



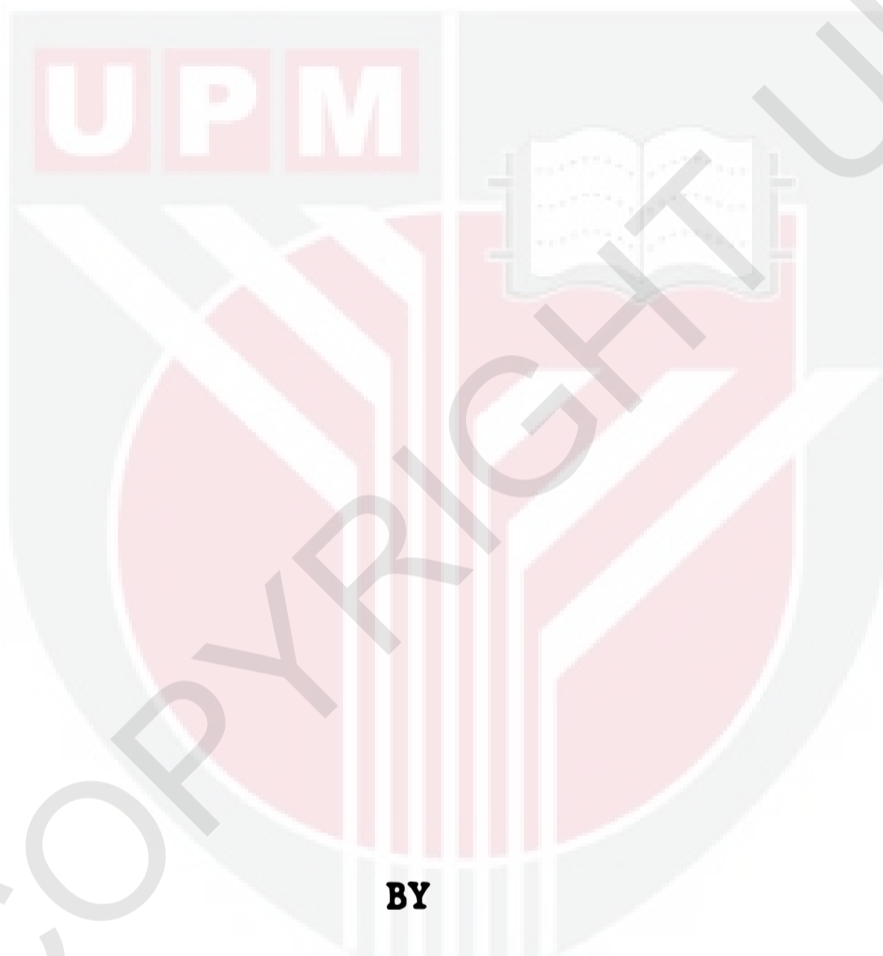
UNIVERSITI PUTRA MALAYSIA

**A STUDY ON THE PATHOLOGY OF LIVERS OF SWINE CONDEMNED
AT SLAUGHTER AT SHAH ALAM ABATTOIR**

SITI SALMIYAH BTE TAHIR

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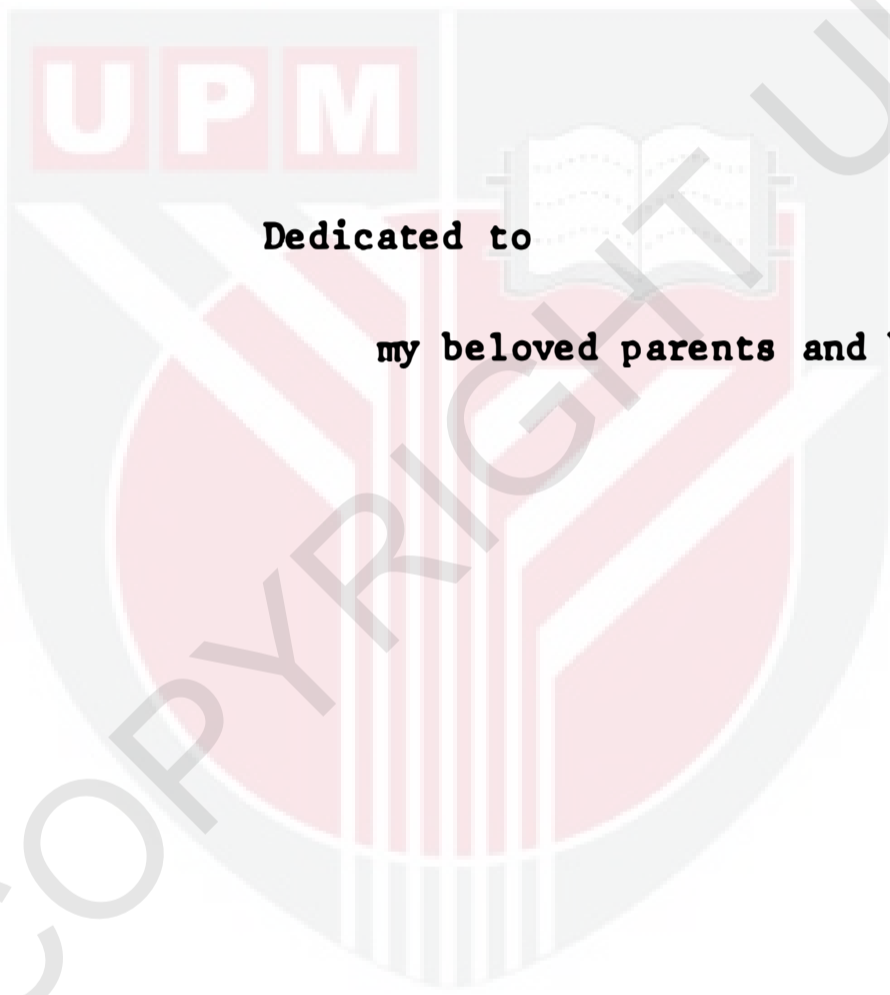
**A STUDY ON THE PATHOLOGY OF LIVERS OF
SWINE CONDEMNED AT SLAUGHTER AT
SHAH ALAM ABATTOIR**



**BY
SITI SALMIYAH BTE TAHIR**

**THIS PAPER WAS SUBMITTED AS A PARTIAL FULLFILMENT
OF A DEGREE OF VETERINARY MEDICINE (DVM) AT THE
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JANUARY 1985



Dedicated to

my beloved parents and brother

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ABSTRAK

Satu kajian mengenai patologi hati babi yang telah 'condemn' dijalankan selama lima minggu di rumahsembelih Shah Alam, dimana 100 keping hati telah dikumpulkan, 75 keping daripada babi jantan muda dan 25 keping daripada babi betina tua. Lesi-lesi yang diperolehi termasuk 36 'milkspots', 16 peritonitis/perihepatitis, 12 abses hepa, 11 kolangiohepatitis, 8 kolangitis, 5 hiperplasia nodular, 4 nekrosis hepa, 3 'post necrotic scarring', 2 lipidosi hepa dan satu 'cystic bile duct hyperplasia', 'massive necrosis' dan hepatokarsinoma. 'Milkspot' akibat dari perpindahan larva *Ascaris suum* adalah satu masalah besar terutama dalam babi jantan muda (41%). Empat belas daripada hati 'milkspot' di atas (39%) diberikan nilai lesi sebanyak 4+ dimana semua lobanya terlibat. Peritonitis/perihepatitis dilihat hanya dalam babi jantan muda dan hiperplasia nodular hanya dilihat dalam babi betina tua. *Escherichia coli* dan *Klebsiella* sp. adalah dua spesis bakteria yang kerap diasingkan daripada 12 abses hepa. Penyakit-penyakit yang mungkin menyebabkan lesi-lesi yang diperolehi telah dibincangkan.

