Chapter 4

Material and Methods
The current study on life cycle, epidemiology, prevention, control and treatment with special reference to chemotherapy in the diagnosed cases of Taeniasis with Nitazoxanide in human population was carried out between December 2004 and March 2007 to present the status of *Taenia saginata* infection in two Districts (Srinagar and Baramulla) of Kashmir Valley, India.
Plate 1. Study sites of District Baramulla and Srinagar, Source: Census 2001
The following protocols were undertaken for the detailed epidemiological, experimental and chemotherapy aspects of the *T. saginata* infection in Kashmir Valley to meet the objective of the problem satisfactorily.

**A. Life cycle of *Taenia saginata* (experimental trials)**

i) Demonstration of life cycle particularly metacestode stage (*Cysticercus bovis*) in their normal intermediate hosts i.e. cattle. This involved experimentation in calves.

ii) Evaluation of species specificity by observing association of this cestode with other non-host animals i.e. goats and sheep.

iii) Distribution and concentration of cysts (*C. bovis*) in the experimental animal carcasses.

**B. Viability of *T. saginata* eggs in various media like normal saline (0.9 %), dextrose solution (5 %), sludge containing naturally collected soil, leaves and water, and sewage of septic tank. Using methylene blue staining technique. Role of local fowl in the dispersal of *T. saginata* eggs.**

**C. Epidemiology**

The epidemiological studies involved the following parameters

i) Prevalence of *T. saginata* infection in human population

ii) Taeniasis and Cystercercosis due to *T. saginata* a major medical and veterinary problem in Kashmir respectively

iii) Common causes for the transmission of this zoonotic disease in Kashmir Valley

iv) To find out any social, geographical and religious barrier for this infection

**D. Chemotherapy in diagnosed cases of *T. saginata* or taeniasis**

i) To observe the efficacy of drug Nitazoxanide (NTZ) against *T. saginata* fresh (who had not taken any taenicide before) and resistant (who had taken several doses of taenicide like niclosamide and praziquantel, but persisted with infection) taeniasis.
A view of infected goats and sheep feeding in enclosure

Experimental goats and lambs being examined

Three infected calves

Caged local fowl inoculated separately with T. saginata eggs to demonstrate their role in the dispersal of infection

Plate 5: Slides showing various scenes of Experimental animal, used in current research study
E. To device various prophylactic measures for *Taenia saginata* infection in Kashmir including capacity building surveillance.

A. Life Cycle of *Taenia saginata* (Experimental trials)

*Taenia saginata* have their evolution in two different hosts, the ultimate one are the humans and the intermediary are cattle including cow (*Bos taurus*); buffalo (*Bos buffelus*); Zebu (*Bos indicus*), Yak (*Bos gruminens*). However, Machul Skii (1941) diagnosed cysticerci found in the cardiac and spinal musculature of the gazelle (*Gazella gutturosa*) as *Cysticercus bovis*. Shpilko (1956) evaluated reindeer as a possible intermediate host of *T. saginata*.

So, to evaluate the relation as an intermediate with normal (usual) host and non host animals, in this regard an experimental trial was carried out with the following methodology.

I. Demonstration of life cycle metacestode stage of *Taenia saginata* (*Cysticercus bovis*) in calves and experimental trials in other non-host animals (goats and sheep) to demonstrate development if any:

Ten healthy parasite free animals including three young sheep, 3 goats and 4 calves ranging all in the age group of 2-6 months were used for this experimental study. All animals belonged to local breeds. The minimum age of animals were 2 months, calf (n = 1), goat (n = 1) and rest animals (n = 8) including 3 calves, 2 goats and 3 sheep were above 4 months but less than 6 months.

II. Preparation of inoculum of *Taenia saginata* eggs

Gravid proglottids of *Taenia saginata* were obtained from non-treated local taeniasis patients, who were enrolled for epidemiological study, at temporary constituted laboratory, at Safapora Manasbal of District Baramulla, Kashmir, India.

Around 55 segments from different patients were identified as *T. saginata* by compression between two glass plates and the microscopic analysis of uterine ramification (15 to 32 ramifications) in *T. saginata* (João Carles et al., 2002; Fan et al., 1992; Hayunga et al., 1991; WHO. 1983; Dorny et al., 1999). These
identified segments were opened with the small sterilized needles of different sizes (micro dissection) and were put into 0.85 \% NaCl solution. The egg number was estimated with a Neubauer chamber. An aliquot of $2 \times 10^5$ eggs were put in test tubes containing 50 ml of saline solution (Joao Carlos et al., 2002; Smith et al., 1991; Kyvsagaard et al., 1991; Fan et al., 1989, 1992; Hayunga et al., 1991).

