RESEARCH ARTICLE

Risk Factors Analysis for Haemoparasitic Infections and Evaluation of Detection Methods for Ehrlichiosis in Dogs

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Abstract

The frequency of occurrence of canine haemoparasitic diseases was analysed in relation to associated risk factors by examining the Leishman-stained thin blood smears of 400 dogs (pet 352 and stray 48) from the Ludhiana district of Punjab state, India. To compare the effectiveness of classic-immuno-molecular techniques, 50 samples suspected of ehrlichiosis were tested by buffy coat smear examination, Immuno-chromatographic lateral flow assay (LFA-Ec) and Polymerase chain reaction (Ec-PCR) based diagnosis by amplification of a fragment of the *E. canis virB9*. The occurrence of haemoparasites was 6.00% with the highest prevalence of *B. gibsoni* (3.75%), followed by *H. canis* (1.25%), *E. canis* (0.5%), *B. canis* (0.25%) and *Trypanosoma evansi* (0.25%) with higher prevalence of haemoparasites in stray dogs (8.33%) than pet dogs (5.68%). Exercise intolerance and fever were significantly ($p \leq 0.01$) associated with occurrence of haemoparasites. The kappa value revealed "slight" and "fair" strength of agreement for LFA-Ec & Ec-PCR with buffy coat smear examination. A sensitivity of 100% and specificity of 4.26% & 68.09% was observed respectively for LFA-Ec & Ec-PCR when compared with buffy coat smear examination. The complete sequence of the *virB9* gene of *E. canis* showed the closest homology with the isolates obtained from the USA strain. This study underscores the importance of accurate diagnostic techniques and the influence of various risk factors on the prevalence of haemoparasitic diseases in dogs.

Key words: Dog, Ehrlichia canis, Haemoparasites, Immuno-molecular diagnosis, Punjab.

Ind J Vet Sci and Biotech (2026): 10.48165/ijvsbt.2026.22.06.15

Introduction

Arthropod-borne canine haemoparasitic infections are very common in Indian sub-continent. Common haemoparasites of canines are *Babesia gibsoni, Babesia canis, Ehrlichia canis* and *Hepatozoon canis,* and are primarily transmitted by *Rhipicephalus sanguineus (*Megat Abd Rani *et al.,* 2010). Babesiosis is caused by *Babesia canis* (large form) and *Babesia gibsoni* (small form), that is clinically characterized by pyrexia, anorexia, epistaxis, petechiae and splenomegaly along with haemoglobinuria, anaemia, and thrombocytopenia (Solano-Gallego *et al.,* 2016). Age wise the disease is more severe in younger dogs (Irwin and Hutchinson, 1991). *Hepatozoon canis* is transmitted by accidental ingestion of an infected brown dog tick (Otranto *et al.,* 2011). Canine monocytic ehrlichiosis is caused by rickettsia *Ehrlichia canis,* which parasitizes the monocytes and granulocytes resulting in pancytopaenia specially thrombo-cytopaenia (Kledmanee *et al.,* 2009). Irreversible bone marrow destruction is related to the chronic form of ehrlichiosis (Skotarczak, 2003). *Trypanosoma evansi* is primarily transmitted mechanically by Tabanid flies along with the consumption of infected meat. Infected dogs show corneal opacity, change of voice, malaise and fever (Irwin and Jefferies, 2004).

The haemoparasitic diseases are routinely diagnosed based on the conventional microscopic examination of Romanowsky-stained thin blood smears that are reliable for the clinical cases. Although considered the gold standard, conventional parasitological methods fail to detect low-grade infections. Specific diagnostic tests for canine ehrlichiosis include a demonstration of intracytoplasmic *E. canis*-morulae within monocytes. The *E. canis* morulae are mainly observed during the acute phase of the infection (Hibler *et al*., 1986). Various immunological tests, e.g., Indirect Fluorescent Antibody Test (IFAT), rapid immuno-migration test can be used to diagnose canine ehrlichiosis (Davoust *et al.,* 2014). Further, DNA based diagnostics can be used with high sensitivity and specificity for confirmatory

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How to cite this article: Eravaramkunnath, F., Singh Bal, M., Kaur, P., Mahajan, V., Singla, L. D., & Gupta, T. (2026). Risk Factors Analysis for Haemoparasitic Infections and Evaluation of Detection Methods for Ehrlichiosis in Dogs. Ind J Vet Sci and Biotech, 22(6), 86-91.

