

LEPTOSPIRA INTERROGANS SEROVAR POMONA ASSOCIATED WITH ABORTION IN CATTLE: ISOLATION METHODS AND LABORATORY ANIMAL HISTOPATHOLOGY

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ABSTRACT

HERR, S., RILEY, A. E., NESER, J. A., ROUX, D. & DE LANGE, J. F., 1982. *Leptospira interrogans* serovar *pomona* associated with abortion in cattle: isolation methods and laboratory animal histopathology. *Onderstepoort Journal of Veterinary Research*, 49, 57-62 (1982).

Leptospira interrogans serovar *pomona* was successfully isolated from cattle urine in the western Transvaal after an abortion storm had occurred. Direct inoculation of EMJH medium proved the most successful method. The selective agent, 5-fluorouracil, was most effective in controlling contamination when used at the 0.4 mg/ml level. The strain isolated was pathogenic in hamsters, but specific lesions and the leptospires were seen only where overwhelming infection occurred.

INTRODUCTION

To date only limited reports of isolations of leptospires have been recorded in the Republic of South Africa. Malherbe & Kaschula (1953) presented serological and microscopic evidence for the presence of *Leptospira canicola* infection in dogs but were unsuccessful in culturing the organism. Five cases of leptospirosis were diagnosed in humans on the Witwatersrand by Gear, Wolstenholme, Jackson, Chesler & Brueckner (1958) and serological evidence was presented for infection by *L. canicola* in dogs belonging to 2 of these patients. No attempts were made to isolate the causative organism in any of these cases. *L. icterohaemorrhagiae* (*copenhageni*) and/or *L. sejroe* leptospirosis was diagnosed on serological as well as on cultural evidence in 2 human cases in Cape Town (Newman & Cohen, 1962). The presence of *L. icterohaemorrhagiae* (*copenhageni*) was demonstrated in dock rats in Cape Town, while strong serological evidence was presented for the occurrence of the disease in dogs (Rademan, Steytler & Wright, 1964). *L. canicola* was isolated also from dogs in Cape Town (Beyers, 1965) and from a dog and also pigs in the western Cape (Van Rensburg, 1973).

Serological surveys in cattle (Botes & Garifallou, 1967; Du Plessis, 1966) indicated that the disease was widespread in South Africa and that its incidence was high. Subsequent to these surveys, serological evidence for the occurrence of both *L. pomona* and *L. hardjo* infections in cattle was demonstrated. In one case leptospiral organisms were seen in centrifuged, formalinized urine specimens submitted from a western Transvaal herd during an abortion storm. The outbreak was dramatically terminated by herd treatment with 25 mg/kg of dihydrostreptomycin⁽¹⁾. However, attempts at culture were unsuccessful (Herr, unpublished data, 1980).

Although *L. pomona* is a recognized abortifacient in cattle (Blood, Henderson & Radostits, 1979), this syndrome had not so far been described in the Republic of South Africa. Consequently, when the opportunity arose, particular efforts were made to make a thorough study of the outbreak, giving special attention to the procedures used for bacteriological confirmation.

A variety of *Leptospira* serotypes may cause disease in man as well as in laboratory and domestic animals, liver and kidney lesions usually being part of such infections. Liver lesions may not be recognized grossly but infected animals will show characteristic microscopic changes (Benirschke, Garner & Jones, 1978). Since histopathological changes caused by leptospirosis in hamsters and guinea-pigs have not been described in any detail, the histopathology in these two laboratory species is described in the present study.

MATERIALS AND METHODS

Animals and locality

The farm selected for the investigation is situated in the western Transvaal bushveld (Zeerust district) and the farming method is extensive, a high density/short period, multi-camp, grazing system being used. The herd consisted of 340 Bonsmara × Afrikander breeding animals, 14 of which had aborted towards the end of the last calving season. The aborted foetuses were approximately 8 months gestation. The abortions occurred over a 3-week period 6-9 weeks before urine specimens were taken. Ten of the animals which aborted had been together in the only camp with an earthen dam as its water source. All the other camps were supplied by concrete watering points. A particularly dry season preceded the abortions. Serum samples were initially submitted on 26 November 1980 from the 14 animals which had aborted as well as from 6 randomly selected animals. Subsequently, on 10 December 1980, serum and urine samples were collected from 13 abortants and from 7 other randomly selected animals. Unfortunately, the record system was such that, of the cows that did not abort, it could not be established whether they had delivered live calves or whether or not they had been pregnant.