III. Animals

All the ten animals were raised in the temporary set laboratory attached with an animal house at Safapora, Manasbal of District Baramulla Kashmir. Initially they were supplemented with milk ($n = 2$): 1 goat and 1 calf and rest ($n = 8$) animals; 2 goats, 3 calves and 3 sheep were fed with leaves of locally available forage plants and pellets ration was later introduced as feeding supplements for the animals.

IV. Infection to experimental animals

Animals ($n = 9$) including calves ($n = 3$), sheep ($n = 3$) and goats ($n = 3$) were infected with *T. saginata* eggs and one non-infected calf was used as control. Each animal was given an oral dose of $2 \times 10^5$ *T. saginata* eggs kept ready in separate test tubes containing 50 ml each of saline solution (0.85\%). Then following the inoculation, they were kept for 5 days in individual stalls. During this time, their faeces/pellets were collected and kept until the fermentation and decomposition of the organic matter. The biophysico-parameters (body weight, rectal temperature, pulse) were recorded before and after inoculation.

V. Slaughter of animals

All the ten experimental animals were slaughtered commonly as the animals in Kashmir are being slaughtered in slaughter houses. First day one sheep, one goat and one calf were slaughtered, then after one week animals ($n = 3$), including one sheep, one goat and one calf were slaughtered. Then finally after one more week remaining animals ($n = 4$), including one infected calf, one sheep, one goat and one non-infected controlled calf were slaughtered. The whole slaughtering took place after 9th, 10th and 11th week of infection. The carcasses and organs, except the
Plate 3: Comparison of gravid proglottid of *T. saginata* and *T. solium*. Note the number of uterine branches in each segment; this was used as main diagnostic tool in differentiating the species during current research study.
intestinal viscera, were removed and brought to locally set laboratory where a
careful inspection took place using the total slicing technique.

VI. Muscular and organ necropsy

All organs and animal muscles were carefully sliced each 0.5 cm as earlier
adopted by Joao Carles et al. (2002); Fan et al. (1989, 1992), Hayunga et al.

First a careful observation of whole carcass was made using hand lenses
after deskinning the animal to detect any surface nodule, cyst, or lesion.

Then the carcasses were cut in individual organs. The predilection sites
were examined more carefully as being the most favourite regions for development
and concentration of cysts. Head, particularly tongue and masticating region, heart
and skeletal muscles were screened then the rest of organs were examined. For
slicing very thin, sharp razor edged knives, cutters were used for smooth and thin
slice cuts. The collected cysts were counted for each organ and site.

VII. Classification and identification of recovered cysticerci

All recovered cysticerci were classified as alive or degenerated during the
necropsy. Fully transparent cysts were considered as viable and other as
degenerated, degenerated cysts were of cheesy type (its contents were yellowish
and smooth) (Joao Carlos et al., 2002; Fan et al., 1992; Walter and Koske, 1980;
Kyvsgaard et al. (1989). All the collected cysticerci were preserved in 4 % formalin
for future scientific use.

B. Viability of Taenia saginata eggs in various media and observe role of
local fowl in the dissemination of infection

Ten T. saginata segments were collected from non treated patients and were
identified by counting uterine branches (WHO, 1983; Fan et al., 1988) These
segments were then put in separate dishes containing different media. The media
include, NaCl solution of 0.9%, dextrose 5 %, sludge containing soil, leaves and
water (naturally obtained) and sewage from septic tank.
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For each dish containing separate media two segments were first opened with sharp needles, razors and then put into these separate dishes, a rough estimate of 1, 10,000 to 1, 50,000 eggs were put in each five separate dishes. Viability was checked with methylene blue staining at weekly intervals.

Methylene blue staining technique: (Knaus and Lange, 1987): One gram of methylene blue powder was dissolved in 100 ml of distilled water to make 1% solution of methylene blue.

