3   Materials and Methods

3.1   Materials

3.1.1   Animals

Cows were enrolled in this study by the clinic of cattle, faculty of veterinary medicine, Free University, Berlin, Germany when they were diagnosed for abomasal displacement or claws diseases from February 2002 to February 2003. A total number of 480 milk cows of different ages and disease conditions were used in this study. Clinical examination of animals revealed that most cows suffered from abomasal displacement complicated with either disease, mostly mastitis and/or metritis. According to the clinical examination, cows were classified into three main groups, groups consisting of left abomasal displacement (n=345), right abomasal displacement (n=40) and other diseases conditions like claws affection, peritonitis, indigestion, mastitis, pneumonia (n=95).

3.1.2   Blood sampling

Two blood samples were taken from each animal at the time of admission or one day after admission by puncture of the middle coccygeal vein using clean and dry tubes. The first blood sample (whole blood sample) was obtained by allowing about 3 ml of blood to flow freely in a clean dry vial which contained EDTA as anticoagulant. This anticoagulated blood was used for the determination of the packed cell volume (PCV %), hemoglobin (Hb g %), erythrocyte count (RBCs $X10^6$ μl), total leucocytes count (WBCs $X10^3$μl**)** and differential leucocytes count. The second blood sample was taken for obtaining serum by allowing the blood to flow gently on the inner surface of a clean dry tube. After complete coagulation, it was cooled for half an hour, and then centrifuged at 3000 rpm for 10 minutes. Clean non-haemolysed serum was transferred to a clean dry plastic vial, and eventually used for blood chemistry analysis.

3.1.3 Liver samples

Liver samples were taken from each cow by liver biopsy one or two day of admission after. The samples were divided into three parts, the first part was used for the copper sulfate test, the second part was put in formalin 4% for histopathological examination and the third part was kept under deep freezing (-20°C) until used for determination of total lipid and triglyceride.

3.1.4 Copper sulfate solutions

Copper sulfate solutions were made with hydrated copper sulfate and distilled water and the preparation of solutions was as follow:

- Saturated solution of copper sulphate was prepared by adding hydrated copper sulphate in a flask containing distilled water and mixed till the copper sulfate powder precipitated in the bottom of the flask.
- Hundred ml from the solution was weighed and then its weight was divided on its volume to give the specific gravity of solution. If the specific gravity was high then removing some milliliters from solution and adding distilled water was done till adjust the specific gravity of the solution was adjusted.
- These procedures were repeated to obtain the different specific gravities of copper sulfate solutions.

3.1.4 Mixture of chloroform and methanol

Two parts of chloroform (Merck®) were mixed with one part of methanol (Merck®) (2vol: 1vol) and strongly shaked to dissolve the fat.

3.1.5 Mixture of hexane and isopropanol

Three parts of hexane (Merck®) were mixed with two parts of isopropanol (Merck®) (3vol: 2vol) and strongly shaked to dissolve fat for the estimation of triglyceride.

3.1.6 Tissue lysine buffer (ATL)

Readily prepared QIAGEN® was used. These buffers might form precipitates during storage. If a precipitate has formed in the buffer, the buffer was incubated at 55°C until the precipitate had fully dissolved.

3.1.7 H&E stain:

- One gm of hematoxylin was dissolved in 10ml ethanol (100%) and left for 24 hours.
- Twenty grams of potassium aluminium sulfate-12-hydrate were dissolved in 200ml warm distilled water. After that the solution was cooled and filtered.
- One gram potassium permanganate was dissolved in 16ml-distilled water.
- Hematoxylin was mixed with potassium aluminium sulfate-12-hydrate solution.
- Under constant agitation 3ml of potassium permanganate solution was poured slowly and then heated for 30-60 seconds. After that the stain was hold on heater temperature under agitation.
- The stain was rapidly cooled and then filtered in cleaned, closed bottles.

3.1.8 Sudan III stain

- Two to three hundred milligrams of Sudan III was put with 100ml 70% hot alcohol in a closed bottle.
- The solution was shaken very well, then incubated at 60°C for some hours.
- The stain was cooled and filtered.
- For good quality staining, 20ml of stain solution can be used with 2-3ml distilled water.
- Soonly, the solution might become turbid and can be used after a short time.