Source of support: No specific funding was received for this study.

Conflict of interest: None

Submitted 22/05/2026 **Accepted** 14/07/2026 **Published** 10/11/2026

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diagnosis of ehrlichiosis. Early diagnosis of haemoparasitic diseases results in administration of early specific treatment which is crucial for canine health. Keeping in view the importance of canine haemoparasites, the present study was envisaged to explore the prevalence of haemoparasitic diseases in dogs in and around Ludhiana, Punjab in relation to associated risk factors and comparative analysis of different diagnostic techniques for *E. canis*.

Materials and Methods

Sample Collection

The study was conducted with IAEC approval (IAEC/2021/294-315; 27.10.2021). A total of 400 EDTA blood samples (2-3 mL) were collected from dogs in and around Ludhiana, Punjab, comprising 352 pet dogs presented at the Multi-Specialty Veterinary Hospital, GADVASU, and 48 stray dogs sampled at the Animal Birth Control Centre, Ludhiana. Data on potential risk factors, including breed, sex, clinical signs, tick history, deworming, and vaccination status, were recorded using a structured questionnaire. Age, exercise intolerance, and epistaxis were not assessed in stray dogs due to field constraints; these dogs were considered unvaccinated and non-dewormed. Among these, 50 samples (47 pet and 3 stray dogs) from dogs suspected of ehrlichiosis based on high fever and epistaxis were selected to evaluate the diagnostic methods for the same.

Parasitological Examination

Thin blood smears were prepared, Leishman-stained, and examined under oil immersion microscope (Olympus CX21i) for haemoparasites. Buffy coat smears from 50 dogs suspected of ehrlichiosis were similarly examined for characteristic *E. canis* morulae in monocytes.

Sero-Molecular Assay for *Ehrlichia canis*

Fifty suspected ehrlichiosis cases were tested using the qualitative immuno-chromatographic lateral flow assay (LFA-Ec), QuickVET Canine Ehrlichia Antibody Rapid Test (Ubio Biotechnology Systems Pvt. Ltd., Cochin, India), for rapid detection of *E. canis* antibodies in plasma according to the manufacturer's protocol.

Genomic DNA was extracted from 50 blood samples suspected of ehrlichiosis using a QIAamp DNA extraction kit (Qiagen, Germany) following the manufacturer's protocol. A fragment of the *E. canis virB9* gene was amplified by PCR using species-specific primers as described by Kledmanee *et al.* (2009). PCR was performed in a 25 µL reaction mixture containing 12.5 µL 2× Taq PCR Master Mix, 1.0 µL each of forward (Ehr1401F) and reverse (Ehr1780R) primers (10 pmol), 2.0 µL DNA template, and 8.5 µL nuclease-free water. Cycling conditions comprised initial denaturation at 95°C for 15 min, followed by 30 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. Amplified products were analyzed by electrophoresis on 1.5% agarose gel.

Purified PCR amplicons (HiMedia, India) were custom sequenced by Barcode Biosciences, Bangalore. The obtained *virB9* sequences were aligned using Clustal X and compared with GenBank sequences through NCBI-BLASTn. Phylogenetic analysis was performed using the neighbour-joining method with 1000 bootstrap replicates in MEGA 6.0, with *virB9* sequences retrieved from GenBank (Kumar *et al.,* 2018)

Statistical Analysis

The data was analyzed using Win Episcape 2:0 software (used to evaluate diagnostic test performance by estimating sensitivity, specificity, and test agreement.) and SPSS (Statistical Package for Social Sciences) for Windows version 11.0.1 © SPSS Inc., USA Computer Software Programs.

