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Full Length Research Paper

Challenges in determining the pathogenicity status of *Leptospira* isolates with phenotypic methods: The need for a polyvalent approach

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Understanding the pathogenic status of leptospires, the causative agents of leptospirosis, is important for successful laboratory diagnosis and control programmes of this zoonosis. Leptospires are difficult to differentiate morphologically; therefore, their pathogenic, intermediate or saprophytic status is currently determined based on both phenotypic tests like growth response in medium containing 8-azaguanine and growth at low temperature (13°C), and genotypic methods. The present study reports on the pathogenic *versus* saprophytic characterization of nine *Leptospira* isolates from animal hosts (rodents and dogs) and humans, with specific interest on a canine isolate coded "Dog109", which showed an ambiguous or intermediate *status* according to conventional (phenotypic) and molecular (genotypic) tests. The results strongly indicate the need of a polyvalent analytical approach for improving the differentiation of the pathogenic status of circulating serovars, particularly of fresh *Leptospira* isolates with an intermediate or controversial taxonomic position.

Key words: Characterization, isolates, *Leptospira*, leptospirosis, pathogenic, saprophytic.

INTRODUCTION

Leptospires cause leptospirosis which is among the neglected tropical diseases. They grow poorly or not at all in ordinary bacterial media. Therefore, routine cultural, morphological, and biochemical tests are not used in characterizing leptospires. The characterization for diagnosis and control measures depends on other approaches (Faine et al., 1999). The genus *Leptospira* is antigenically divided into two complexes, *L. interrogans* sensu lato (s.l.), comprising all pathogenic serovars, and *L. biflexa* s.l., containing the saprophytic strains. However, the new genomic classification system has revealed pathogenic species, which can contain both pathogenic and nonpathogenic serovars (Brenner et al., 1999) as well as intermediate species such as *L. meyeri*, *L. inadai* and *L. fainei* (Levett, 2003; Morey et al., 2006). The conventional classification of leptospires relies mainly on growth responses in medium containing 8-azaguanine (225 µg/ml) (Johnson and Rogers, 1964), and growth at low temperature (Johnson and Harris, 1967). The 8-azaguanine is an inhibitor of induced enzyme formation and in some instances this inhibitory effect may be reversed by guanine, guanosine or xanthine (Creaser, 1956). The low temperature test makes use of the fact that the minimum growth temperature ranges from 13-15°C for pathogenic leptospires and 5-10°C for saprophytes; however this criteria could be misleading as some pathogenic *Leptospira* like serovar Icterohaemorrhagiae can also grow at 10°C (Kmety et al., 1966). Inclusion of *in vivo*

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animal inoculation (guinea pigs and hamsters) and molecular PCR technology has been successfully used to differentiate *Leptospira* (Brenner et al., 1999; Murgia et al., 1997; Perolat et al., 1996, 1998).

This study reports typical controversies on the pathogenicity status characterization of *Leptospira* fresh isolates revealed by a polyvalent approach that included phenotypic tests (8-azaguanine and growth at 13°C), molecular (PCR) analyses and animal inoculation.

MATERIALS AND METHODS

Strains

Nine fresh *Leptospira* isolates from: a human patient (H1) and mammalian hosts (three rodent isolates from Madeira island coded MD4, MD7 and MD11, and five canine isolates from Mainland Portugal named Dog12, Dog109, Dog133, Dog150 and Dog342, collected under the Project IMUNOPOR, PPS4 "Optimization of a leptospiral canine vaccine in Portugal" funded by the PEDIP II Programme (INETI/IAPMEI; 1996-2000).

Four pathogenic *L. interrogans* reference serovars of Icterohaemorrhagiae serogroup: Copenhageni (strain Wijnberg), Mwogolo (strain Korea), Lai (strain Lai), and serovar Sokoine (strain RM1) from one Tanzanian bovine isolate.