3.1.9 Ultrasonography set

The liver of a cow was examined ultrasonographically using a 3.5 MHz linear scanner (Echokamera SSd-650, Aloka Deutschland GmbH, Mollsfeld 5, and 40670 Meerbusch®).

3.2   Methods

3.2.1   Clinical examination

All cows underwent a clinical examination according to the methods of **Rosenberger (1990).** The appetite and general health condition were examined by inspection. The temperature was measured rectally. The respiration was counted by observation of abdominal movement and the number of breaths per minute. The examination of the heart was done through auscultation on the left side, about a feast of hand above the elbow and then was counted per minute. Abomasal displacement was diagnosed by hearing a characteristic ping sound on either the left or right side by simultaneous percussion and auscultation. The rumen was also examined by auscultation of the left flank and by count per two minutes.

According to the clinical examination, cows were classified into three main groups: the first group consisted of left abomasal displacement, the second group contained right abomasal displacement and the third group other diseases conditions.

3.2.2   Liver biopsy technique

Liver samples were obtained from 480 live cows according to the method described by **Gröhn and Lindberg (1982)** by percutaneous needle biopsy. A site is selected on the right side of the cow in the 11th intercostals space under the level of tuber coax. This site corresponds to the caudate lobe of the liver (picture 1). The site was shaved and disinfected by 70% alcohol and betadine solution, then local anaesthesia (3-5ml procaine hydrochloride) was applied. A small stab incision was made through the skin with a pointed scalpel and incision was only in the skin and subcutaneous tissue to facilitate introduction of biopsy needle. A liver biopsy needle (Berlin Model, d=2,5mm. 25cm, Eickemeyer® Medizintechnik für Tierärzte, Tuttlingen, Germany) (picture 2) was inserted in the abdominal cavity and directed to the controversial elbow until feeling the liver texture, which resembles a snowball. After that the needle was removed and the canule pushed inward and outward; the canule was closed during the outward pushing. The