ABSTRACT

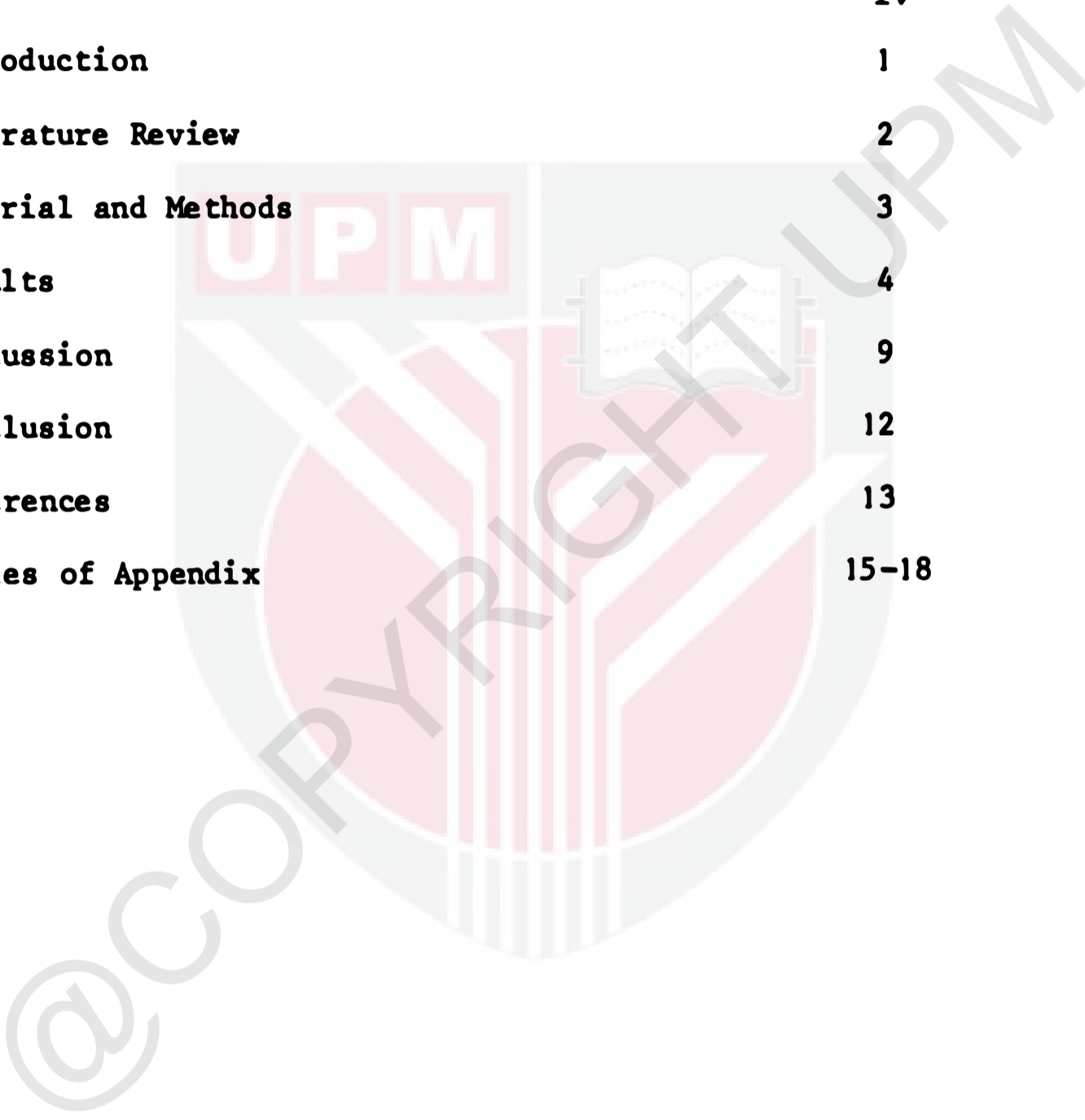
A five weeks study on the pathology of condemned livers of 100 pigs, 75 porkers and 25 sows at Shah Alam abattoir was conducted. The lesions seen included 36 milkspots, 16 peritonitis/perihepatitis, 12 abscessation, 11 cholangiohepatitis, 8 cholangitis, 5 nodular hyperplasia, 4 local hepatic necrosis, 3 post necrotic scarring, 2 hepatic lipidosis and one case each of cystic bile duct hyperplasia, massive necrosis and hepatocarcinoma. Milkspots due to migration of *Ascaris suum* larvae was a big problem especially in porkers (41%). Fourteen of the milkspot livers (39%) were severely affected with a 4+ score for all the lobes affected. Peritonitis/perihepatitis was seen only in porkers and nodular hyperplasia was seen only in sows. *Escherichia coli* and *Kliebsiella* sp. were the common bacteria isolated from 12 liver abscessations. The possible causes of the lesions were discussed.

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INTRODUCTION

Liver is the principal organ of metabolism for many endogenous and exogenous substances and as a result is one of the most frequently damaged organs in the body. The damage may arise from a wide range of vascular, metabolic, toxic, obstructive and neoplastic derangements. Morphological changes in the gross and minute form as well as their arrangement and course of the liver are largely dependent on events in the afferent blood vessels and bile ducts (8). Liver however, has a large capacity to regenerate in response to injury for as much as 70 percent of the liver can be removed surgically without much adverse effects (8). Therefore, only a severe damage beyond the regenerative capacity will lead to liver dysfunction.

In a study on the causes of condemnation of carcass and organs of livestock slaughtered at the Shah Alam abattoir, 0.47 percent of 5,466 pigs slaughtered had their livers condemned and most of them was due to milkspots (24). Tham and Sheikh Omar (22) also reported that liver was the fourth most common organ at 0.35 percent condemned in pigs at Shah Alam abattoir. Helminthiasis is common in Malaysia and about \$193,030.32 was spent in 1977 in treatments of pigs for parasitism (13). Thus, condemnation and cost of treatments was of economic importance.

This study conducted on pigs slaughtered at Shah Alam abattoir has the following objectives:

1. to study the gross and histopathology of the condemned livers, and
2. to establish the etiological agents in liver abscesses observed on bacterial isolation.