Weekly the four dishes containing *T. saginata* eggs in their respective media were first stirred with a sterilized glass rod and at random one drop of this suspension was placed on the slide following one drop of methylene blue solution, then after few seconds a cover glass was laid on it. Then eggs were observed under microscope without any concentration or floatation technique as the suspension of various media was concentrated with known quantity of *T. saginata* eggs. The eggs taking stain were considered as non viable and non staining eggs as viable (Knaus and Lange, 1987). The percentage of viable eggs was calculated by counting total number of non staining eggs by total number of eggs seen (Parvaiz et al., 1999).

The similar technique was also employed to observe the viability of *T. saginata* eggs which were fed to two local fowl; kept in separate cages for two weeks after inoculation of *T. saginata* two gravid segments each. Following the inoculation of segments, Coprological examination was done to evaluate the role of birds in the dispersal of eggs and dissemination of infection.

C. Epidemiology of *Taenia saginata* infection in Kashmir

The state of Jammu and Kashmir consists of 14 districts, 59 tehsils, 119 blocks, 3 municipalities, 75 towns, and notified area committee, and 6,652 villages, out of which 6, 41 are inhabited and 235 are un-inhabited villages. The total population of the state is 1,01,43,700. Males: 53,60,926. Females: 47,82,774; Sex ratio: 900(females per 1000 males). (Source: census 2001)
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Baramulla, the largest district of Kashmir valley in terms of area covers 4588 sq. km and is enclosed between Kupwara in the North, Budgam and Poonch in the South, parts of Srinagar and Ladakh in the East and has the line of control in its west. As per the 2001 Census Baramula has recorded a population of 11,69,780. population density :254 per sq. km and has 8 tehsils, 7 towns, 14 blocks, and 660 (646 inhabited) villages. This district ranked first also in terms of population has an economy where people primarily focus on agriculture, horticulture and industries for their subsistence. (Source: census 2001)

The Srinagar has an area of 2228 sq. Kms, the district is situated in the centre of the valley, is surrounded by five districts. It comprises of three tehsils/towns viz Srinagar, Ganderbal, and Kangan, four blocks, besides 175 villages. The population of the district is 12, 02,447 with a literacy rate 59.31%, population density is 401 per Square Km which is highest in the state. (Source: census 2001)

Epidemiology is the scientific study of the distribution and determinants of health-related events in specified populations, and the application of this study to the control of health problem (Lawlor, 2004).

The world Health Organization (WHO, 1983) has classified the prevalence of *Taenia saginata* in three different groups: highly endemic countries or regions with their presence in human population above 10%; moderate prevalence with infection rate between 0.1 and 10%; low prevalence with infection rate below 0.1% or the total absence of endemic organism. According to WHO classification, South American Countries are included among the moderate prevalence of *Taenia saginata*.

In India there was no conclusive data about the prevalence of *Taenia saginata* and cysticercosis in bovines and particularly for Jammu and Kashmir State. This epidemiological study is the first of its kind in this region as past data pertaining to this infection is almost nil. So, this study has been carried out with utmost care and responsibility.

Between September 2004 and March 2007, an epidemiological survey was carried out in various population sites (rural and semi rural communities) of Tehsil Sonawari of District Baramulla and Tehsil Kangan of District Srinagar of Jammu and Kashmir State involving a total population sample of 12,404 subjects including
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male (n=6,364) and females (n=6,040). There were 8,894 persons from Baramulla District including, subjects from semi urban (n=2500) and rural (n=6394) origin, males (n=4,567) and females (n=4,327), and from Kangan Tehsil of District Srinagar a population of 3,510 subjects of rural status were screened for Taenia infection, these include males (n=1,797) and females (n=1,713).

The identification of species of human tapeworms is crucial and important because the consequences of infection by T. solium and T. saginata are very different (Jerl et al., 1999). It was very difficult to evaluate the epidemiological prevalence of Taenia saginata taeniasis because the coproscopical methods if used for the survey were not completely adequate and usually cannot differentiate between T. solium and T. saginata infection (Dorny et al., 2003). Therefore, a comprehensive method of house to house survey was adapted to study the epidemiology of T. saginata infection in selected populations and people were enquired for the passage of proglottids in faeces or spontaneously. In this regard a preformed Proforma were made and were filled after interviewing every subject of the selected population. The Proforma giving detail of various epidemiological parameters is given on next page:

While recording every possible related parameter of T. saginata in free living Kashmiri population, much stress was laid on history and patterns of passage of gravid proglottids either spontaneously or within the feces; beef eating habits source of meat, taenicides used, resistance if any, etc.