procedures were repeated 3 to 4 times. A piece of liver obtained by this procedure was usually weighing approximately 100-200mg.
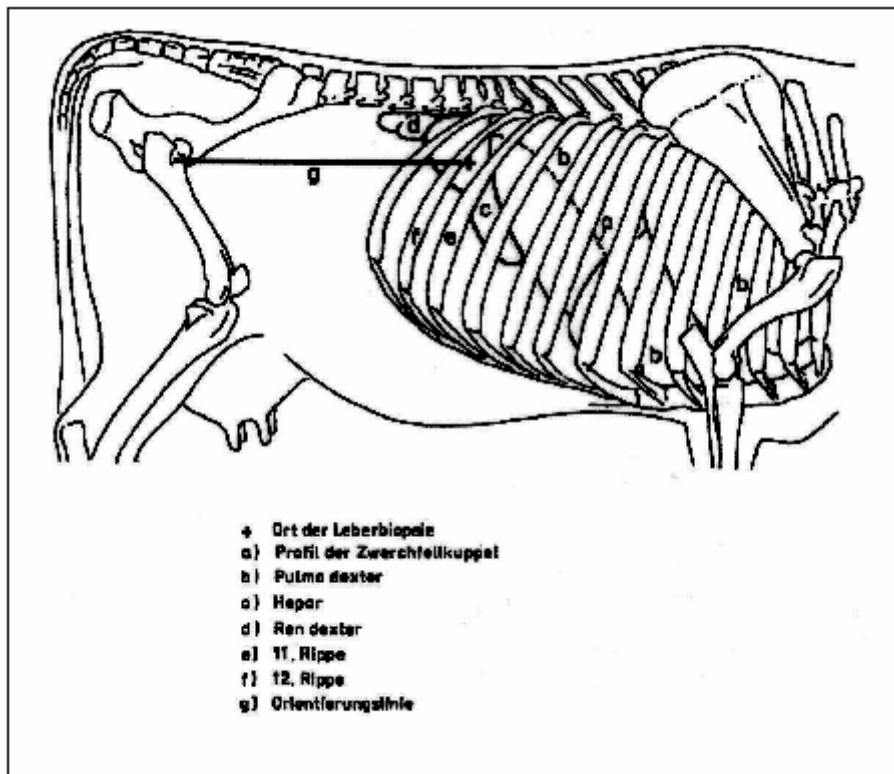
Picture 1:   Site of liver biopsy
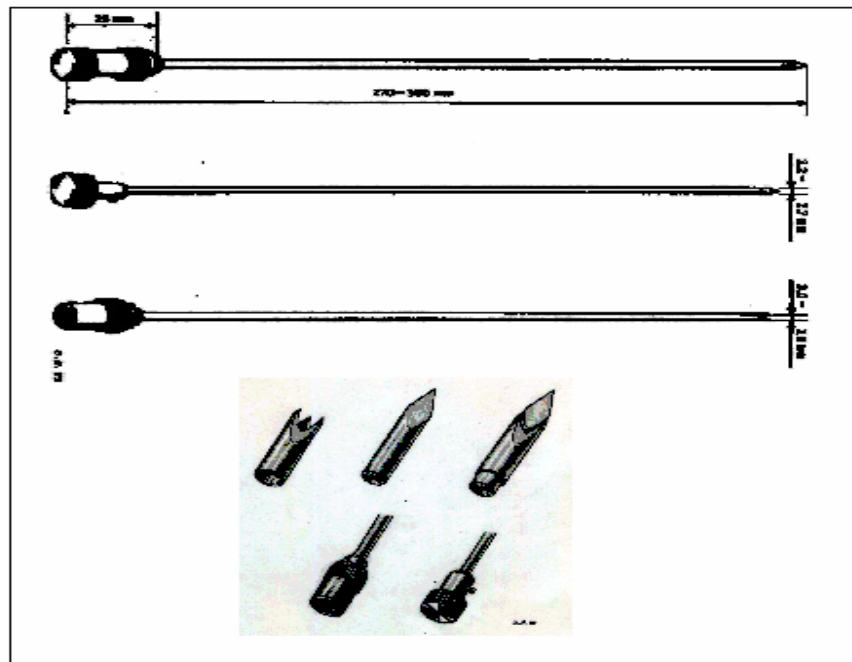
Picture 2: Liver biopsy needle "Berlin model"

3.2.3 Estimation of total lipid

Total lipid was estimated in liver biopsies either by different specific gravity of copper sulfate solutions or gravimetrically as follows:

3.2.3.1 Copper sulfate test

This test is rapid, practical and a quantitative test for bovine hepatic lipid content. It is suitable for field application in the diagnosis of bovine hepatic lipidosis. It depends on buoyancy. The test was as described by **Herdt et al. (1983)**. A piece of liver biopsy was placed in vials containing either water or copper sulfate solutions with different specific gravity (1010, 1020, 1030,..1100) (picture 3). The specimen was submerged in the solutions and its buoyancy was observed. If the piece floats above the surface, it would be transported to the next low specific gravity test tubes until it sinks to the bottom as shown in picture 3. Then the fat content was registered as shown in table (1).

3.2.3.2 Gravimetric method

The preparation of liver samples was made by weighing approximately 100mg of fresh liver in a clean glass homogenizator. The sample was homogenized with 1ml tissue lysine buffer (Qiagen GmbH, Germany) until complete cutting of the liver tissue into small parts. The homogenized sample was incubated overnight in a water bath at 55 °C. Total lipid was extracted by a chloroform-methanol-water system as mentioned by **Veenhuizen et al. (1991)**. The homogenized sample was mixed with the chloroform-methanol mixture and incubated for 30 minutes in a refrigerator. After that, the sample was centrifuged for 10 minutes at 5000 rpm. The solution divided it into two layers. The above layer was methanol and the lower layer was chloroform containing fat.

The chloroform layer was drawn up by using a cerebrospinal fluid canule. Fat with chloroform was put in a clean evaporating glass flask with known weight. Chloroform was evaporated by leaving the flask at room temperature or by acceleration on a heater at 50°C.