Two saprophytic reference serovars: Patoc (strain Patoc I) of *L. biflexa* and Semaranga (strain Veldrat Semarang 173) of *L. meyeri*.

Characterization of *Leptospira* isolates by conventional methods

Pathogenic and saprophytic leptospires were respectively differentiated by: a) inhibition or growth response in the synthetic EMJH medium (Ellinghausen and McCullough, modified by Johnson and Harris) containing 8-azaguanine (225 µg/ml) according to Johnson and Rogers (1964), and b) inhibition or growth response at low temperature (13°C), according to Johnson and Harris (1967) and Hartskeerl et al. (2001). Duplicates of the test isolates and controls (pathogenic serovar Copenhageni, and saprophytic serovar Patoc) in EMJH medium with and without 8-azaguanine were incubated at 30°C and checked at interval of 2-5 days up to four weeks using dark field microscopy and spectrophotometer optical density reading (cells/ml at OD_{420nm}). Fresh EMJH medium with and without 8-azaguanine served as blanks. Similarly, for low temperature test, duplicate cultures of test isolates and reference strains were incubated at 13 and 30°C and checked as above. Plain - sterile EMJH medium served as blank.

In both 8-azaguanine and low temperature tests, the initial culture densities were set on an optical density (OD_{420nm}) of 0.000, whereas an OD_{420nm} of 0.100 was the cut-off point separating growth inhibition (OD_{420nm} < 0.100) and no inhibition (OD_{420nm} > 0.100), meaning a positive growth with normal multiplication of leptospires.

Characterization of *Leptospira* isolates by PCR

Leptospira DNA was extracted according to Boom et al. (1990). Four PCR with primers and procedures previously described for the identification of pathogenic and saprophytic leptospires were used. The PCR primers used for pathogenic leptospires were PCR 1 (Murgia et al., 1997)-330 bp; PCR 2 (Perolat et al., 1998)-1008 bp and PCR 3 (Perolat et al., 1998)-420 bp. The PCR for saprophytic leptospires (PCR 4) was according to (Murgia et al., 1997) -240 bp.

Animal inoculation

Three of the nine *Leptospira* isolates (Dog109, Dog133 and Dog150) (≈$3x10^8$ cells/ml) were intra-peritoneally inoculated into approximate one-month-old guinea pigs (*Cavia sp*) with an average weight of 360 g, according to Faine (1957). All animals were negative for leptospiral antibodies including serovar Sokoine that is prevalent in Morogoro (Mgode et al., 2006) where the guinea pigs used in the present study were obtained. The animals were monitored for signs of illness, or death, for 35 days.

RESULTS

Conventional (phenotypic) characterization

Growth in medium with 8-azaguanine (225 µg/ml)

Seven out of nine *Leptospira* isolates were inhibited (OD_{420nm} < 0.100) in EMJH medium containing 8-azaguanine (225µg/ml), but not in medium without 8-azaguanine (OD_{420nm} > 0.110). The reference pathogenic serovar Copenhageni was also inhibited by 8-azaguanine (OD_{420nm}=0.020, *versus* OD_{420nm}=0.209 in medium without 8-azaguanine). The mean optical densities of leptospires grown in the medium with and without 8-azaguanine were different (0.045±0.017 and 0.194±0.012 respectively), which corresponds well to the cut-off value for distinguishing growth status. This result indicates that the seven isolates (H1, MD4, MD7, MD11, Dog12, Dog109, and Dog342) are pathogenic strains. The growth of two canine isolates (Dog133 and Dog150) was not affected by the presence of 8-azaguanine (OD = 0.156 and 0.133, respectively). These ODs were similar to those of the reference saprophytic strain Patoc I in the medium with 8-azaguanine (0.113) and without 8-azaguanine (0.182). The growth trends of the representative saprophytic and pathogenic leptospires are shown in Figure 1.