LITERATURE REVIEW

Many abattoir studies have been done on swine livers. In a study of 8,558 swine from Shah Alam abattoir, Malaysia, the causes of condemnations of livers comprised 0.12 percent milkspots, 0.06 percent cirrhosis, 0.05 percent fatty change, 0.02 percent abscess and 0.01 percent others (22). In a similar study in the same abattoir, the majority (71.4%) of livers of 5,466 swine was condemned due to milkspots (24). A study in Ipoh abattoir of 118 partially condemned organs from swine showed that livers were the most common organ being condemned (71.4%). Cirrhosis (61%), abscessation (12%), congestion (10%), infiltration with polymorphs and mononuclear cells (9%), necrosis (8%), infiltration with eosinophils (7%), haemorrhages (5%), bile duct hyperplasia (4%) and fatty degeneration (2%) were the pathological changes observed (9).

Milkspots are also commonly observed in livers of pigs slaughtered overseas. In Saskatchewan, Canada, of 2500 market weight swine, 37 percent were infected with adult *Ascaris suum* and 44 percent had milkspot lesions and approximately 12 percent of the latter had severe lesions (17). In Southwestern United States of America, 95 percent of condemned livers had lesions associated with infections by ascarid and or swine kidney worms (1). Studies on sequential development of hepatic lesions of ascaridiosis in colostrum-deprived swine, classified milkspots into lymphonodular white spots and diffuse white spots with an opaque centres (2).

Neoplasia of swine livers have been observed in abattoirs. Moulton (12) described many neoplasm of livers and concluded that hepatic carcinoma was the most common liver neoplasm in swine although the incidence was low. Hyperplastic liver nodules have been studied in slaughtered swine (7) and hepatic carcinoma have also occurred at the same time (20).

MATERIALS AND METHODS

Collection of samples

One hundred condemned livers of 7,778 pigs slaughtered at Shah Alam abattoir were collected on several visits to the abattoir from 9th May, 1984 to 11th June, 1984. Most of the pigs slaughtered came from the states of Selangor and Negeri Sembilan and occasionally from Province Wellesly and Perak. The condemned livers were collected into a plastic drum and stored in cold room at post mortem room at Universiti Pertanian Malaysia for a detailed examination later.

Histological method

Tissue samples were taken from three or four sites in each liver that showed pathological changes. Specimens were then fixed in 10 percent neutral buffered formalin embedded in paraffin wax and 4 μ m sections were stained with Harris haematoxylin and eosin (Appendix I). Selected sections were also stained by Masson's trichome method (Appendix II) for connective tissue and Gram's stain (Appendix III) for gram positive and negative bacteria.

Bacteriological culture method of abscesses

The surface of each abscess was flamed and carefully incised using an aseptic technique. Pus was sampled aseptically with cotton swab and subcultured aerobically at 37°C for 24 hours on blood agar and MacConkey agar and anaerobically in an anaerobic chambers at 37°C for 48 hours. The organism isolated were identified using standard biochemical technique (3) for aerobes and based on gram reaction and microscopic morphology as well as colonial morphology and characteristics. Ten samples were incubated anaerobically and if negative results were obtained, only aerobic incubation was conducted subsequently.

RESULTS

Of 100 condemned livers examined, 75 were from porkers and 25 were from sows. The most predominant lesions seen in each liver are presented in Table 1. The common lesions were milkspots (36%), peritonitis (16%), Abscessation (12%) and cholangiohepatitis (11%). In porkers, the most common lesions were milkspots (41%) and peritonitis (21%) while in sows cholangiohepatitis (20%), nodular hyperplasia (20%) and milkspots (20%) were the most common.

Milkspots was easily the most common lesion seen. Grossly, they were small stellate white spots, 0.1 cm to 1.5 cm diameter. In a few livers, some of bigger spots had a nodular cystic centre of 0.1 cm diameter. The right lateral lobes, left and right medial lobes were the most commonly affected. The most common were livers with 4+ score in which all the lobes were affected. The lesions were scored according to the number of lobes affected (Table 2).

Table 1: Lesions observed in condemned livers

Lesion	Number of livers		
	Porker	Sow	Total
Milkspots	31	5	36
Peritonitis/perihepatitis	16		16
Abscessation	9	3	12
Cholangiohepatitis	6	5	11
Cholangitis	7	1	8
Nodular hyperplasia		5	5
Focal hepatic necrosis	3	1	4
Post necrotic scarring	1	2	3
Hepatic lipidosis	2		2
Cystic bile duct hyperplasia	-	1	1
Massive necrosis	-	1	1
Hepatocarcinoma		1	1
Total	75	25	100

Table 2: The severity of milkspot lesions in 36 pigs

Liver score*	Number of livers affected
4+	14
3+	8
2+	3
1+	11
Total	36

* 4+ - all lobes affected

3+ - three lobes affected

2+ - two lobes affected

1+ - one lobe affected

Microscopically, there was mild to marked focal fibrosis, biliary hyperplasia and cellular infiltration with mainly eosinophils, also lymphocytes and mononuclear cells.

Peritonitis was seen in 16 animals (16%), all porkers. The lesions were easily diagnosed grossly as thickened and cloudy capsule sometimes with fibrin tags causing fusion of the liver lobes and adhesion to the diaphragm. In severe cases, the fusion was complete and the liver becomes one large mass (Figure 1). One liver was severely affected and adhered to the diaphragm, lungs and heart. Microscopically, there was capsular and subcapsular fibrosis.

Abscessation was seen in 12 animals, 9 porkers and 3 sows. The abscesses ranged from 0.1 cm to 1.5 cm diameter appearing as one focus or multifoci and affecting all lobes. The abscesses contained caseated whitish, creamy greenish yellow, sometimes dry and calcified material.

Cholangiohepatitis was diagnosed microscopically in 11 animals (11%), 6 porkers and 5 sows. Histopathology included fibrous tissue replacement of liver lobules, biliary hyperplasia and periportal infiltration with mononuclear cells and eosinophils.

Eight livers had cholangitis, 7 porkers and one sow. Histologically mild to moderate fibrosis with marked eosinophils infiltration in the portal triads were present.

Nodular hyperplasia was only observed in sows. The affected liver had a few multinodular firm nodules usually at the edge of

the lobes. On cut surface, the nodules were reddish separated by a thin capsule. Histologically, the nodules consisted of normal hepatocytes without the normal lobular architecture surrounded by a thin fibrous tissue.

Four livers had focal hepatic necrosis, microscopically seen as focal areas of hepatocytes undergoing pyknosis, karyorrhexis and karyolysis.

Post necrotic scarring seen in 3 livers had fibrous tissue infiltration of hepatocytes thus compressing the latter, marked periportal and portal fibrosis with eosinophils infiltration.

Two porkers and livers with lipidosis which grossly were pale yellow and friable while histologically the hepatocytes had vacuoles in the cytoplasm.

One case each of cystic bile duct hyperplasia, massive necrosis and hepatocarcinoma were also observed in sows. The former was a cystlike dilation of bile duct with hyperplastic cuboidal cells. Massive necrosis was seen in a brown and friable liver which histologically showed necrosis of hepatocytes of many lobules filled with red blood cells. The liver with hepatocarcinoma was enlarged and had a single raised nodule which appeared homogeneously brown and multilobulated on the cut surface (Figure 2). Histologically, there was loss of normal liver arrangement, tumor cells were present as a solid mass separated by connective tissue septa and the cells were round with large, round or ovoid hyperchromatic nuclei.