Serology

Sera were tested, using the microscopic agglutination microvolume technique of Sulzer & Jones (1978). Their method was modified to be read by manual transfer of test material onto microscope slides with coverslips under dark field illumination. The antigens used were cultures of *L. canicola*, *L. grippityphosa*, *L. hardjo*, *L. hyos* (*tarrasovi*), *L. icterohaemorrhagiae* (*copenhageni*), *L. pomona* and *L. pyrogenes* maintained in the dark on liquid modified Stuarts' medium⁽²⁾ at 29 °C. The end-point titre was taken as the dilution where 50% of the organisms, as compared with the negative control, were either absent or visibly agglutinated and where a marked difference existed between it and the immediately preceding lower dilution.

In addition to the serology done on cattle sera above, sera from the hamsters used in biological isolation and from the guinea-pigs used in pathogenicity testing were also tested when these animals were sacrificed. In all these cases a titre of less than 1/80 was regarded as negative, 1/80 as suspicious and 1/160 or higher as positive.

Urine collection

Five cattle at a time were confined in a crush and one labourer per animal was provided with a sterile 250 ml nylon beaker fitted with a tinfoil cover for collecting midstream urine specimens. The perineal area was wiped

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clean of faecal material with dry cotton wool. The first 5 animals were treated intravenously (i/v) with 500 mg of frusemide⁽³⁾. Subsequent batches of animals were kept in the crush for 10 minutes and only those that had not urinated by this time were treated with frusemide. Immediately after collection, the specimen was covered with tinfoil and taken to a vehicle parked in the shade 30 m from the crush. This was considered necessary to avoid the dust contamination of the air in the vicinity where specimens were being processed.

Inoculation of media

Semi-solid media containing 0,15% Bacto agar⁽²⁾ were used. The first was a modified Stuarts' medium⁽²⁾ with 10% locally produced rabbit serum, 0,5% haemoglobin solution and 0,2 mg 5-fluorouracil⁽⁴⁾/ml medium added. The second was EMJH medium with EMJH enrichment⁽²⁾ and 0,1 mg 5-fluorouracil/ml medium added. The media were dispensed in 5 ml quantities into screw-capped tissue culture tubes, incubated for 48 h at 37 °C and 72 h at 29 °C to check for contaminants. A *L. pomona* culture was inoculated in 4 tubes of each medium in 0,05, 0,1, 0,5 and 1,0 ml quantities to check growth-supporting properties.

Each urine specimen was inoculated into 4 tubes of each medium, 2 with 0,025 ml and 2 with 0,05 ml of inoculate by means of sterile plastic disposable tips and micropipettes⁽⁵⁾. No attempt was made to mix the inocula with the media, and the cultures were kept at ambient temperature but protected from direct sunlight for up to 8 h before being placed in an incubator at 29 °C.

Examination and subculturing of inoculated media

All media were examined macroscopically from the 4th day for the presence of a Dinger's zone and/or contamination. Wet smears from these cultures were examined microscopically. Contaminated cultures negative for leptospirae were discarded, while 4 tubes of semi-solid enriched EMJH⁽²⁾ medium containing 0,4 mg 5-fluorouracil/ml were inoculated from all positive cultures, whether visibly contaminated or not. One heavily contaminated *Leptospira* positive culture was inoculated (1 ml i/p) into a hamster in an attempt at biological purification. After 25 days all cultures were examined microscopically and those in which no leptospirae were found were discarded. Positive cultures were again subcultured as above.

These subcultures were examined daily for the presence of a Dinger's zone and then examined microscopically. Where good growth was seen the subcultures were subinoculated into various media, including the semi-solid EMJH described above, a liquid enriched EMJH⁽²⁾ and a commercial⁽²⁾, as well as a locally prepared, liquid modified Stuart's medium (Cruickshank, Duguid, Marmion & Swain, 1975).

Typing of isolates

When a 4–14 day culture with good growth was achieved by subculture on liquid Stuart's medium from specimen No. 9, it was typed, using various antisera. These included commercial⁽²⁾, locally prepared antisera and antisera kindly supplied by WHO Collaborating Centres for Leptospirosis in Australia and the USA⁽⁶⁾. In addition, a culture was sent to the WHO Collaborating Centre, Australia, for typing.

