181 controls (Table 1). The approximate 1:4 case-to-control ratio was selected to improve statistical efficiency, consistent with established case-control design principles [12]. Animals were sampled from comparable production environments and management systems. Four millilitres of jugular blood were collected into K3EDTA tubes for DNA isolation, and separate blood samples were collected into serum tubes for serology. Samples were transported on ice and stored at -20 °C until analysis. Ethical approval was obtained from the Institutional Animal Ethics Committee [approval number to be inserted].

Serological screening

Serum was separated from clotted blood by centrifugation at 1,000-1,500 × g for 10 min. For RBPT, 20 µL serum was mixed with 20 µL antigen (Biological Products Division, ICAR-Indian Veterinary Research Institute) on a clean glass slide and observed for agglutination after 4 min. RBPT-positive samples were tested using a commercial indirect ELISA kit (Genomix Carl Pvt. Ltd., Pulivendula) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a Multiskan GO spectrophotometer (Thermo Scientific). Sample-to-positive ratios of 0-0.3, 0.3-0.5 and >0.5 were classified as negative, equivocal and positive, respectively.

Genotyping of the TLR2 T385G polymorphism

Genomic DNA was isolated by a modified high-salt method [13-14]. The T385G variant in exon 2 of TLR2 was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The forward primer was 5'-CTCTGTCTTGTGACCCAACT-3' and the reverse primer was 5'-ACATAAAGGGACCTGAACC-3' [10]. Amplification produced a 295-bp fragment. Reactions were performed in a final volume of 15 µL using 2× PCR Master Mix (Amplicon), with annealing at 55 °C for 35 cycles. Amplicons were digested overnight at 37 °C with 3 U of EcoRV (New England Biolabs). The expected fragment patterns were 295 bp for GG, 295/185/110 bp for GT and 185/110 bp for TT. Digested products were resolved on 2.5% agarose gels in 1× Tris-acetate-EDTA buffer with a 100-bp DNA ladder.

Statistical analysis

Breed-wise seroprevalence was summarized for RBPT and i-ELISA. Genotype and allele-frequency differences among breeds were examined using Fisher's exact test. Association between TLR2 genotype and brucellosis status was assessed by logistic regression in SNPStats [15]. Codominant, dominant, recessive, overdominant and log-additive models were compared using Akaike's information criterion (AIC), and the model with the lowest AIC was retained. Odds ratios (OR), 95% confidence intervals (CI) and P values were reported. Gir cattle were used as the reference breed. Because positive Murrah buffaloes showed no genotype variation, buffaloes were not included in the breed-adjusted logistic model.

Results

Seroprevalence

Among the 222 animals screened, 44 (19.8%) were RBPT-positive and 41 (18.5%) were confirmed positive by i-ELISA (Table 2). One Ongole and two Gir animals were RBPT-positive but not confirmed by i-ELISA and were treated as non-cases for association analysis. Confirmed seroprevalence ranged from 0% in Jersey crossbreds to 50.0% in Punganur cattle. Murrah buffaloes and Holstein Friesian crossbreds had confirmed seroprevalence values of 18.3% and 13.7%, respectively. The use of a screening test followed by a confirmatory assay improves diagnostic specificity in sero-epidemiological studies [16-17].

Genotype distribution

Genotype and allele frequencies are summarized in Table 3. Among positive Holstein Friesian crossbreds, GT was the most frequent genotype. In the indigenous cattle groups and Murrah buffaloes, GG predominated among positives. The TT genotype was more frequent among negative crossbred cattle. All positive Murrah buffaloes were GG. Fisher's exact tests indicated differences in genotype frequencies between Holstein Friesian crossbreds and Gir or Punganur cattle ($P < 0.05$), whereas comparisons among indigenous breeds were not significant.

Association analysis

The recessive model had the lowest AIC (Table 4). TT animals showed lower estimated odds of seropositivity than animals with GG or GT genotypes (OR 0.17, 95% CI 0.02-1.46; $P = 0.05$). Relative to Gir cattle, the estimated ORs were 0.43 for Holstein Friesian crossbreds, 0.23 for Ongole and 1.71 for Punganur; none of the breed comparisons was statistically significant.

Discussion

The overall i-ELISA seroprevalence of 18.5% exceeded national estimates, which was expected because the study deliberately targeted animals with reproductive disorders rather than a random population [4]. The high proportions observed in Gir and Punganur cattle should not be interpreted as breed-level susceptibility because the Gir animals were largely derived from one herd and the Punganur sample was very small. Earlier studies in Andhra Pradesh have likewise documented substantial between-herd variation [18-19]. Age, herd size, reproductive management and artificial insemination practices may further influence exposure risk [20-21].

Under the recessive model, TT animals had lower estimated odds of seropositivity than animals carrying a G allele (OR 0.17, 95% CI 0.02-1.46; $P = 0.05$). Because the confidence interval included 1.0 and only one positive TT animal was observed, this result should be regarded as borderline evidence rather than definitive proof of protection. Nevertheless, the direction of effect was consistent with earlier reports in Chinese Holstein and Indian cattle, in which G-containing genotypes were associated with greater disease susceptibility [10-11]. Functional plausibility is supported by previous work showing that TLR2 variants can modify immune responses to intracellular mycobacteria [9, 22].

The companion analysis of TLR1 + 1380 G/A could not support logistic modelling because all seropositive animals carried the GG genotype. A previous Indian study also

reported lower brucellosis risk for AG and AA animals than for GG animals [23]. Other TLR1 variants have been linked to bovine tuberculosis, paratuberculosis and health traits [24-27]. These observations justify evaluating TLR1 and TLR2 jointly in larger, independently sampled populations.