Table 3: Bacteria isolated from 12 hepatic abscesses

Species	Frequency of isolation*
<i>Escherichia coli</i>	7
<i>Kliebsiella sp.</i>	5
<i>Staphylococcus aureus</i>	3
<i>Citrobacter freundii</i>	2
<i>Proteus sp.</i>	2
<i>Corynebacterium pyogenes</i>	1
<i>Citrobacter intermedius</i>	1
<i>Pseudomonas pseudomallei</i>	1
<i>Proteus rettgeri</i>	1
<i>Salmonella sp</i>	1

* in mixed cultures

One liver had an abscess with fibrin tags on its capsular surface while similar abscesses were present in the spleen (Figure 3) which was found attached to the liver. *Escherichia coli* and *Kliebsiella sp.* were cultured from these abscesses. One liver abscess was positive for *Pseudomonas pseudomallei*. The abscess contained thick greenish material and histologically showed many epitheloid and mononuclear cells in the fibrous capsule. All abscesses had mixed cultures. Bacteria species isolated and their frequency are shown in Table 3. The most common bacteria isolated were *E. coli* and *Kliebsiella sp.* One bascess was positive for *Salmonella sp.* when tested with polyvalent O antigen and showed

pink colonies on brilliant green agar. The culture was however lost before serotyping was done.

DISCUSSION

The high frequency of livers with milkspots in this study is consistent findings of other workers (1,9,17). This means that *Ascaris suum* infections is a problem in porkers in Malaysia and could be due to improper deworming programmes and poor quarantine measures at the farms. However work done overseas has shown that *Ascaris suum* infection still approaches 100 percent despite widespread use of anthelmintics and confined housings (5). Eggs of *Ascaris suum* are very resistant to environmental stress and have been found to remain viable in manure collection pit up to 14 months (21). Untreated pigs infected with ascaris act as an important source of infection to other pigs by contaminating pig stalls with shedding 'infective' eggs when they reach 8 weeks of age (5).

We assume that all milkspots observed in this study are due to ascarid larval migration but there are other parasites that can produce similar lesions such as *Toxocara canis*, *Toxocara cati*, *Stephanurus dentatus* and *Parascaris equorum* (17). It is possible that the first two parasites mentioned, do occur in pigs in this country and confirmative work need to be carried out such as examination of adults of ascaris from the entire small intestines.

The pigs studied were clinically healthy and presumably had reached market weight without any retardation in body weight. They were infected probably at a grower phase and were able to acquire

immunity against the parasites. Had the infection developed during starter and prestarter phases, high mortalities and growth retardation would be prominent (5). Segments of ascaris larvae and aggregations of lymphoid cells were not observed histologically in this study because the lesions had been already going on for some time. Such segments have been detected after two subsequent infections with ascaris at 21 days interval (2).

It was interesting to find peritonitis/perihepatitis on this study but the cause are not very well understood. Copeman and Gaafar (2) described the possibility of ascarid larval migration causing clouding of the capsule 24 hours after infection and progressing to be opaque by the 12th day. *Mycoplasma hyorhinis* and *Haemophilus parasuis* and chronic cholangiohepatitis are other possible causes but rare (8, 10). The latter can cause the liver capsule to be thickened, bear fibrous villi or adherent to adjacent viscera. However in this study, all the affected livers had marked eosinophils infiltration suggestive of parasitic involvement.

Isolates of bacteria from liver abscesses in mixed cultures consisting mainly of *Escherichia coli*, *Kliebsiella sp.* and *Staphylococcus aureus* are similar findings to other workers (5,9,14). Since aseptic culture techniques were strictly followed, these isolates were unlikely contaminants. Anaerobic bacteria were not studied for they were not easily isolated. The liver abscess due to *Pseudomonas pseudomallei* microscopically resembled the lesion of melioidosis in pigs (16,23,15). In another study in this abattoir, most cases of melioidosis seen involved abscessation in the spleen, sometimes

the lungs (24) which were not observed in this case.

The isolation of *Salmonella* sp. from one liver abscess was not surprising since the organism is commonly isolated from the gall bladder with bile (18). Since studies have shown that mesenteric lymph nodes, gall bladder with bile and the intestinal tract are frequently infected with *Salmonella* (18), improper evisceration and dressing of the carcasses at abattoirs would provide a source for its spread to other organs like livers.

Nodular hyperplasia which was seen in sows was consistent with the findings of Hayashi et al., (7) who reported an incidence of 40 per million pigs and suggested hepatocarcinogens as a possible cause.

Focal hepatic necrosis in growing pigs is usually associated with bacteremia or viremia (10). Damaged enzyme systems within the hepatocytes also result in focal hepatic necrosis (20).

Hepatic lipidosis as seen in this study has been reported by Yap et al., (24), at a higher frequency (11.9%). This problem usually arises when the fat is mobilised too rapidly from various fat depots when the animal is undernourished (8). It is also seen in pigs suffering from severe protein malnutrition causing great reduction in hepatic phospholipid and disturbance in lipid metabolism (6). It may also occur as a result of 'unmasking' of fat normally present in hepatic cells following their injury (8).

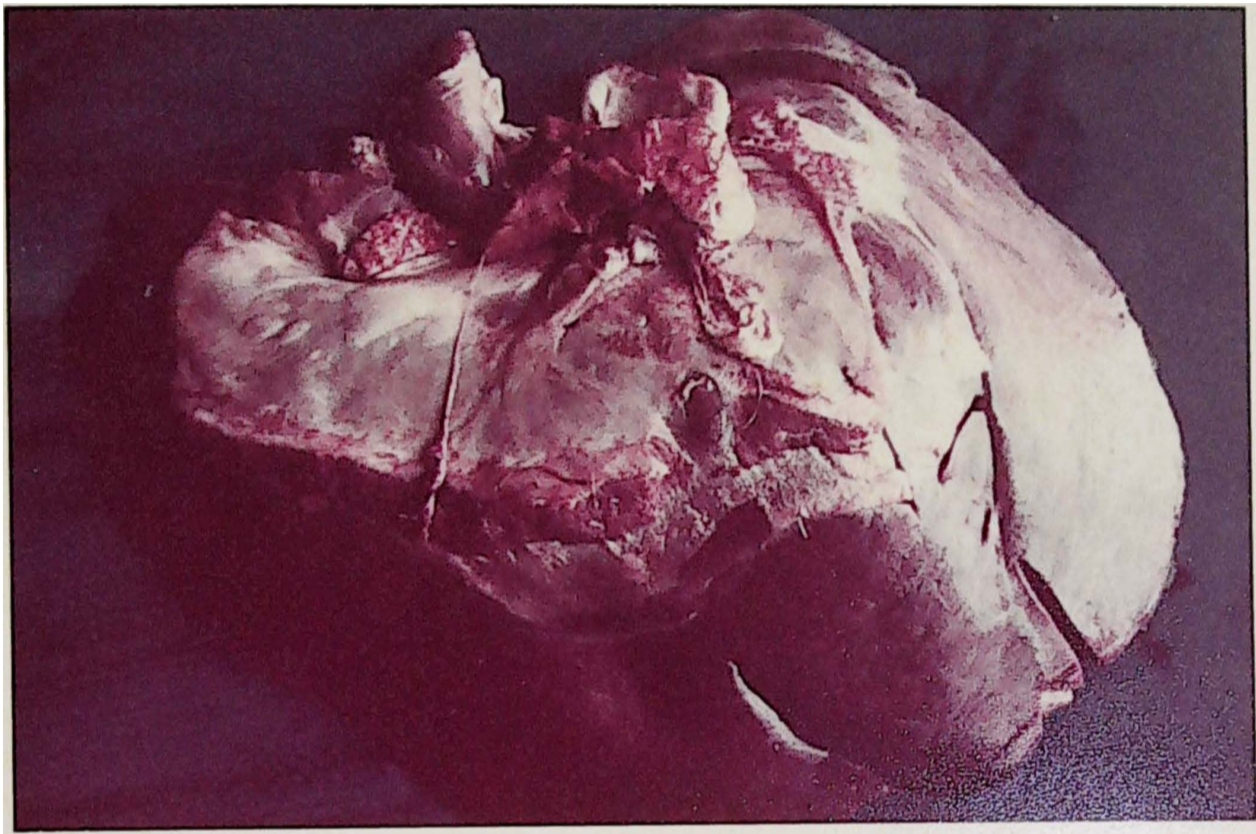
The one case of massive hepatic necrosis seen in a sow could have been induced by a simultaneous administration of carbon