Hamster inoculation

Hamsters of various ages and both sexes were used. The abdomen was shaved, swabbed with 70% alcohol and 1 ml of urine injected intraperitoneally (i/p), using a blunt 20 gge × 2,5 cm needle. A single hamster per specimen was inoculated.

The hamsters were observed at 4-hour intervals for outward signs of illness. On Day 7 hamsters No. 5 and 11 were sacrificed, as at that stage other specimens from the urine samples of these 2 cattle had proved positive on direct examination and/or culture. At the same time hamster No. 20 (negative to the other examinations) was also euthanized. Immediately after death the hamsters were pinned to a board in a vertical laminar flow cabinet⁽⁷⁾. The abdomen was swabbed with 70% alcohol, the skin reflected, using a sterile forceps and scalpel, and pinned to the board, while a 2nd set of sterile instruments was used to reflect the abdominal musculature and remove the required tissue specimens. One kidney was cut into appropriate sections, while approximately 5 × 2 × 2 mm segments of liver tissue, pinched off with a forceps, were inoculated into the variety of semi-solid Stuart's and EMJH media as previously described. The remaining kidney and a 15 × 5 × 4 mm liver segment were placed in 10% formalin for histopathological examination.

On Day 8 another 2 hamsters, No. 2 and 9, which had also proved positive on other examinations, were sacrificed. Hamster No. 1 died on Day 11. The rest of the hamsters were euthanized on Day 13. Specimens for culture and histopathology were taken as described above. In all these cases a subculture onto semi-solid EMJH/0,4 mg 5-fluorouracil medium was done on Days 5 and 17 and the original cultures from tissue specimens were examined microscopically at the same time. In the case of specimen No. 1 this subculture was done on Day 1.

Direct examination of formalinized urine specimens

One hundred ml of the balance from each urine specimen was formalinized by adding 2,5 ml of a filtered 40% formalin solution.

The formalinized urine specimens were first centrifuged at 1 000 g for 10 minutes for the sedimentation of any larger particles present. The supernatant fluid was carefully decanted and recentrifuged at 3 000 g for 60 min. The resultant pellet was resuspended in a large drop after the supernatant was decanted, and wet smears were examined microscopically under 128 × and 320 × dark-field illumination. The supernatant was then recentrifuged at 34 000 g for 30 min, and the sediment re-examined as above. Smears from sediments of the 3 000 g centrifugation were stained by a modified Giemsa staining technique (Ris & Hamel, 1978), and microscopically examined as above.

Testing for pathogenicity

A subculture on semi-solid EMJH/0,4 mg of 5-fluorouracil medium which came originally from the urine culture of specimen No. 9 was used to inoculate 2 weanling hamsters and 2 weanling guinea-pigs (1 ml i/p) when this tube showed good growth on the 5th day post-subculturing. Six days later 2 weanling hamsters were injected with 1 ml of uninoculated semi-solid EMJH/0,4 mg of 5-fluorouracil medium i/p to act as negative controls. Tissue specimens were inoculated into media as described above when these animals showed signs of disease or died. Serum samples from the animals that survived as long were examined serologically on Day 21. The negative controls were sacrificed after 25 days.

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Histopathology

Liver and kidney specimens were fixed in 10% buffered formalin solution, processed in a routine manner and embedded in paraffin wax. Sections were cut at 3-5 μm and stained with haematoxylin and eosin (HE). In addition, both liver and kidney sections were stained by the silver impregnation method of Warthin-Starry (Young, 1969) for identification of spirochaetes.

RESULTS

Serology

In the original serological investigation (26 November 1980; Table 1), 13 out of the 14 cows that aborted were classified as serologically positive, whereas out of the 6 that did not abort 3 were positive. The 2nd test (10 December 1980; Table 1) showed that all 13 cows that aborted were serologically positive, while amongst the 7 non-aborters 2 were positive and one suspicious. Serology performed on hamster sera after euthanasia was negative in all cases. The 2 guinea-pigs had titres of 1/160 to *L. pomona* on Day 21.

TABLE 1 The correlation between seropositive reactors, abortions and isolations

Category of animal	Total	% seropositive	% isolation	
			Biological	Bacteriological
Aborters	14	92,8	21,4	64,3
Non-aborters	13	38,5	0	7,7

TABLE 2 The efficacy of detection of leptospire in sediments from urine subjected to different relative centrifugal force (g) in wet and stained smear preparations

No. of positive isolations	% positive		
	Wet smears		Stained smears
	3 000 g	34 000 g	3 000 g
10	40	20	10

5-fluorouracil as a selective agent

Contamination occurred in 47,5% of the urine inoculations in which 0,1 mg of 5-fluorouracil/ml was used, in 23,75% where 0,2 mg levels were present and, in the subcultures with 0,4 mg, only 13,4% (Table 3). Subculturing onto the higher level of 5-fluorouracil was successful in a large number of cases in purifying already contaminated cultures. No difference in the growth of leptospirae was seen between the different levels of selective agent used.