The remaining solution was washed by 1ml chloroform and centrifuged. Then the chloroform layer was drawn up and put in an evaporating flask in order to evaporate chloroform as mentioned before. Dried fat was weighed to calculate the total fat content of the liver sample.

Table 1. The relationship between the specific gravity and fat content of liver

Specific Gravity mg/ml		Fat content %	Specific Gravity mg/ml		Fat content %
1000	Floating	>33	1060	Floating	>12
	Sinking	33 - >29,5		Sinking	12 – >8,5
1010	Floating	>29,5	1070	Floating	>8,5
	Sinking	29,5 - >26		Sinking	8,5 - >5
1020	Floating	>26	1080	Floating	>5
	Sinking	26 – >22,5		Sinking	5 - >1,5
1030	Floating	>22,5	1090	Floating	>1,5
	Sinking	22,5 - >19		Sinking	1,5 - <1,5
1040	Floating	>19	1100	Floating	<1,5
	Sinking	19 – >15,5		Sinking	1,5 - 0
1050	Floating	>15,5			
	Sinking	15,5 - >12			
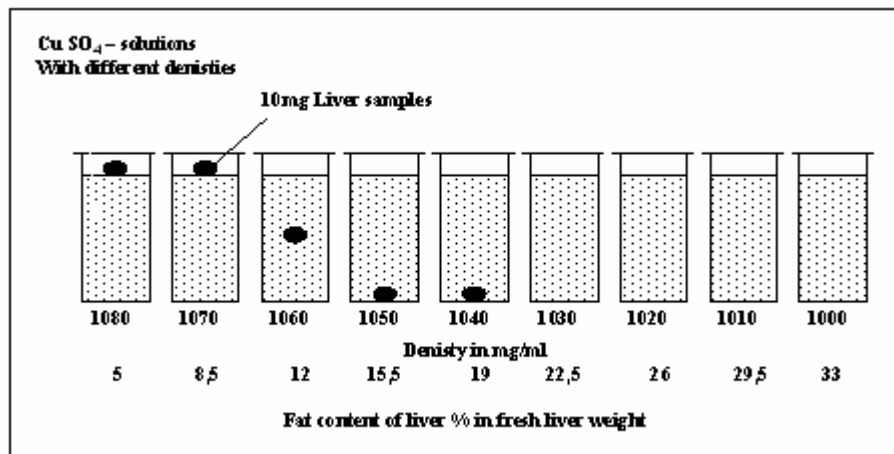
Picture 3. Copper sulphate test

3.2.4 Estimation of triglyceride

The lipid extract was redissolved in hexane-isopropanol mixture as recorded by **Drackley et al. (1992)** and the concentration of triglyceride was estimated using a LP 700 spectrophotometer with kit (Labor + Technik). The total lipid was dissolved in a mixture of hexane and isopropanol which was readily prepared. Ten microliters from the dissolved lipid solution were taken into a clean test tube then 1ml from kit reagent was add. The blank was prepared by taking ten microliters of distilled water in a clean test tube then adding one milliliter of kit reagent. The standard was prepared by taking ten microliters of the standard solution at the kit package into a clean test tube, then adding 1ml reagent. The different solutions were mixed well then incubate at 20-25°C for 20min. The triglyceride was measured by the automatically adjusted spectrophotometer against blank at 546nm wavelength.

3.2.5 Blood chemistry

Serum magnesium and calcium were determined simultaneously using flame atomic absorption (Philips, PU 9200®) with standard quality control (Merck®). Phosphorus, bilirubin, creatinin, protein, albumin, ASAT (aspartate aminotransferase), CK (creatininkinase) and GLDH (glutamate dehydrogenase) were measured by using the Automatic Analyser (Hitachi, 704®) with Kits (Boehringer Mannheim®). Sodium, Potassium and Chloride were estimated through the electrolyte analyser (Beckmann, Synchron EL-ISE® Electrolyte system).

3.2.6 Hematology

Packed cell volume (PCV%), hemoglobin (Hb g%), erythrocyte count (RBCs $X10^6$ μl), total leucocytes count (WBCs $X10^3$μl), blood platelets and blood indices (Mean corpuscular volume (MCV), Mean corpuscular Hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC) were determined by using the Haematology analyser for veterinary medicine (NIHON KOHDEN, MEK-6108G®). The differential leukocytic count was done through the blood film

stained with Giemsa stain and examined under the oil immersion objective as described by **Jain (1986)**.