As microscopic faecal examination would only reveal Taenia species (Dorny et al., 2003) So, the species difference was purely made on the identification of gravid proglottids by examining these segments after compressing them between two glass slides after washing these segments in distilled water, the number of uterine ramifications were counted as (15-32) in case of T. saginata which are low in other species, as (7-11) in T. solium (WHO, 1983) and presence of vaginal sphincter muscle, bilobed ovary in T. saginata would confirm the species (Verster, 1967). Thus every specimen was screened on the basis of above mentioned species difference criterion for T. saginata given in figures 6, 7.
Proforma

Name and address: .................................................................
Age: ............................................................................ Sex: ..............................................................
Occupation: .....................................................................
Educational qualification: ......................................................
Marital status: ..................................................................
Weight: ................................................................. Height: ..............................................................
Abdominal girth: ..............................................................
Hip girth: ........................................................................

Presentation of cases:

A. Symptoms and complications
1. Whether passing segments proglottids, if yes mention the period from

2. Abdominal discomforts if any; / Heart burn / Diarrhea / Constipation / Epigastric pain

3. Abdominal swelling / Distension

4. Nausea / Vomiting if any

5. Weight loss if any Yes / No. Weight gain. Yes / No

6. Anaemic if any

B. Beef history/ source of infection
1. History of Beef / Beef steaks / Beef Handler / Tasting while Kneading / Grinding Raw Beef

2. Method of Preparation, Roasting / Boiling / Partial Frying / Deep Frying / Traditional Habit Of Eating Raw Beef if any

3. Source of beef

C. Number of courses of Anthelmintic / Taenicides taken, if yes
1. Name of the drug. No. of courses taken

2. Recurrent infection after chemotherapy OR passage of segments after 1/2/4/8/10/12 weeks of chemotherapy or any other kind.

3. Observation after chemotherapy with Nitazoxanide

4. Naked eye examination of the expelled worm

5. Microscopic examination of the worm

6. Scolex present if any

7. Past H/O of infection or passage of segments Duration, whether treated spontaneously/with drug, or others

8. Family history of passage of Taenia segments or related complications

9. Date of chemotherapy with Nitazoxanide

10. Dosage

Date ..............................................................

Signature of the patient .....................................................

Signature of the Researcher ...................................................

Participation in the drug trial
(Patients consent)
Fig. 6 Sketch Showing Comparison of gravid proglottids of *T. saginata* (A) and *T. solium* (B); Other comparative details are given in Table 2 (manual)

Fig. 7 Sketch Showing Comparison of genital atria in *T. saginata* (C) and *T. solium* (D) Note absence of vaginal sphincter muscle in *T. solium* (after Verster. 1967)

**D. Chemotherapy with Nitazoxanide:** From a total screened population of 12,404 subjects, including 8,894 from Baramulla and 3510 from Srinagar, a total of 201 and 140 cases of *Taenia saginata* were detected purely on the basis of passage of gravid proglottids alone either passing spontaneously or via feces from Baramulla and Srinagar Districts respectively.

**Baseline characteristics of the patients enrolled for the drug trial are as:**

1. Mean age±SD : 28.08±16.56 cm  
2. Mean Height±SD : 138.20±28.14 cm  
3. Mean Weight±SD : 45±16.99 kg  
4. Mean Abdominal girth±SD : 64.5±17.1 cm  
5. Mean Hip girth±SD : 73.6±33.1 cm  
6. Duration of infection: Range : 15 days-24 Yrs,  
   Mean = 29.04 months
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From all these confirmed 341, cases of taeniasis of species specific *Taenia* infection (*Taenia saginata*) of Baramulla and Srinagar Districts, only 295 were enrolled for the study of drug trial of Nitazoxanide, after their written consent to participate in the study. Rest (n=46) were excluded from the study on the basis of following criterion.

N=11 were pregnant; N=17 were lactating women; N=3 had renal impairment; and N=15 did not co-operate to participate in the study.