TABLE 3 The efficacy of different levels of 5-fluorouracil in controlling contamination

	Medium and 5-fluorouracil level		
	Semi-solid EMJH 0,1 mg/ml	Semi-solid Stuart's 0,2 mg/ml	Semi-solid ⁽¹⁾ EMJH 0,4 mg/ml
Total	80	80	82
Contaminated	38	19	11
% Contamination	47,5	23,75	13,4

⁽¹⁾ Used exclusively to subculture both contaminated and clean cultures emanating from the original urine inoculations as well as inoculations of liver and kidney tissues from hamsters

Efficacy of media

Despite the higher incidence of contamination experienced in the EMJH medium with only 0,1 mg of 5-fluorouracil per ml, the majority of successful isolations was

achieved in this medium. Leptospirae were isolated from 24/80 tubes of EMJH representing 10 different animals, while single tubes from 2 of these animals proved positive in Stuart's medium.

Typing

Cultures on liquid Stuart's medium, showing good growth between Day 4 and Day 14 when titrated against a battery of antisera (Table 4), gave titres of 1/640-1/2560 with *L. pomona* antisera, 1/80 with *L. autumnalis* and were negative for the other antisera used. The WHO Collaborating Centre for Leptospirosis in Australia confirmed the isolate as being serovar *pomona*.

TABLE 4 Typing of isolate against various antisera

Antisera	Titres
<i>pomona</i> (OP) ⁽¹⁾	1/640
<i>pomona</i> (Difco) ⁽²⁾	1/640
<i>pomona</i> (Australia) ⁽³⁾	1/2560
<i>pomona</i> (U.S.A.) ⁽⁴⁾	1/2560
<i>hardjo</i> (Australia)	—
<i>hardjo</i> (OP)	—
<i>hardjo</i> (Difco)	—
<i>australis</i> (U.S.A.)	—
<i>autumnalis</i> (U.S.A.)	1/80
<i>bataviae</i> (U.S.A.)	—
<i>sejroe</i> (U.S.A.)	—
<i>canicola</i> (Difco)	—
<i>hyos</i> (Difco)	—
<i>grippityphosa</i> (Difco)	—
<i>icterohaemorrhagiae</i> (Difco)	—
<i>pyrogenes</i> (Difco)	—

⁽¹⁾ Antiserum prepared by Veterinary Research Institute, Onderstepoort

⁽²⁾ Commercial antiserum supplied by Difco

⁽³⁾ Antiserum kindly supplied by WHO Collaborating Centre for Leptospirosis, Australia

⁽⁴⁾ Antiserum kindly supplied by WHO Collaborating Centre for Leptospirosis, Atlanta, Georgia, USA

Biological isolation

Leptospirae were successfully isolated from liver and kidney tissue of hamsters in 3 specimens. Subculturing on Day 1 or Day 5 proved successful in all 3 cases, but was only successful in 1 specimen when the process was delayed to Day 17. By Day 17 organisms could no longer be demonstrated in any of the other original cultures, whereas the subcultures showed good growth. At this stage 8/38 (21%) of the original cultures were contaminated, while none of the subcultures had this problem. In all, 10 positive culture cases were seen amongst the 15 serologically positive animals.

Biological purification of contaminated cultures

One tube from specimen No. 5, which was very heavily contaminated, was successfully purified by passing 1 ml i/p in a hamster, sacrificing the animal 5 days later, inoculating liver and kidney tissue into EMJH with 0,4 mg of 5-fluorouracil/ml and subculturing into fresh media on Day 3. Subculturing the original contaminated specimen in this instance was unsuccessful in purifying the culture.

Direct examination of formalinized urine specimens

Sediments from urine centrifuged at 3 000 g for 60 min were the most successful in demonstrating the organisms (Table 2), but in the case of specimen 9 a remarkable concentration of organisms was achieved by recentrifugation of the supernatant at 34 000 g for 30 min. This was not the case in the other specimens. Stained smears proved less reliable in demonstrating organisms than wet smears. In all, 4 specimens, representing 40% of the culture positive cases, proved positive in this examination (Table 2).