Growth at low temperature (13°C)

Leptospires incubated at low temperature (13°C) showed growth inhibition (mean OD_{420nm} = 0.086±0.017) for six (H1, MD4, MD7, MD11, Dog12, and Dog342) out of nine isolates, and the reference pathogenic strain Wijnberg (OD_{420nm} = 0.001). These growth densities were much lower than those of the same strain incubated at 30°C indicating no inhibition (OD_{420nm} > 0.100), which is consistent with a pathogenic status. Three isolates (Dog109, Dog133 and Dog150) grew well at this low temperature with mean optical densities of 0.151, 0.165, and 0.180 respectively. These values exceeded the growth density of OD420nm=0.091 with the reference saprophytic strain Patoc I at the same growth condition. This is consistent with a saprophytic status. Initially, Dog109 isolate and reference saprophytic strain Patoc I were inhibited at low temperature on the first 6 days of

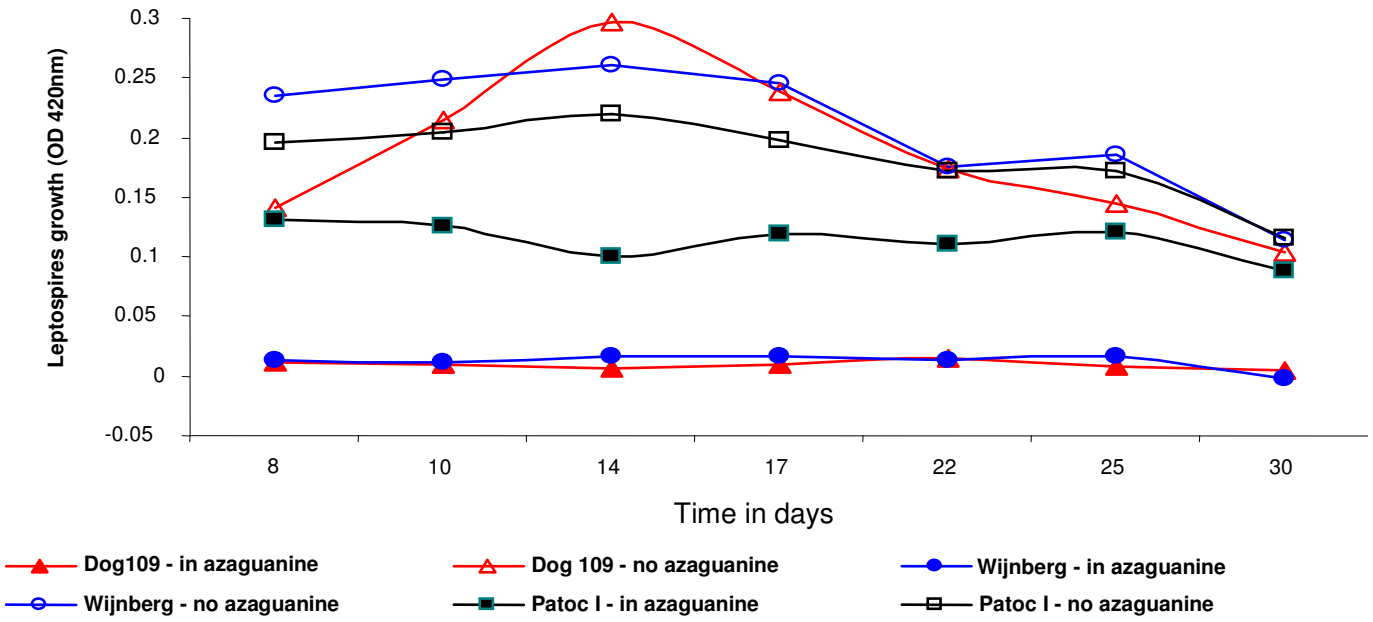

Figure 1. Growth trends of *Leptospira* in EMJH medium with 8-azaguanine (solid pictograms) and without 8-azaguanine (open pictograms) showing growth inhibition (OD_{420nm} < 0.100) and no inhibition (OD_{420nm} > 0.100). Dog109 and Wijnberg strains in 8-azaguanine show growth inhibition (OD_{420nm} < 0.100) unlike similar strains in medium without 8-azaguanine which had reached stationary phase (full growth = OD_{420nm} > 0.100) within 8 days.