tetrachloride and phenobarbitone in pigs as has been reported (11). Other possibilities include malnutrition, systemic diseases and administration of arsenicals, tetracyclines, phosphorus and halothane (20).

Hepatocarcinoma was the only neoplasia seen. Its occurrence is low in pigs, occurring at the rate of one per 5.5 million pigs.

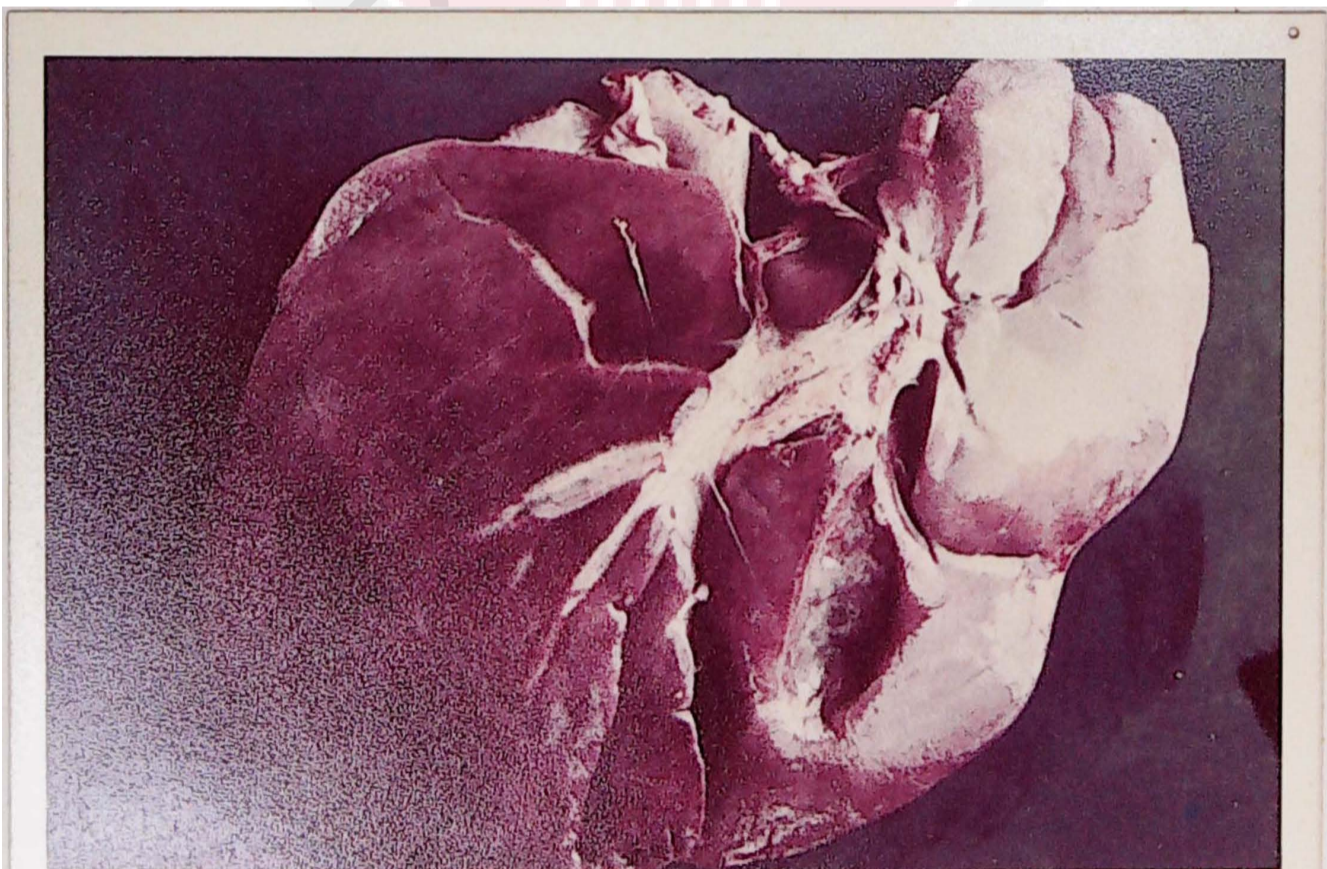
CONCLUSION

Many constraints were encountered in conducting this project such as limited time available, transport problem and late night slaughter. Better results could have been obtained if a larger number of livers had been studied for some epidemiological inferences could be made. However, this study do show the spectrum of lesions seen in the liver of healthy pigs slaughtered at an abattoir. If it was possible to trace the source of the pig it will help the farmer to know what kind of problems he had in his farm. Lastly, in view of the high frequency of milkspot livers as was seen in this study, this is an aspect of pig health that merits further study.



MAR

Fig. 1(a) Chronic peritonitis showing complete fusion of the liver lobes on its diaphragmatic surface



MAR 85

Fig. 1(b) Same liver showing partial fusion of its lobes on its visceral surface



Fig. 3(a) Liver showing multifocal abscesses.