Pathogenicity

Of the 2 hamsters inoculated with 1 ml of culture, one was euthanized in *extremis* 103 h post-inoculation and the other died between 112 and 116 hours. *Leptospirae* were isolated from liver and kidney tissues in both cases. The control hamsters inoculated with clean medium showed no untoward effects up to 25 days.

Of the 2 guinea-pigs inoculated with culture one died on Day 30 and no attempt at culture was made because of the more than 6 h that had elapsed between death and post-mortem. The other had a staring haircoat and appeared ill on Day 30 and was euthanized. *Leptospirae* were cultured from this latter case. Serology performed on the sera of the 2 guinea-pigs on Day 21 showed both were positive to *L. pomona* at 1/160 dilution and negative to the other serotypes.

Gross pathology

A moderate anaemia, icterus and enlargement of the kidneys were seen in the 2 hamsters infected with *Leptospira*-positive subculture material from urine specimen No. 9. No other significant gross changes could be seen at autopsy in the remaining hamsters and guinea-pigs.

Histopathology

The most important microscopic changes in the liver and kidneys of the hamsters and guinea-pigs are summarized in Table 5, while the number of cases in which organisms could be microscopically demonstrated are indicated in Table 6.

TABLE 5 Incidence of microscopic changes in the liver and kidneys of hamsters and guinea-pigs infected with suspected *Leptospira*-positive bovine urine and culture

Liver lesions	No. of cases positive	Kidney lesions	No. of cases positive
Marked congestion of vessels and sinusoids	6/24	Marked congestion and disseminated foci of haemorrhage	16/24
Marked round cell infiltration into portal tracts	7/24	Foci of interstitial round cell infiltration	3/24
Disruption of hepatic cell cords and individualization of hepatocytes	11/24	Eosinophilic homogeneous and granular tubular casts	7/24
Hydropic degeneration and single cell necrosis of hepatocytes	13/24	Hydropic degeneration and single cell necrosis of tubular epithelium cells	17/24

TABLE 6 Incidence of *Leptospira* organisms demonstrated by microscopic examination of liver and kidney specimens in relation to isolation of organisms from organ and urine culture specimens

Organ	Microscopic identification No. of cases positive	Isolation from organs No. of cases positive	Isolation from urine culture No. of cases positive
Liver	2/24	6/24	7/20
Kidney	4/24	6/24	10/20

Liver

Moderate, diffuse, degenerative changes, accompanied by varying degrees of round cell infiltration into portal triads, characterized the appearance of the liver in both hamsters and guinea-pigs. The sinusoidal structure appeared disturbed because of swelling and in some cases there was individualization of hepatocytes (Fig. 3). Congestion of central veins, sinusoid and interlobular vessels appeared conspicuous in certain areas. Single or

small foci of 2 or more necrotic hepatocytes with eosinophilic cytoplasm and pycnotic nuclei were scattered throughout the liver (Fig. 1). Varying numbers of plasmacytes, lymphoblasts and mature lymphocytes were present within the portal triads. No significant correlation between the intensity of portal round cell infiltration and the isolation of *leptospirae* from the liver and kidneys could be found (Table 5).

After specific staining of liver sections with the silver impregnation method of Wharthin-Starry (Young, 1969), no distinct *Leptospira* organisms could be identified in specimens from hamsters infected with suspected *Leptospira*-positive bovine urine (Table 6). Numerous *Leptospira* organisms, however, could be seen in specimens from both the 2 hamsters and the 2 guinea-pigs that had been infected with 1 ml of positive subculture from urine specimen No. 9 (Table 6). Large numbers of black-staining, spiral to L shaped organisms, varying in length from 5–15 μ m, could be seen in sinusoids and blood vessels as well as in the cytoplasm of hepatocytes (Fig. 2).

Kidneys

In most cases moderate congestion and small focal disseminated haemorrhages were present in the cortex and medulla of the kidneys in both hamsters and guinea-pigs (Table 5). Small foci of interstitial, round cell infiltration were present in the cortex of some cases. As in the liver, there was no correlation between the presence of interstitial round cell foci and the isolation of *Leptospira* organisms from the kidneys and liver (Table 5).

Cloudy swelling, hydropic degeneration and single cell necrosis of tubular epithelial cells were regularly present in the cortex and medulla. Some tubules in both the cortex and medulla contained eosinophilic hyalin and granular casts (Fig. 3).