incubation (OD_{420nm}=0.087±0.017 and 0.005±0.017 respectively). Around the 10th day Dog109 adapted to the "*in-vitro*" conditions showing no inhibition (OD_{420nm}=0.171±0.035) and the saprophytic strain adapted between the 13th and the 18th days with no inhibition (OD_{420nm}>0.100) differing from the pathogenic strains (OD_{420nm}<0.100).

Molecular (PCR) analyses

PCR 1 - pathogenic leptospires (Lepat 1/Lepat 2)

Six isolates (H1, MD4, MD7, MD11, Dog12 and Dog342) out of nine were PCR positive (330 bp), in accordance to the pathogenic control (serovar Mwogolo strain Korea). Three isolates (Dog109, Dog133 and Dog150) and the reference saprophytic serovar Semaranga strain Veldrat Semarang 173 were PCR negative with Lepat 1 and Lepat 2 (Table 1).

PCR 2 - pathogenic leptospires (LP1 / *a*1190)

With PCR 2, six isolates (H1, MD4, MD7, MD11, Dog12 and Dog342) were again positive (1008 bp) (pathogenic) whereas three isolates (Dog109, Dog133 and Dog150) and the reference saprophytic strain (Veldrat Semarang 173) were PCR negative (saprophytic) (Table 1).

PCR 3 - pathogenic leptospires (LU/rLP)

PCR 3 showed a similar pattern; six isolates (H1, MD4, MD7, MD11, Dog12 and Dog342) were PCR positive (420 bp) (pathogenic), while the three canine isolates (Dog109, Dog133 and Dog150) and the reference saprophytic serovar Semaranga were negative (Table 1).

PCR 4 - saprophytic leptospires (Sapro 1/Sapro 2)

In this PCR, the three canine isolates (Dog109, Dog133 and Dog150), and the reference saprophytic strains (Veldrat Semarang 173 and Patoc I) were positive (240 bp) (saprophytes), while six isolates (H1, MD4, MD7, MD11, Dog12 and Dog342) and the reference pathogenic serovar (Mwogolo and Lai) were negative (Table 1).

Animal inoculation

Leptospira strains (Dog109; Dog133; Dog150; reference saprophytic strain Patoc I; and local serovar Sokoine) inoculated into guinea pigs did not cause death or produce any noticeable signs of disease up to 35 days. However, a guinea pig inoculated with Dog109 isolate showed increased urination (polyuria), indirectly assessed by observing the degree of wetness of bedding materials in its cage, whereas in other cages were drier

Table 1. Summarized findings of the polyvalent characterization of *Leptospira* isolates.