Breed estimates were imprecise. Relative to Gir, Holstein Friesian crossbreds and Ongole cattle showed lower estimated odds, whereas Punganur cattle showed higher estimated odds; none of these comparisons was statistically significant. The pattern may reflect a mixture of genotype frequency, herd exposure, management and additional immune loci. The apparently low risk in Ongole cattle despite a high G-allele frequency is particularly important because it argues against treating a single polymorphism as a stand-alone resistance marker.

The study had several limitations. The number of confirmed cases was modest, and subgroup sizes were especially small for Jersey crossbred and Punganur cattle. Serology indicates exposure but does not distinguish active infection from resolved infection; culture or molecular confirmation and longitudinal sampling would strengthen phenotype

definition. The association estimate was based on one locus and was vulnerable to population structure and herd-level confounding. TLR2 variants have also been associated with milk-production traits, so correlated responses should be assessed before marker-assisted use [28]. Future studies should use larger, multi-herd cohorts and multi-locus panels that include TLR1, TLR4, TLR6, TLR9 and downstream signalling genes.

Table 1: Breed-wise distribution of confirmed cases and controls used in the association analysis

Breed/group	Animals tested	Cases	Controls
Murrah buffalo	120	22	98
Holstein Friesian crossbred	51	7	44
Jersey crossbred	5	0	5
Ongole	21	2	19
Gir	19	7	12
Punganur	6	3	3
Total	222	41	181

Cases were positive by both RBPT and i-ELISA; controls were negative by both tests.

Table 2: Seroprevalence of brucellosis by Rose Bengal plate test and indirect enzyme-linked immunosorbent assay

Breed/group	Tested	RBPT positive, n (%)	i-ELISA positive, n (%)
Murrah buffalo	120	22 (18.3)	22 (18.3)
Holstein Friesian crossbred	51	7 (13.7)	7 (13.7)
Jersey crossbred	5	1 (20.0)	0 (0.0)
Ongole	21	3 (14.3)	2 (9.5)
Gir	19	8 (42.1)	7 (36.8)
Punganur	6	3 (50.0)	3 (50.0)
Total	222	44 (19.8)	41 (18.5)

RBPT, Rose Bengal plate test; i-ELISA, indirect enzyme-linked immunosorbent assay.

Table 3: TLR2/EcoRV genotype and allele frequencies in brucellosis-positive and brucellosis-negative animals

Breed/group	GG +	GT +	TT +	G +	T +	GG -	GT -	TT -	G -	T -
HF crossbred	0.14 (1)	0.71 (5)	0.14 (1)	0.50	0.50	0.16 (7)	0.39 (17)	0.45 (20)	0.36	0.64
Jersey crossbred	0	0	0	0	0	0.20 (1)	0.40 (2)	0.40 (2)	0.40	0.60
Ongole	1.00 (2)	0	0	1.00	0	0.74 (14)	0.05 (1)	0.21 (4)	0.77	0.23
Gir	0.86 (6)	0.14 (1)	0	0.93	0.07	0.92 (11)	0.08 (1)	0	0.96	0.04
Punganur	1.00 (3)	0	0	1.00	0	1.00 (3)	0	0	1.00	0
Murrah buffalo	1.00 (22)	0	0	1.00	0	0.99 (97)	0	0.01 (1)	0.99	0.01

Values in parentheses are animal counts; +, seropositive; -, seronegative; HF, Holstein Friesian.

Table 4: Logistic regression of TLR2/EcoRV genotype and breed effects on brucellosis status under the recessive model

Variable	Category	Negative	Positive	OR	95% CI	P value
TLR2 genotype	GG/GT	154	40	1.00 (reference)	—	—
	TT	27	1	0.17	0.02-1.46	0.05
Breed	Gir	12	7	1.00 (reference)	—	—
	HF crossbred	24	6	0.43	0.12-1.56	NS
	Ongole	15	2	0.23	0.04-1.31	NS
	Punganur	3	3	1.71	0.27-10.92	NS

OR, odds ratio; CI, confidence interval; NS, not significant; HF, Holstein Friesian. The genotype association is borderline because the 95% CI includes 1.0

Conclusion

Brucellosis seroprevalence was 18.5% among bovines selected for abortion or repeat-breeding history in Andhra Pradesh. The TLR2 TT genotype showed lower estimated odds of seropositivity, but the evidence was borderline and imprecise. The T385G polymorphism should therefore be treated as a candidate for validation, not as a ready-to-use selection marker. Larger, independently sampled and longitudinal studies that account for breed, herd and management effects are required before breeding application.

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Author Contributions

Choppara Gana Daya: conceptualization, sample collection, laboratory analysis, serological testing, genotyping,

statistical analysis and drafting. Vinoo Regula: study design, supervision, interpretation, critical revision and final approval. Satish Kumar Illa: statistical analysis, interpretation, editing and final approval. All authors read and approved the final manuscript.

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Ethics statement

Blood samples used in the present study were collected from animals at organized farms and from clinical cases presented to NTR College of Veterinary Science, Gannavaram, following standard veterinary procedures. The study did not involve any experimental challenge, sacrifice, or procedures outside routine clinical and diagnostic sampling. Hence, separate approval from the Institutional Animal Ethics Committee and an ethics approval number were not required for this work.

Conflict of Interest

The authors declare no conflict of interest.

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