Results and Discussion

Parasitological Examination

The overall prevalence of haemoparasites was recorded as 6.00% comprising of *B. gibsoni* (3.75%), *H. canis* (1.25%), *E. canis* (0.5%), *B. canis* (0.25%) and *Trypanosoma evansi* (0.25%)(Table 1, Fig. 1). The higher prevalence was observed in stray dogs (8.33%) as compared to pet dogs (5.68%). Using buffy coat examination, *E. canis* morulae were detected in 6% of dogs, including 6.38% of pet dogs, while no morulae were detected in stray dogs (Table 2). This was in accordance with various prevalence reports ranging from 1.12% to 22.09% (Thirunavukkarasu *et al*., 1993; Kumar *et al*., 2020) reported from India. The concentration of white blood cells in the buffy coat facilitates morulae detection, particularly during the acute phase of *E. canis* infection, whereas detection is more difficult in the chronic phase.

The geo-climatic conditions of India are very conducive for the propagation of vector and vector borne pathogens, with a prevalence of 6.54-15.4% for *B. gibsoni* and 0.26-0.93% of *B. canis* in Punjab (Singh *et al*., 2014; Singla *et al.,* 2016). In the current study, the prevalence of these parasites was relatively comparable with the earlier reports in the region. Prevalence rates from 0.26% to 5.78% were reported for *Hepatozoon canis* from the same region (Singla *et al.,* 2016; Singh *et al.,* 2017). *T. evansi* also has been reported from the region of Punjab (0.40 %)(Kaur *et al.,* 2025). When the prevalence of *E. canis* in Punjab is considered, prevalence ranged 0.93 to 3.33% (Milanjeet *et al*., 2014, Kaur *et al*., 2020) in the last decade. Consonantly, the prevalence revealed in this study (0.5%) was compatible with previous studies from Punjab.

Table 1: Prevalence of haemoparasites in Ludhiana district by blood smear examination

Source of dogs	Samples examined	Positive samples (%)					
		B. gibsoni	*H. canis*	*E. canis*	*B. canis*	*T. evansi*	Total
Pet	352	14 (3.97)	2 (0.56)	2 (0.56)	1 (0.28)	1 (0.28)	20 (5.68)
Stray	48	1 (2.08)	3 (6.25)	0	0	0	4 (8.33)
Total	**400**	**15 (3.75)**	**5 (1.25)**	**2 (0.5)**	**1 (0.25)**	**1 (0.25)**	**24 (6.00)**

Table 2: Detection percentage of *E. canis* by different diagnostic techniques

Parameter	Total examined	Positive by buffy coat (%)	Positive by LFA-Ec	Positive by Ec- PCR
Pet dogs	47	3 (6.38%)	45 (95.74%)	16 (34.04%)
Stray dogs	3	0	3(100%)	2 (66.66%)
Total	**50**	**3 (6%)**	**48 (96%)**	**18 (36%)**

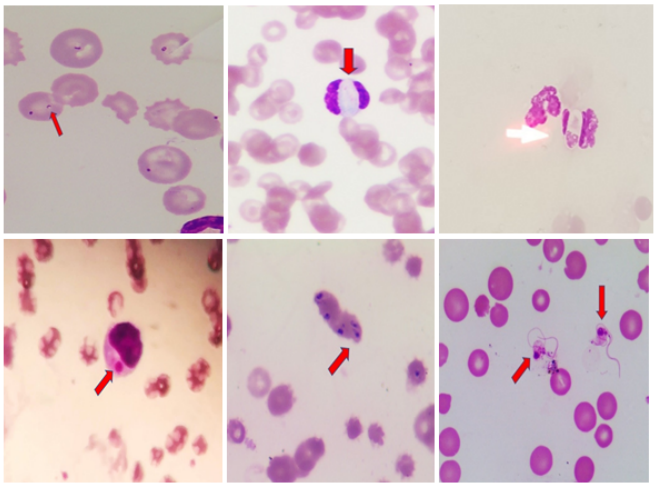


Fig. 1: Leishman-stained thin blood smears showing A. *Babesia gibsoni* in RBC, B. Gelatine capsule shaped gamonts of *Hepatozoon canis* in neutrophils of dog, D. *Ehrlichia canis* morula in monocytes of dog. E. Rosette shaped piroplasm of *B. canis* in RBC of dog and F. *Trypanosoma evansi* extracellular hemoflagellate haemoparasite.