Characterization methods (polyvalent approach)									
Conventional (phenotypic) tests			**Molecular (genotypic) tests**				**Animal inoculation**		
***Leptospira* Strains**	**Growth in 8-azaguanine**	**Growth at 13°C**	**PCR – pathogenic strains**			**PCR with saprophytic strains**	**Debility, illness or death**	**Pathogenic status**	**Reference**
			PCR 1 [c]	PCR 2 [d]	PCR 3 [e]	PCR 4 [f]			This study
H1 (human [a])	-	-	+	+	+	-	-	Pathogenic	This study
MD4 (rodent [a])	-	-	+	+	+	-	-	Pathogenic	This study
MD7 (rodent [a])	-	-	+	+	+	-	-	Pathogenic	This study
MD11 (rodent [a])	-	-	+	+	+	-	-	Pathogenic	This study
Dog12 (canine [a])	-	-	+	+	+	-	-	Pathogenic	This study
Dog109 (canine [a])	-	+	-	-	-	+	-	Intermediate	This study
Dog133 (canine [a])	+	+	-	-	-	+	-	Saprophytic	This study
Dog150 (canine [a])	+	+	-	-	-	+	-	Saprophytic	This study
Dog342 (canine [a])	-	-	+	+	+	-	-	Pathogenic	This study
Sokoine (cattle [a])	-	-	+	+	+	-	-	Pathogenic	Mgode et al., 2006
Patoc I [b]	+	+	-	-	-	+	-	Saprophytic	Faine et al., 1999
Semarang [b]	+	+	-	-	-	+	*	Saprophytic	Faine et al., 1999
Wijnberg [b]	-	-	+	+	+	-	*	Pathogenic	Faine et al., 1999
Korea [b]	*	*	+	+	+	-	*	Pathogenic	Faine et al., 1999
Lai [b]	*	*	+	+	+	-	*	Pathogenic	Faine et al., 1999

[a] = fresh *Leptospira* isolate; [b] = reference *Leptospira* strain; [c] = 330 bp fragment; [d] =1008bp fragment; [e] =420bp fragment; [f] = 240 bp fragment; * = not subjected to this test.

including that of the pathogenic strain.

DISCUSSION

The present study shows the typical challenges in characterizing *Leptospira* isolates with non-polyvalent or conventional phenotypic methods which may lead to incorrect identification of the pathogenicity status of new isolates. An analysis of nine fresh *Leptospira* isolates using phenotypic methods (8-azaguanine and low temperature) showed conflicting pathogenicity status (intermediate) in one isolate (Dog109). Dog109 isolate growth was inhibited by 8-azaguanine suggesting that it is pathogenic (Johnson and Rogers, 1964), but grew well at low temperature corresponding to saprophytic leptospires (Johnson and Harris, 1967). This controversy was resolved by genetic analyses (PCR) (Murgia et al., 1997; Perolat et al., 1998) that consistently assigned Dog109 isolate to saprophytic *Leptospira*.

Growth of this isolate at low temperature was ambiguous by being initially inhibited mimicking pathogenic strains and after six days adapted to this temperature with full growth similar to saprophytic strains. Similarly, the reference saprophytic strain (Patoc I) was also initially inhibited by low temperature and after 13 days adapted with full growth (Figure 2). Prolonged incubation of cultures at low temperature appears to be crucial in overcoming variable (plasticity) responses of leptospires to low temperature. With shorter incubation the growth of the canine (Dog109) isolate and the Patoc I strain at low temperature would be incorrectly considered as totally inhibited. Variable responses or plasticity is also reported in 8-azaguanine studies whereby the inhibitory effect of 8-azaguanine on induced enzyme formation could be reversed (Creaser, 1956). The observed conflicting pathogenic status (intermediate) on Dog109 isolate in the present study corroborates previous reports on other *Leptospira* strains (Brenner et al., 1999; Postic et al., 2000). Molecular analyses were only consistently congruent with those of the low temperature test despite varying responses of saprophytic leptospires to low temperature. This demonstrates the necessity for polyvalent analytical approach in determining the pathogenic status of leptospires isolates.

Pathogenicity establishment using guinea pigs was unsuccessful due to difficulties in assessing the effects in infected animals. All animals survived and remained healthier with increasing body weights from average of 360 g prior to inoculation to 500 g post inoculation although increase in body weight could also be due to good care provided to the experimental animals. Failure of leptospires to cause death or noticeable illness/debility in the experimental animal as well as asymptomatic