No *Leptospira* organisms could be seen after careful microscopic examination of silver-stained kidney specimens from hamsters infected with suspected *Leptospira*-positive urine (Table 6). As in the case of the liver, numerous organisms could be seen in the specimens from the 2 hamsters and 2 guinea-pigs infected with 1 ml of positive subculture from a urine specimen. Large aggregations of organisms could be seen in the lumen of tubules as well as in the cytoplasm of epithelial cells (Fig. 4).

DISCUSSION

Significant serological titres

An arbitrary level from which to judge serological titres as positive is often taken as 1/100 (Turner, 1968), although recently lower levels have been suggested (Milner, Wilks & Calvert, 1980; Little, Hussaini & Jones, 1980; Mackintosh, Marshall & Broughton, 1980). In our results the 1/160 end-point proved significant, as no positive cultures were achieved below this level and 10 out of 15 cultures were successful where the titre was 1/160 or higher (Table 1). An important criterion for finding a large percentage of high titres in cattle indicating leptospirosis following an abortion storm (Sullivan, 1974) was also seen in this series, although our titres were of the order 1/320–1/1280 as against his 1/3000. This level of discrepancy in end-point titres loses much of its significance when one considers the absence of standardization of antigens and the variability of interpretation of results between laboratories.

The failure of the hamsters used in biological isolation from urine to develop detectable titres can best be explained by the early termination of the experiment on Day 13. Seroconversion in guinea-pigs after urine inoculation was present on Day 21 (Doherty, 1966). Other

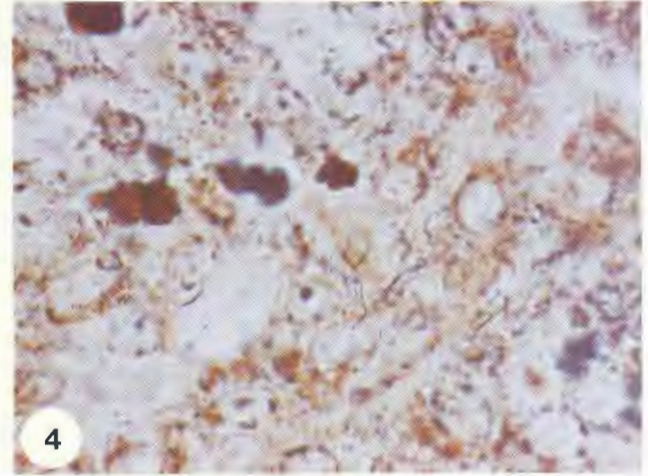
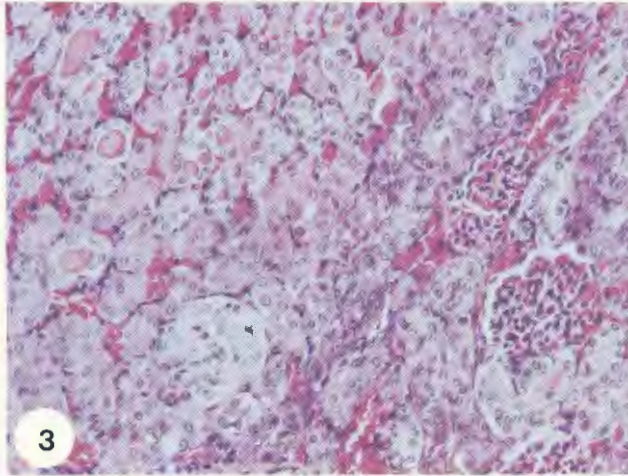
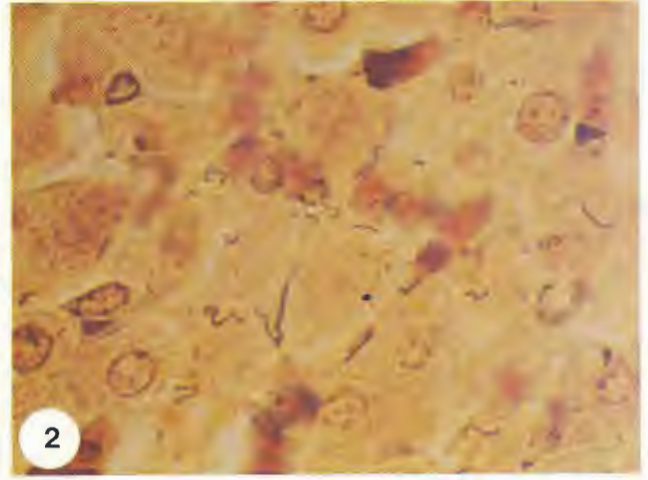
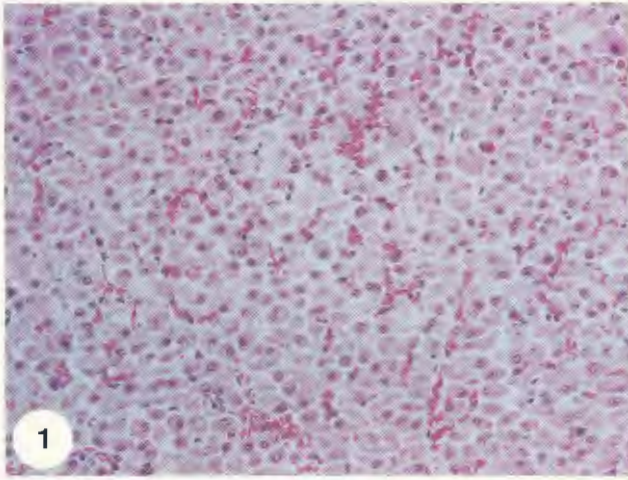