Patients (n = 70) had received 2-7 courses (median 3) of Niclosamide including patients (n =11) had received both 1-3 courses of Praziquantel (median 2) and 2-7 courses (median 3) of Niclosamide and but had continued to pass proglottids with stool or spontaneously. All had demonstrated a relapse 3 - 8 weeks after initiation of treatment. All these subjects (n = 70) were categorized as resistant for taenicides. And (n = 225) had not taken any drug (taenicide) before and were categorized as fresh cases. The resitants were given a dose of Nitazoxanide 500 mg b.i.d (twice daily) for three days in adults and a dose of 15-20 mg /kg body weight /day for three days was administered in subjects with less than 15 years of age. The drug which was available in 500 mg tablets, 200 mg tablets and in suspension form was given orally with food to increase its absorption, and ensure its safety and efficacy. (Rossignol, 1984).

After chemotherapy their stool specimens were examined and, collected worms were screened for scolices and other morphological characteristics. And then following routine faecal examination adapting floatation technique, egg viability was tested with methylene blue technique. The egg count was performed by Stoll's technique. All the patients whether fresh (n = 225) or resitants (n = 70) were followed at 1, 2, 4, 8, 10 and 12 weeks for the passage of proglottids and there faecal samples were examined for the presence, number and viability of *Taenia* eggs. All responders stopped passage of proglottids with median of three days.
(range 1-5) days. All patients underwent physical examination including analysis of haemogram, serum chemistry (Liver function tests, Glucose, Urea, Sodium and Potassium), and urine and electrocardiogram before and seven days after treatment.

**Egg counting technique (Stoll’s technique):**

**Principle:** To obtain accurate information with regard to the severity of an infection, egg counting methods were carried out in order to determine number of eggs per gram of faeces (EPG).

The flotation fluids used was saturated salt solution. Its composition is

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>400 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.200</td>
</tr>
</tbody>
</table>

**Procedure**

- 3g of faeces were put into a container and mixed with 50ml of water
- The contents were mixed thoroughly and the resulting faecal suspension was strained through a double layer of cheese cloth to remove coarser material
- Flotation tube was filled with the faecal sample up to 1/3rd of its capacity
- Flotation fluid was poured into the flotation tube to the full capacity of the latter leaving a convex meniscus at the top of the tube
- A cover slip was carefully placed on top of the flotation tube. After 20-30 minutes of standing in a test tube rack, cover slip was clearly lift off and immediately placed a micro slide
- The slides were examined under the Microscope.
Flotation Technique: Simple test tube flotation or Will's Technique.

Principle: Based on separating of eggs from faecal material and concentrating them by means of a flotation fluid with an appropriate specific gravity, so that eggs rise to the surface and skimmed out of the surface film. This was adopted for nematode and cestode eggs.

The flotation fluids used was saturated salt solution with the following composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
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<tr>
<td>NaCl</td>
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- The slides were examined under the Microscope.
Table 2. Useful features for identification of scolexes and segments of *Taenia* species

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Number of hooks</th>
<th>Large hooks</th>
<th>Small hooks</th>
<th>Number of Testes</th>
<th>Layers of Testes</th>
<th>Cirrus sac Extends to Longitudinal Vessel</th>
<th>Number of uterine Branches</th>
<th>Lobes of ovary unequal in size with small well developed vaginal sphincter. Testes extend to vitellarium, but not confluent behind</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. saginata</em></td>
<td>22-36</td>
<td>139-200</td>
<td>93-159</td>
<td>375-575</td>
<td>1</td>
<td>No</td>
<td>7-16 that re-divide</td>
<td>Lobes of ovary unequal in size with small accessory lobe. No vaginal sphincter. Testes confluent behind vitellarium.</td>
</tr>
<tr>
<td><em>T. solium</em></td>
<td>28-36</td>
<td>191-218</td>
<td>118-143</td>
<td>600-700</td>
<td>1</td>
<td>Yes</td>
<td>6-10 that re-divide</td>
<td>Lobes of ovary unequal in size. No vaginal sphincter. Testes extend to vitellarium, but not confluent behind.</td>
</tr>
<tr>
<td><em>T. multiceps</em></td>
<td>22-30</td>
<td>157-177</td>
<td>98-136</td>
<td>284-388</td>
<td>2</td>
<td>Yes</td>
<td>14-20 that re-divide</td>
<td>Lobes of ovary equal in size. Pad of muscle on anterior wall of vagina. Testes extend to vitellarium, but not confluent behind.</td>
</tr>
</tbody>
</table>

Source: Manual of Diagnostic tests and Vaccines for Terrestrial animals. Updated: 23.07.2004