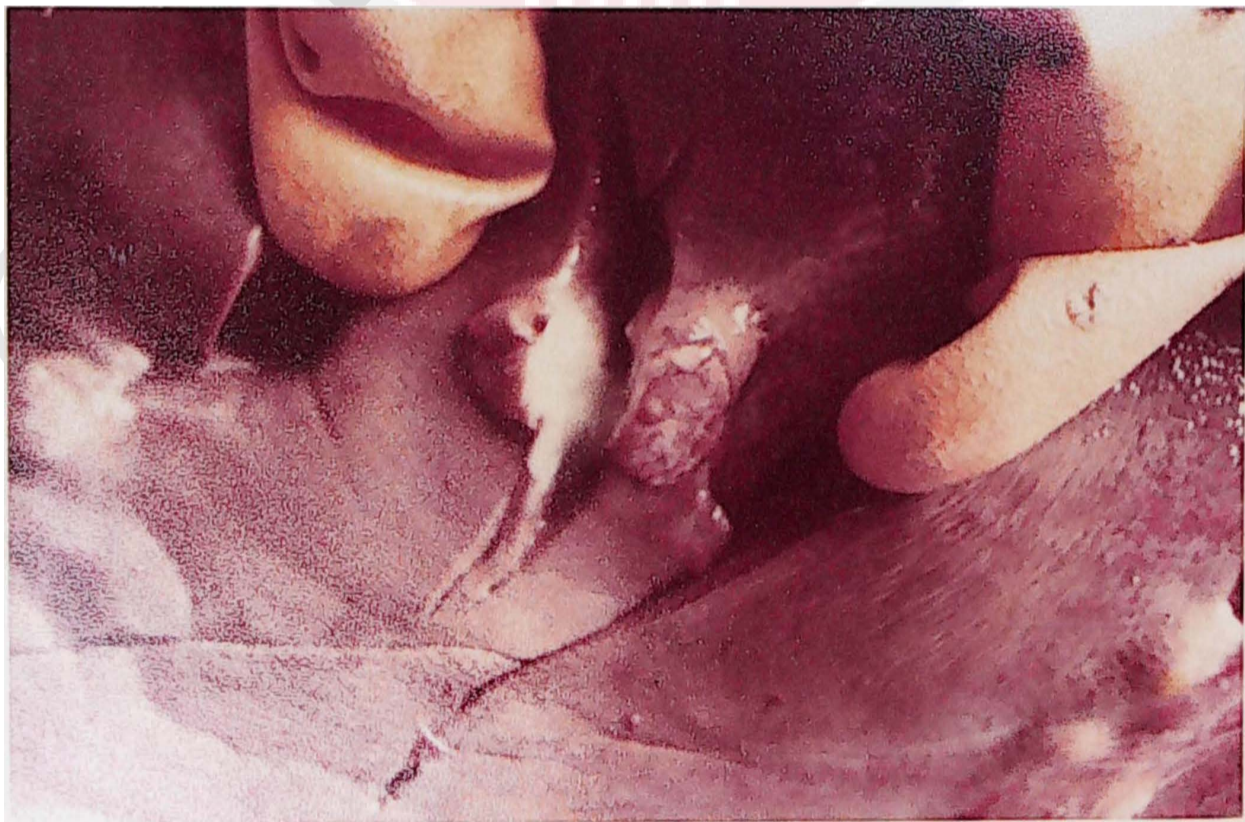


Fig. 3(b) Close up of the abscesses

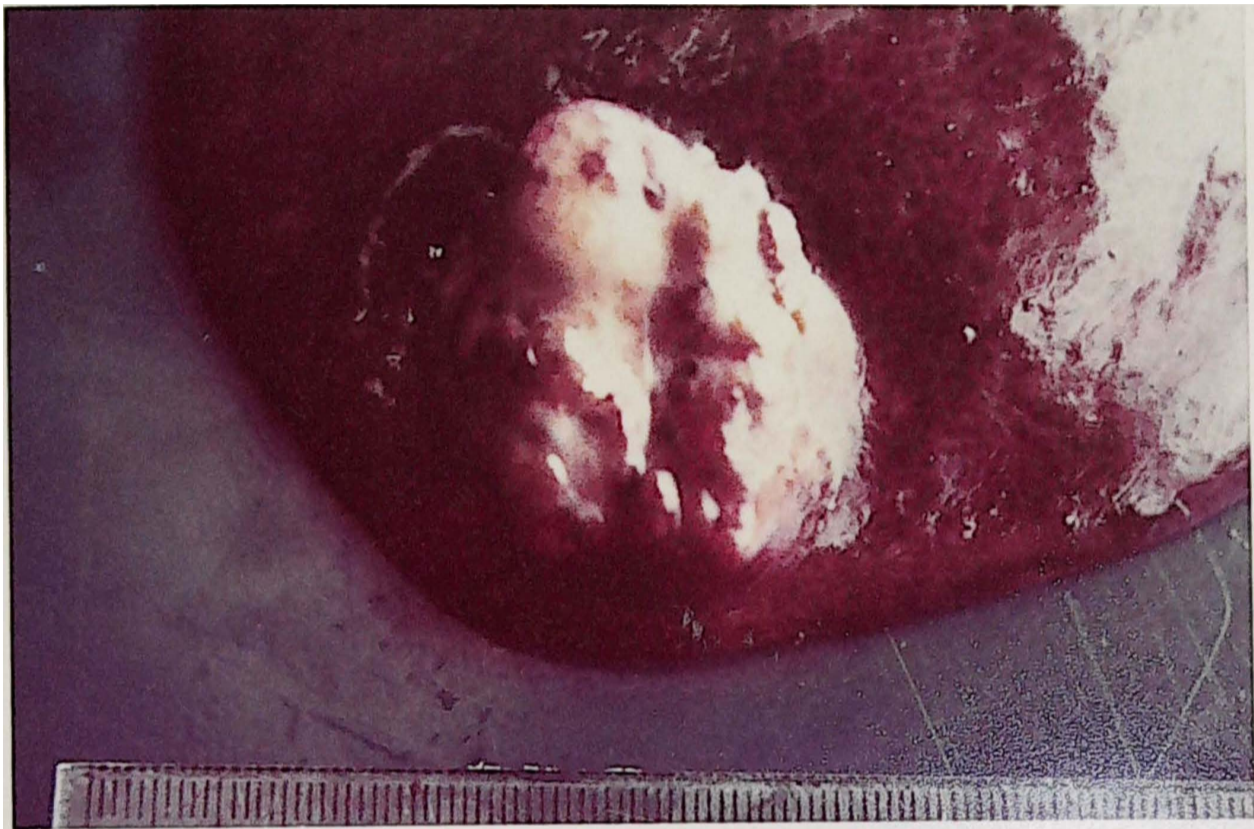


Fig. 2(a) Liver with hepatocarcinoma showing raised nodules at edge of one lobe of the liver