3.2.7   Ultrasonographic examination of the liver

The ultrasonographic examination of liver was performed for 140 cows by the technique described by **Braun (1990)** and **Braun and Gerber (1994)** to evaluate normal hepatic structure. 3.5 MHz linear transducer was applied to the right side of the abdominal cavity of the standing cow. The hair was clipped between the 6th intercostal space to a hand's breadth behind the last rib. The skin was cleaned with alcohol to remove the fat debris. After application of transmission gel to the transducer, the cows were examined cranially from the last intercostal space until the 6th intercostal space.

Each intercostal space was examined from dorsal to ventral with the transducer held parallel to the ribs. The texture and the visceral and diaphragmatic surfaces of the liver were scanned and the portal veins and the biliary system were examined.

The actual measurements were made as seen on a schematic cross-section. The dorsal and ventral liver margin, dorsal margin of portal vein and dorsal margin of gall bladder were measured in relation to the midline of the back as described by **Braun (1990)**.

The dorsal margin of the liver was determined by measuring the distance between the dorsal margin of the liver and the midline of the back. The visible size of the liver in a given intercostal space was determined by subtracting the distance between the dorsal liver margin and the midline of the back from the distance between the ventral liver margin and the midline of the back.

The depth and diameter of the portal vein were measured through the ultrasonographic image that was electronically stored at maximal inspiration of the cow. The appropriate measurements were then made electronically on the ultrasonogram by means of the two cursors.

 By the same route, the dorsal margin of the gall bladder and its dimensions could be measured. The measurements of the gall bladder were applied only in the 10th intercostal space in which they could be assessed. The portal vein can be measured also at the 9th to 10th intercostal spaces.

3.2.8   Histopathological examination of the liver

Small pieces ($1mm^3$) of the liver biopsy were put in 4% formaldehyde until histological examination.

3.2.8.1 H&E staining

Ten samples from each fat content were cut and fixed in 4% formaldehyde buffered with phosphate buffer. The block were rinsed in several changes of distilled water and kept overnight in 70% ethanol. After dehydration and embedding in paraffin, they were left leave until solidified sections (5-7µm thickness) were cut by use of a Tetrandermikrotom (Jung, Heidelberg). After that, sections were mounted on a clean glass slide, then left to dry for several hours in air. After that incubation at 60°C for 24-36 hours. Followed the dried sections were put in xylol for 20 minutes to remove paraffin.

After removal of paraffin, the sections were put in a descending sequence of alcohol from 100% to 50% for rehydration of samples; after that they were transported in distilled water and sections were stained using H&E stain according to **Romeis (1989)** and examined under a light microscope.

3.2.8.2 Sudan III staining

Some samples were fixed in 4% formalin buffered with phosphate buffer and then frozen. Frozen sections were cut using a frozen-microtome (cryocut E, Reichert-JUNG). The sections were put in a petri dish filled with water.

After that, the sections were transported and spread on glass slides impregnated with protein-glycerine together. Frozen sections were stained with Sudan III stain according to **Romeis (1989)** and examined under the microscope.

3.2.9   Statistical analysis

Data for all variables were tabulated in Excel tables for easy transfer to a statistic program.

The data were analysed using the general linear model of SPSS, version 11.5 programs. This program was used for estimation the correlations between the results of two methods used for estimation of total fat content of livers. It also was used to estimate the correlation between total fat and total triglyceride content and triglyceride content of total fat.

Moreover, the relationships between fat content of livers and different disease conditions were estimated.

The correlation of total fat of liver and total triglyceride and triglyceride contents of livers on one side and results of blood chemistry, haematology and the ultrasonographic dimension of portal veins and gall bladder on the other side was determined statistically.

Statistical comparison between the survival rate of animals and both total fat content, total triglyceride content and triglyceride content of total fat were made.

The significance of correlation between different traits was estimated by using the t-test at $p < 0.05$ or $p < 0.01$.