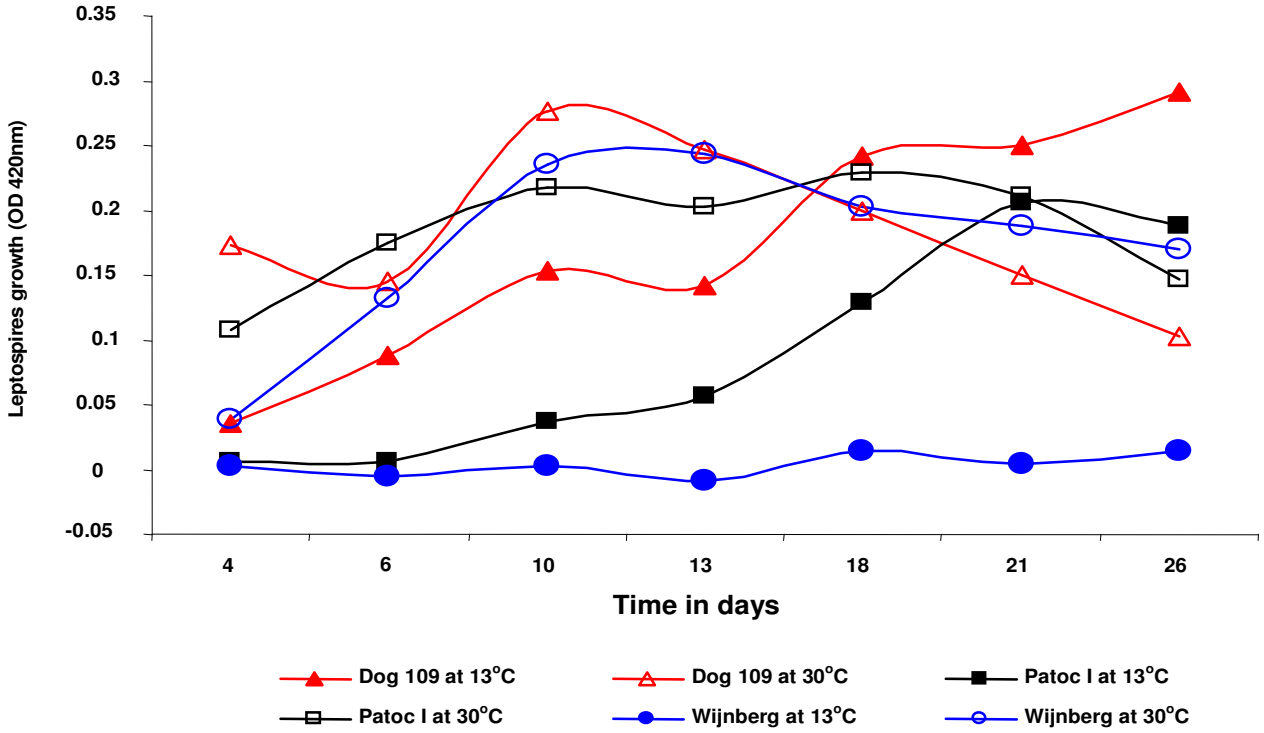

Figure 2. Growth trends of *Leptospira* cultures incubated at low and high temperatures (13 and 30°C; solid and open pictograms, respectively) showing inhibition of pathogenic strains at low temperature unlike the saprophytic strains.

leptospirosis has been reported elsewhere (Ashford et al., 2000). And most recently, the study in the Amazonian human population reported a high prevalence of asymptomatic leptospira colonization (Ganoza et al., 2010). This shows the difficulties in establishing thepathogenicity status of new *Leptospira* isolates in animal models. However, Dog109 isolate (intermediate pathogenicity) induced high polyuria in the guinea pig unlike the pathogenic strain. It should be noted that, unlike oliguria, polyuria is not a common clinical manifestation of leptospirosis and therefore might not be due to the experimentally injected leptospires. It is unfortunate that post-mortem examination was not performed to assess pathological features of the target organs. From the present research, it is evident that the correct characterization of leptospiral pathogenicity is strengthened by using a polyvalent analytical approach that minimizes uncertainties encountered from individual tests especially when phenotypic analysis does not strictly equate with genotypic speciation.

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