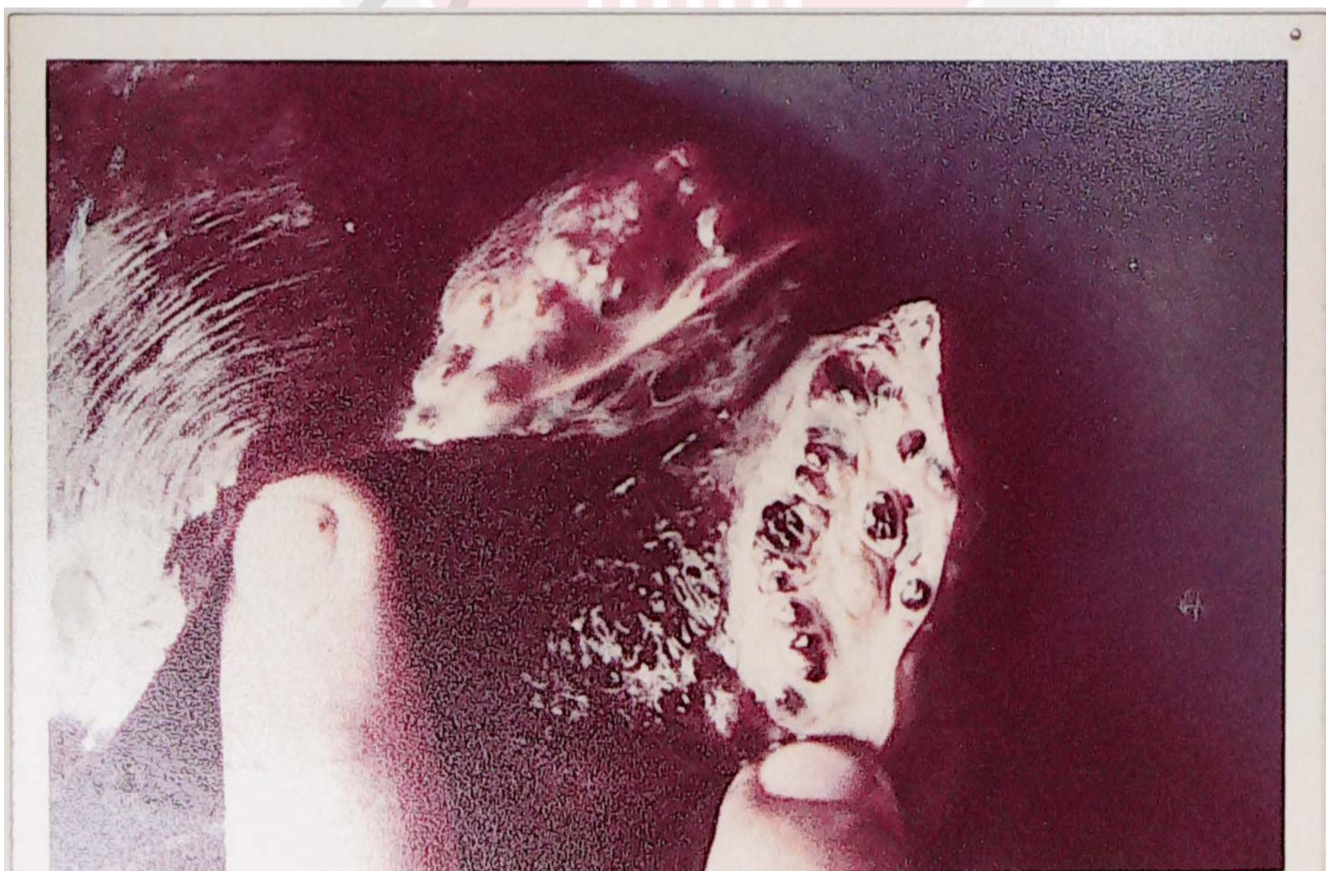


Fig. 2(b) Cut surface of the tumor appeared homogeneously brown and multilobulated.

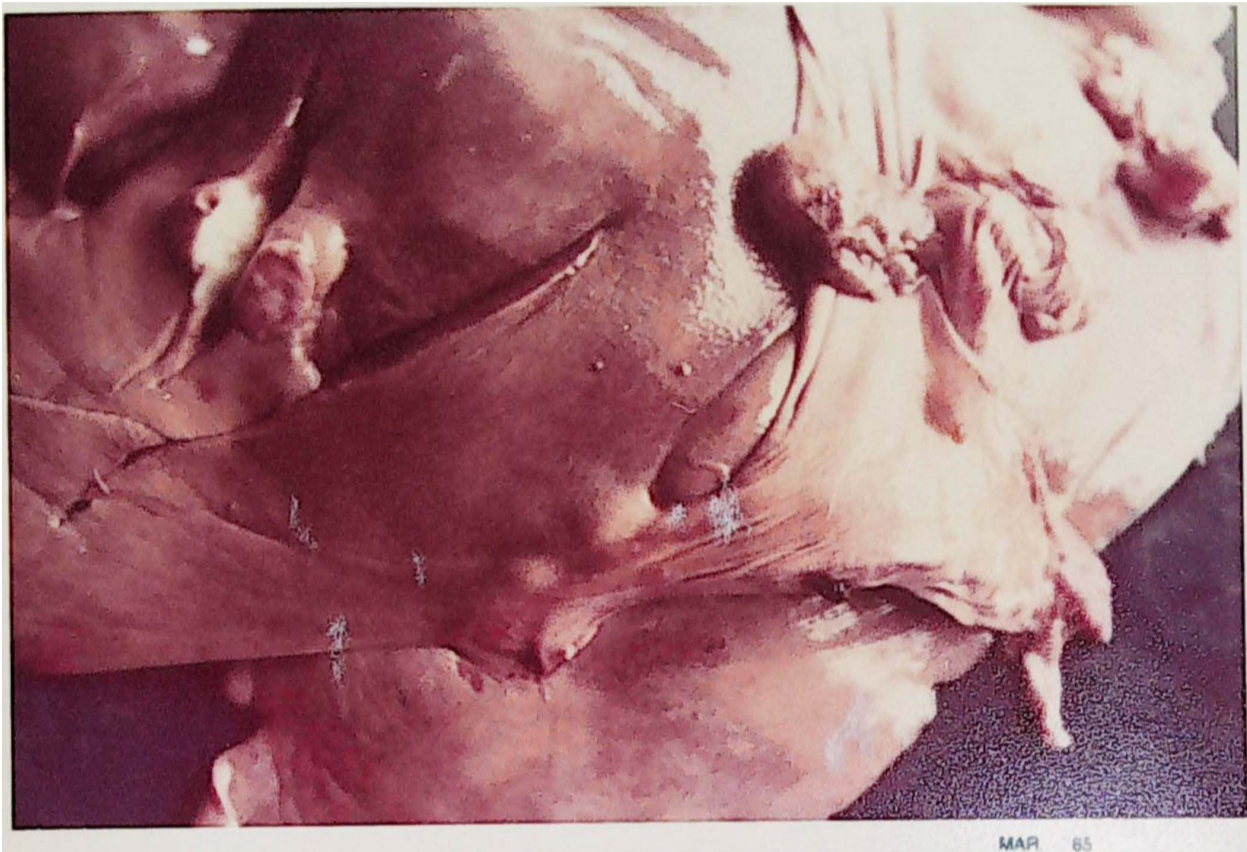


Fig. 3(a) Liver showing multifocal abscesses.



Fig. 3(b) Close-up of the abscesses.