Risk Factor Analysis

The association between haemoparasite infection and associated risk factors is presented in Table 3. Haemoparasite prevalence was higher in puppies below one year (8.93%) than in older dogs (5.07%) and in males (7.38%) than females (3.85%). Prevalence was also higher among stray dogs, particularly those with tick infestation (8.33%) The lower prevalence in pet dogs with a history of ticks may be attributed to recent acaricidal application. The highest breed-specific prevalence was recorded in American Bully (17.39%) and the least in non-descript pet dogs (2.27%). A large majority of the dogs were vaccinated and dewormed, and the prevalence was found to be more in non-vaccinated dogs (8.82%) and non-dewormed dogs (10.34%). Haemoparasites were more prevalent in dogs without epistaxis (6.60%), and prevalence was highest during summer (9.26%) and lowest during winter (2.78%). The higher prevalence in summer may be attributed to favourable conditions for the survival and proliferation of the vector tick, *Rhipicephalus sanguineus*.

Although exercise intolerance was absent in most dogs, haemoparasite prevalence was significantly higher ($p \leq 0.01$) in dogs with exercise intolerance (10.85%;) consistent with findings of De Tommasi *et al.* (2013). All haemoparasite-positive dogs presented with fever, with a prevalence of 11.42%, which was significantly higher in stray dogs (26.60%) than in pet dogs (10.25%). Fever was found to be associated in concomitant tick-borne infections in dogs (Sarma *et al*., 2015).

Sero-Molecular Diagnosis of Ehrlichiosis

Positive samples (Fig. 2) showed both control (C) and test (T) bands, whereas negative samples showed only the control band, confirming the use of a lateral flow assay for antibody detection. Of 50 dogs tested, 48 (96.0%) were positive for *E. canis* antibodies, including 45/47 (95.74%) pet dogs and all 3 stray dogs (100%). Previous reports from Punjab recorded *E. canis* antibody prevalences of 86.90% (Kottadamane *et al.,* 2017) and 80% (Singla *et al.,* 2011) using enzyme-labelled antibody detection and ImmunoComb® Dot-ELISA, respectively.



A B

Fig. 2: (A) Immuno-chromatographic Lateral Flow Assay (LFA-Ec) with positive sample (B) Immuno-chromatographic Lateral Flow Assay (LFA-Ec) with negative sample

Globally *E canis* has been detected from various countries and different states from India. PCR amplification yielded the expected 380-bp amplicon, confirming *E. canis* DNA (Fig. 3), with an overall molecular detection of 36 % (18/50). Positivity was 34.04% (16/47) in pet dogs and 66.67% (2/3) in stray dogs. This detection percentage falls within

Table 3: Prevalence of canine haemoparasites in relation to associated risk factors

Risk Factor	Group/Parameter	Samples	Positive (%)	Negative (%)	χ^2	Df	P value
Age	Group 1 (Less than 1 year)	56	5 (8.93)	51 (91.07)	1.310	1	0.252
	Group II (More than 1 year)	296	15 (5.07)	281(94.93)			
Gender (Male n=244)	Pet	214	15 (7.01)	199 (92.99)	2.104	1	0.147
	Stray	30	3 (10.00)	27(90)			
	Total	244	18 (7.38)	226 (92.62)			
Gender (Female n=156)	Pet	138	5 (3.62)	133 (96.38)			
	Stray	18	1 (5.55)	17 (94.45)			
	Total	156	6 (3.85)	150 (96.15)			
Breed	American Bully	23	4 (17.39)	21(82.61)	8.696	6	0.191
	Golden Retriever	30	2 (6.66)	28(93.34)			
	Labrador Retriever	82	4 (4.88)	80 (95.12)			
	Pomeranian	33	3 (9.09)	30 (90.91)			
	Non-descript (Pet)	44	1 (2.27)	43 (97.73)			
	Non- descript (Stray)	48	4 (8.33)	44 (91.67)			
	Others	140	6 (4.28)	134 (95.72)			
Vaccination status	Vaccinated	332	18 (5.42)	314 (94.58)	1.158	1	0.282
	Non-vaccinated	68	6 (8.82)	62 (91.18)			
Deworming status	Dewormed	313	15 (4.79)	298 (95.21)	3.721	1	0.054
	Non-dewormed	87	9 (10.34)	78 (89.66)			
Exercise intolerance	Present	129	14 (10.85)	115 (89.15)	10.160	1	0.001*
	Absent	223	6 (2.69)	217 (97.31)			
Fever (Present n=210)	Pet	195	20 (10.25%)	175 (89.75)	23.100	1	0.000*
	Stray	15	4 (26.6%)	11(73.4)			
	Total	210	24 (11.43)	186(88.57)			
Fever: Absent (n=190)	Pet	157	0	157 (100)			
	Stray	33	0	33 (100)			
	Total	190	0	190 (100)			
Epistaxis	Present	64	1 (1.56)	63 (98.44)	2.477	1	0.116
	Absent	288	19 (6.60)	269 (93.40)			
History of ticks: (n=221)	Pet	173	7 (4.04%)	166 (95.96)	0.916	1	0.339
	Stray	48	4 (8.33%)	44 (91.67)			
History of ticks: Absent (n=179)	Pet	179	13 (7.26%)	166 (92.74)			
	Stray	0	0	-			
Season	Autumn	87	3 (3.45)	84 (96.55)	5.503	3	0.138
	Winter	72	2 (2.78)	70 (97.22)			
	Spring	79	4 (5.06)	75 (94.94)			
	Sum	162	15 (9.26)	147 (90.74)			