FIG. 1 Liver. Disintegration of sinusoidal structure caused by swelling and individualization of hepatocytes: HE \times 200

FIG. 2 Liver. Black-staining spiral to L-shaped organisms. Whartthin-Starry method for leptospirae \times 1200

FIG. 3 Kidney. Hydropic degeneration, single cell necrosis, homogenous and granular casts in the renal tubules: HE \times 200

FIG. 4 Kidney. Black-staining spiral to L-shaped organisms. Whartthin-Starry method for leptospirae \times 1200

workers, Sulzer & Jones (1978), stipulate the use of weanling hamsters, but those used by us were the only ones available. The 2 hamsters used in pathogenicity trials died before seroconversion was likely to have occurred. The 2 guinea-pigs used in the latter trial were thus the only laboratory animals to be tested serologically at the 21-day level, and they proved to be positive.

Direct examination of formalinized urine specimens

Although other authors found direct examination of urine to be reliable after centrifugation at 800 g for 45 min (Doherty, 1966), or 3 000 g for 40 min (Ris & Hamel, 1978), we found, both in this series and in a previous examination of infected urine, that leptospirae were difficult to identify amongst the debris present unless a preliminary centrifugation at 1 000 g for 10 min was done to remove the larger particles. In the examination of sediments from this preliminary centrifugation leptospirae could not be identified with any degree of certainty. The sediments from the higher g force centrifugations were vastly superior in this respect. In a single case a remarkable concentration of organisms was seen after recentrifugation at 34 000 g for 30 min, and this could be simply explained if the pellet had been dislodged into the supernatant after the initial centrifugation. Alternatively, the specific gravity of the urine relative to that of the organism may be such that in some cases a higher g force is necessary to ensure pelleting of the bacteria.

The staining technique of Ris & Hamel (1978), which proved as successful as wet smears in their examination, was markedly less so in our cases (Table 2). This may well be due to the fact that we were working with this technique for the first time and that further experience would be necessary.

Bacteriological versus biological isolation and urine examination

Although the hamsters used were not weanlings, bacteriological isolation proved so vastly superior (10/15 serologically positive cases) to biological methods (3/15 serologically positive cases) that the latter method seems hardly worthwhile. Our finding was corroborated by Mackintosh *et al.* (1980). Doherty (1966) found direct examination of urine as effective as serology in guinea-pigs, while Ris & Hamel (1978) found direct examination of urine specimens both as wet and stained smears to be more effective than hamster inoculation but less so than direct inoculation into EMJH semi-solid medium. In our results the same tendency is seen, since direct inoculation into media gave the best results (10 successful isolations), then, in order, direct examination of urine (4 positive cases) and biological isolations (3 positive cases) (Tables 1 & 2).

Pathology

Microscopic changes in the liver of hamsters infected with *Leptospira*-positive urine specimens (Table 5) were not specific enough to be of accurate diagnostic value. There was a considerable amount of overlap between changes present in hamsters infected with positive and negative urine specimens. The most consistent changes, however, appear to be individualization, hydropic degeneration and single cell necrosis of hepatocytes. Hepatic changes in the 2 hamsters and 2 guinea-pigs infected with *Leptospira*-positive subculture material from urine specimen No. 9 were similar but more emphatic and severe. These changes in the 2 hamsters, although not very specific, have also been reported by others in a variety of host species (Smith, Jones & Hunt, 1972).