REFERENCES

1. BOTTLE E.G., MCLAMB R.D., VESTAL T.J. 1975. Swine Parasites: Causes of liver condemnations. *Vet. Med. Small Ani. Clin, Agric. Practi.*, 70:809-813.
2. COPEMAN D.B., GAAFAR S.M., 1972. Sequential development of hepatic lesions of ascaridiosis in colostrum-deprived pigs. *Aust. Vet. J.*, 48:263-268.
3. COWAN S.T., COWAN and STEEL: Manual for the identification of medical bacteria, University Press, Cambridge, 1977.
4. ENGVALL A. and SCHWAN O., 1983. Isolation and characterisation of bacteria recovered from abscesses of normally slaughtered pigs. *Acta. Vet. Scand.*, 24:74-83.
5. FROE II D.L., 1982. Ascarid larval migration. *Mod. Vet. Pract.*, 63:829-832.
6. GUPTA P.P., 1973. A study on quantitative changes in liver lipids of Indian pigs suffering from severe protein malnutrition. *Indian J. Anim. Sci.*, 43:354-858.
7. HAYASHI M., TSUDA H., ITO N., 1983. Histopathological classification of spontaneous hyperplastic liver noudles in slaughtered swine. *J. Comp. Path.* 93:603-612.
8. JUBB K.V.F. and KENNEDY P.C., 1970. Pathology of Domestic Animals. Vol. II, 2nd ed. Academic Press, New York, pp. 191-261.
9. LOGANATHAN P. and A.A. HUSSIN., 1983. A pathological study of condemned organs at Ipoh abattoir. *Kajian Vet. Malaysia* 1983 15:64-68.
10. LEMAN A.D., GLOCK R.D., MENGELING W.L., PENNY R.H.C., SCHOLLE E., STRAW B., 1981. Diseases of swine. 5th ed. The Iowa State University Press, Ames, Iowa, USA.
11. LEENHOFF J.W. VAN, HICKMAN R., SAUNDERS S.J., TERBLANCHE J., 1974. Massive liver necrosis induced in pig with carbon tetrachloride. *South African Medical Journal*, 48:1201-1204.
12. MOULTON J.E., 1978. Tumors in Domestic Animals. 2nd ed. Revised University of California Press Berkely, Los Angeles, London. pp. 273-287.

13. MOHD FADZIL Y., 1977. The economic importance of parasitism in food animals in Peninsular Malaysia. Proceedings of the First Joint Conference of the Association of Cattle Veterinarian Bulletin, Ministry of Agriculture Kuala Lumpur., 146:62-79.
14. MC CRACKEN A. and MC CAUGHEY W.J., 1973. A survey of abscesses in in bacon weight pigs. *Br. Vet. J.*, 129:359-361.
15. OLDS R.J., and LEWIS F.A., 1955. Melioidosis in a pig. *The Aust. Vet. J.* 31:273-274.
16. OMAR A.R., 1963. Pathology of melioidosis in pigs, goats and horse. *J. Comp. Path.*, 73:359-372.
17. POLLEY L.R., and MOSTERT P.E., 1980. *Ascaris suum* in Saskatchewan pigs: An abattoir survey of prevalence and intensity of infection. *Can. Vet. J.*, 21:307-309.
18. QUASIM A., PRASAD N.N., PRASAD C.B., CHAUDHARY S.P., 1982. Incidence of Salmonella in slaughtered pigs. *Indian J. Anim. Sci.*, 21:24-28.
19. RONCUS O., 1966. As cited by POLLEY L.R. and MOSTERT P.E., 1980. Studies on the aetiology and pathogenesis of white spots in the liver of pigs. *Can. Vet. J.*, 21:307-309.
20. ROBBINS S.L., 1974. Pathologic Basic of Disease. W.B. Saunders Company, Toronto. pp. 985-1055.
21. SMITH J.P., 1979. Viability of larvated swine ascarid (*Ascaris suum*) eggs in a conventional non-aerated manure collection pit. *Southwest Vet.*, 32:33-35.
22. THAM K.M. and SHEIKH OMAR A.R., 1981. A study on causes of condemnation of carcass and organs at Shah Alam abattoir. *Pertanika*, 4:43-46.
23. THOMAS A.D., NORTON J.H., FORBES J.C., FAULKNER. 1981. Melioidosis in an intensive piggery. *Aust. Vet. J.*, 57:144-1
24. YAP T.C., MOHAN S.S. and GILL H.S., 1983. A study on causes of condemnation of carcass and organs of livestock slaughtered at the Shah Alam abattoir. *Kajian Vet. Malaysia.*, 15:1-10.

APPENDIX I

HARRIS' HEMATOXYLIN

Hematoxylin arystals	5.0 gm
Alcohol, 100%	50.0 ml
Ammonium or potassium alum	100.0 gm
Distilled water	1000.0 ml
Mercuric oxide (red)	2.5 gm

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Remove from heat and mix the two solutions. Brint to boil as rapidly as possible (Limit this heat to less than 1 minute and stir often). Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel into a cold water until cool. The stain is ready for use as soon as it cools. Addition of 2-4 ml of glacial acetic acid per 100 ml of solution increases the precision of the nuclear stain. Filter before use.

APPENDIX II

MASON'S TRICHOME METHOD

Solution: WIEGERT IRON

Solution A

Hematoxylin crystal	1 gm
Ethanol 90%	100 ml

Allow the solution to ripen in bright daylight for few days.

Solution B

Ferric chloride (30% in distilled water).	4 ml
Hydrochloride acid	1 ml
Distilled water	95 ml

Staining solution - mix equal part of Solution A and B.

PONCEAU - ACID FUCHSIN

Xylidine ponceau crystal	0.7 gm
Fuchsin (acid)	0.37 gm
Acetic acid glacial	1 ml
Distilled water	100 ml

Dilute 1 to 10 with distilled water before use.

1% PHOSPHOMOLYBDIC ACID

LIGHT GREEN

Light green	2 gm
Acetic acid	2 ml
Distilled water	100 ml

STAINING PROCEDURE

1. Stain the sections with Weigert iron haematoxylin (10 min) differentiating until only the nuclei are stained.
2. Wash and immerse in ponceau-acid fuchsin (2 min).
3. Rinse and differentiate in 1% phosphomolybdic acid until the collagen fibres are nearly colourless.
4. Transfer direct to light green and stain for $\frac{1}{2}$ to 2 minutes.
5. Differentiate in water until the green stain is reduced to the required intensity.
6. Dehydrate with ethanol, clear and mount.

RESULTS

Nuclei stained black

Muscle stained red

Connective tissue stained green

APPENDIX III

CALCIUM OXALATE PIZZOLATO'S METHOD

FIXATION : 10% buffered neutral formalin

TECHNIQUE : Cut paraffin at 6 micron

SOLUTION : Silver nitrate hydrogen peroxide solution

Silver nitrate 5% aqueous 25 ml

Hydrogen peroxide 30% 25 ml

NUCLEAR FAST RED (KERNECHTROT) SOLUTION

Dissolve 0.1 gm nuclear fast red in 100 ml of 5% solution of aluminium sulfate with aid of heat. Cool, filter add grain of thymol as preservative.

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Pour silver nitrate-hydrogen peroxide solution onto slides in sufficient quantities to cover section.
Place 60 watt light bulb 6 inches from section for 30 min.
3. Rinse thoroughly with distilled water.
4. Counter stain in Kernechtrot for 5 min.
5. Rinse with distilled water.
6. Dehydrate in 95% alcohol, absolute alcohol and clear in xylene two changes each.
7. Mount with Posmount or Hestoela.

RESULT

Calcium oxalate stained black.

All other tissue element stained red.