the reported range of 3.5-41.59% for Punjab (Milanjeet *et al.,* 2014; Padmaja *et al.,* 2022). Only highly suspected samples were taken for the study, which could be the reason for a much higher molecular detection percentage in this study. Since molecular assays are the confirmatory diagnostic method, the study firmly confirms the prevalence of ehrlichiosis in Ludhiana. This comparatively higher sero-moelcular detection rates reflects widespread circulation of *E. canis* in the Punjab canine population, highlighting the need for strengthened surveillance, tick control, and timely preventive measures to reduce transmission

The unrooted phylogenetic tree was constructed using the nucleotide sequence of representative strains of *E. canis* (Fig. 4). Sequence of the *virB9* gene of *E. canis* (OP471415) formed cluster with other members from foreign countries (USA, China, Australia, Egypt) and India. The sequence showed the closest homology with the isolated obtained from USA AY205339.1 and AF546158.1.

Comparison of Various Diagnostic Tests

Briefly, the results obtained for canine monocytic ehrlichiosis in comparison with various diagnostic tests are summarized in Tables 2 & 4. The diagnostic performance of the LFA-Ec and Ec-PCR was evaluated against conventional buffy coat smear examination. LFA-Ec showed low specificity (4.26%; 95% CI: 0.52–14.54%), with frequent false-positive results, possibly due to circulating antibodies from previous exposure. In contrast, Ec-PCR demonstrated higher specificity (68.09%; 95% CI: 52.88-80.91%), indicating better accuracy in identifying uninfected dogs. Agreement with buffy coat examination was assessed using Cohen's kappa. LFA-Ec showed slight agreement (κ= 0.005±0.005), whereas Ec-PCR demonstrated fair agreement (κ= 0.204±0.104), based on the criteria of Landis and Koch (1977). These

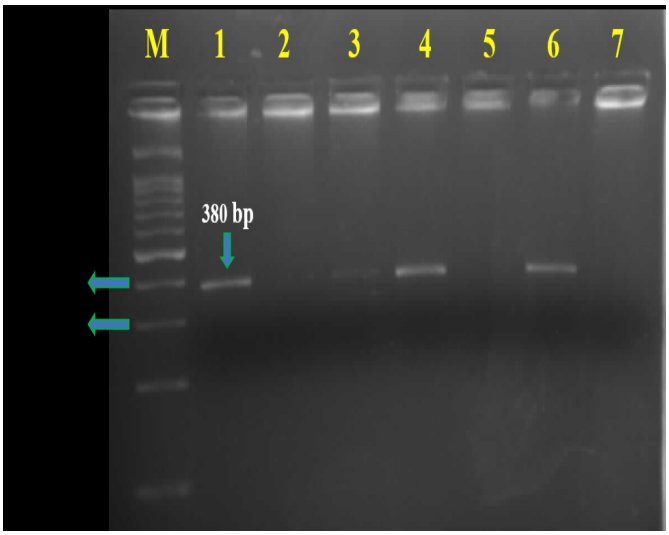


Fig. 3: PCR-based detection of *E. canis* from various clinical and post-mortem samples based on *virB9* amplified 380 bp fragment of genome (A); LM: 100 bp DNA Ladder RTU (Fermentas); L1: Positive control; L2: Negative control; L 3-7: Collected samples; 3,4,6 – Positive samples; 5,7-negative samples