Accentuation of these changes in the latter specimens was probably the result of overwhelming infections with *L. pomona*. The relatively large number of organisms

visible in these specimens further supports this conclusion. The emphatic round cell infiltration into portal tracts appears to be of no significance, since it was present in both infected and non-infected specimens. Emphatic round cell infiltrations into portal tracts caused by leptospirosis have not been recorded in the literature to the best of our knowledge, but these changes may be the result of previous occult bacterial infections of our hamster and guinea-pig colonies.

Microscopic changes in the kidneys of infected hamsters and guinea-pigs were not specific enough to be of reliable diagnostic value either. As in the case of liver specimens, there was considerable overlap between the changes present in infected and non-infected specimens (Table 5). Marked hyperaemia, haemorrhage, tubular degeneration, cast formation and inflammatory cell infiltration have been described as characteristic lesions in rats and mice (De Martino, Belloci & Natali, 1969; Fuzi & Csoka, 1962). The most characteristic changes, however, appear to be degeneration and necrosis of tubular epithelium and eosinophilic homogeneous and granular cast formation in renal tubules. Accentuation of these changes was present in the kidney specimens of the hamsters and guinea-pigs infected with subculture material. As in the case of the liver specimens, this was probably due to the greatly increased infective dosage.

Microscopic demonstration of *Leptospira* organisms by the silver impregnation method of Wharthin-Starry was of limited value. Organisms could be identified with certainty only in the hamsters and guinea-pigs that had been infected with overwhelming numbers of organisms from positive urine subculture material.

The Wharthin-Starry method for the impregnation of spirochaetes with silver in formalin-fixed sections is the most reliable method when performed according to the specifications. Difficulty in demonstrating *Leptospira* organisms by this method implies their absence from sections rather than their failure to stain clearly. The method has several advantages. Sections can be cut and impregnated from paraffin blocks which have been processed in the normal way. It is relatively quick and simple and lends itself well to the impregnation of large numbers of sections (Young, 1969).

CONCLUSIONS

L. pomona as the cause of the abortions

Although Koch's postulates cannot be fulfilled until, in the dosage and route that could be expected in nature, this isolate proved capable of causing abortion in cattle, many of the criteria for satisfying these postulates have been established. *L. pomona* has been isolated from the cows and it has proved pathogenic, at least in hamsters. There is thus little doubt in the authors' minds that this abortion storm was due to leptospirosis.

5-fluorouracil as a selective agent

Various authors have reported on the efficacy of 5-fluorouracil as a selective agent if used in concentrations between 0.05 and 1 mg/ml (Johnson & Rogers, 1964; Hussaini & Ruby, 1976; Sulzer & Jones, 1978; Flint & Liardet, 1980). Levels of 0.4 mg/ml proved best in controlling contaminants, and we would recommend the use of at least this level where contamination is a problem.

Size of inocula in bacteriological isolation

Specifically in the case of urine, but also with inocula such as blood at 1/100–1/200, the dilution factor enhances the success rate, probably because toxic factors in the inoculum itself or from its breakdown products are adequately diluted (Hussaini & Ruby, 1976; Ellinghausen, Deyoe & Nervig, 1977; Sulzer & Jones, 1978; Flint

& Liardet, 1980). No difference in success of isolation could be seen between our series of 1/100 (0,05 ml urine/5 ml medium) and 1/200 (0,025 ml/5 ml).

Efficacy of media

The EMJH media proved vastly superior in field isolation work despite the finding that a laboratory strain of *L. pomona* did equally well on both media in preliminary trials. This finding is in agreement with the published findings of other workers (Orr & Little, 1979; Sulzer & Jones, 1978; Ellinghausen, 1975; Mackintosh *et al.*, 1980).

Pathogenicity

Our finding that the *L. pomona* strain isolated was more pathogenic in hamsters than in guinea-pigs agrees with the recommendation of Ellinghausen (personal communication, 1980) that hamsters be used in vaccine challenge trials.

Histopathological diagnosis of infected laboratory animals

Since changes observed were largely non-specific and organisms only demonstrable in overwhelming infections, this method failed as a diagnostic approach (Tables 5 & 6).

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