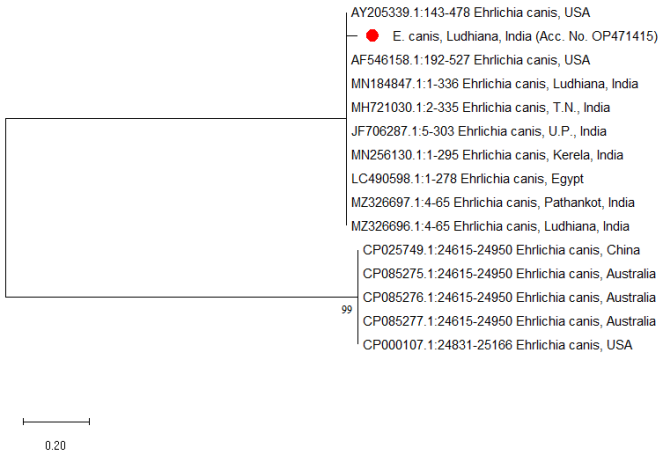


Fig. 4: Neighbour Joining tree showing clustering of present study *E. canis* strain based on nucleotide sequence of *virB9* gene using MEGA X program. (The bootstrap values are shown on the nodes of the tree and genetic distance is shown in 0.20 scale bar. Sequence obtained in this study is shown with highlighted taxon marker).

findings suggest that while both LFA-Ec and Ec-PCR are highly sensitive, their differences in specificity may impact their clinical applicability. The low specificity of the LFA-Ec test raises concerns about potential overdiagnosis, whereas the improved specificity of Ec-PCR supports its use as a more reliable diagnostic tool for Ehrlichiosis.

Conventional microscopy remains widely used for diagnosing canine haemoparasitic infections; however, its sensitivity is limited in subclinical and chronic infections due to low parasitaemia. Molecular assays, particularly PCR, offer greater sensitivity and specificity, with improved species-level differentiation, and diagnostic accuracy is the result of advancements in molecular biology tools. LFA-Ec cannot determine the animal's exposure status and cannot differentiate between current and previous infections. In contrast to PCR and immunochromatographic assays, the results of the current investigation also show reduced microscopic prevalence rates of canine haemoparasites, including buffy coat smear examinations. The low specificity of the LFA-Ec test raises concerns about potential overdiagnosis, whereas the improved specificity of Ec-PCR supports its use as a more reliable diagnostic tool for Ehrlichiosis.

Conclusion

The study highlights the widespread circulation of canine haemoparasites, including *E. canis,* in the Punjab canine population. The combined use of buffy coat microscopy, LFA-Ec and Ec-PCR supports an integrated diagnostic approach, with Ec-PCR offering greater specificity and LFA-Ec enabling rapid screening. The higher serological and molecular detection emphasizes the need for early and accurate diagnosis, particularly in clinically suspected and subclinical infections. Regular tick control, reduced tick exposure, routine surveillance, prompt treatment and preventive veterinary care are essential to limit *E. canis* transmission and disease propagation. Overall, integrated diagnostics, effective tick management and continuous monitoring are crucial for controlling canine ehrlichiosis in Punjab.

Acknowledgement

The authors are thankful to the Director of Research and Dean, Post Graduate Studies, Guru Angad Dev Veterinary & Animal Sciences University, Ludhiana for all the help to carry out this work.

Table 4: Comparison of various diagnostic tests with Ec- PCR

Test	Fischer's Exact Test for Buffy Coat			P value	Kappa value (κ)	SE of Kappa	Strength of agreement	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
LFA-Ec	**+**	**-**	**Total**						
+	3	45	48	1.000	0.005	0.005	Slight	100.00% (29.24 to 100%)	4.26% (0.52% to 14.54%)
-	0	2	2						
Total	3	47	50						
Ec-PCR	**+**	**-**	**Total**						
+	3	15	18	0.041	0.204	0.104	Fair	100% (29.24 to 100%)	68.09% (52.88% to 80.91%)
-	0	32	32						
Total	3	37	50						

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