Observation and its Application of the Helicobacter by the Variable Pressure Scanning Electoron Microscope.

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Observation and its Application of the *Helicobacter* by the Variable Pressure Scanning Electron Microscope.

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Abstract - Observation of *Helicobacter* in the rich mucus of the stomach is often difficult. However use of a variable pressure scanning electron microscope (VP-SEM) with a cooling stage allowed the ultrastructure of *Helicobacter* to be clearly seen. We also applied this method to observation of the Corti's organ, which can easily be influenced by specimen pre-treatments such as chemical fixation, and found that the exact structure of Corti's organ was able to be confirmed.

I. Introduction

To observe hydrous biological specimens by scanning electron microscope (SEM), which detects secondary electrons (SE), chemical fixation of the specimen is necessary. However, the variable pressure SEM (VP-SEM), which has a cooling stage and detects backscattered electrons (BSE), has been developed to avoid even slight changes in the specimen tissue[1]. The merits of the VP-SEM technique are that it;

1. avoids dehydration of the tissue
2. decreases damage from an electron beam
3. requires no chemical fixation of the specimen
4. avoids expansion or contraction of the specimen
5. makes long term observation possible

Helicobacter is known to inhabit in a free state the gastric lumen and the mucus of the gastric surface[2]. Therefore, in high vacuum conditions, as in the case of observation by ordinary SEM, the existence of mucus covering the bacteria makes direct observation difficult.

To examine the details of *Helicobacter* distribution in the stomach or to elucidate the ultrastructure of the bacteria, preparation to remove mucus is required. However, if stomach mucus is removed for observation, the *Helicobacter* will also often disappear with mucus. Therefore, the authors employed a technique using the VP-SEM with the cooling stage to observe the incultivatable bacteria *Helicobacter* in stomach. We also observed the untreated tectorial membrane in the cochlea of guinea pigs, which easily sustains damage during preparation and fixation, by the VP-SEM with the cooling stage and acquired particularly new insights here.

II. Materials and Methods

A. Materials

Canine gastric *Helicobacters*
The stomach of a dog naturally infected with *Helicobacter*.

Cheetah gastric *Helicobacters*
The stomach of a cheetah naturally infected with *Helicobacter*.

Corti's organ of guinea pigs
Corti's organ of adult guinea pigs with normal Preyer reaction

B. Methods

Gastric *Helicobacters* in a dog.
Samples were divided into 4 different groups according to the method of tissue fixation used, as follows;
(1)Formalin fixation only
(2)Formalin fixation →t-butylalcohol dehydration (50%,80%,100%) (t-butyl alcohol-freeze-sublimation technique [3])
(3)Formalin fixation → platinum blue staining →20% DMSO (platinum blue staining method [4])
(4)Formalin fixation → platinum blue staining → t-butylalcohol dehydration (50%,80%,100%)

Gastric *Helicobacters* in a cheetah.
Formalin fixation → platinum blue staining → t-butylalcohol dehydration

Corti's organ of guinea pigs.
Divided into 2 groups as follows;
(1)Chemical fixation performed cochlea (fixation → dehydration → critical point drying)
(2)Untreated cochlea

C. Observation conditions (using the VP-SEM with the cooling stage)

Gastric *Helicobacters* in a dog and a cheetah

Accelerating voltage;10kV
Pressure of sample chamber;30Pa
Temperature of sample stage;-10℃ to –15℃

Corti's organ of guinea pig
Accelerating voltage;10kV
Pressure of sample chamber;30Pa
Temperature of sample stage;-10℃ to –15℃

III. Results

Gastric *Helicobacters* in a dog

Helicobacter is usually demonstrated in the mucus of surface gastric mucosa or of the gastric lumen by microscopical finding as shown in Fig.1.Therefore,the secondary electron observation after routine chemical treatment (chemical fixation →dehydration with a graded series of ethanol → drying), shows that *Helicobacter* hides in the mucus , making it difficult to reveal distribution or to elucidate the details of the bacteria's ultrastructure (Fig.2) .

Observation by the VP-SEM with the cooling stage simplifies and shortens the preparation of the specimen and the result is as follows:

Fig. 1 Warthin-stary stained dog's gastric by microscopical finding

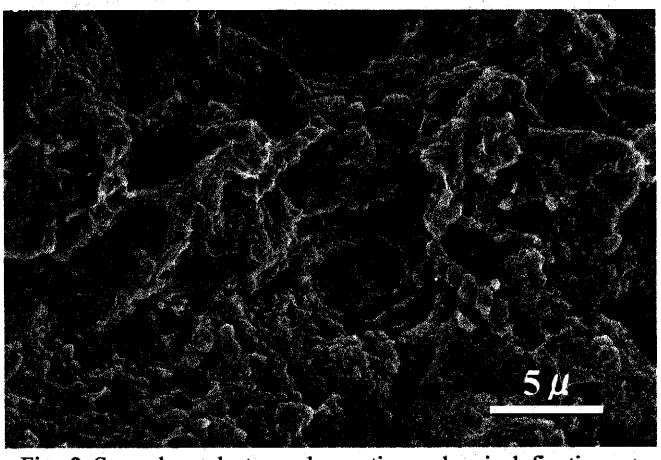

Fig. 2 Secondary electron observation : chemical fixation → frozen vacuum → coating

(1) Only formalin fixation group
Due to the covering mucus, the surface of the *Helicobactor* was indistinct, making it difficult to detect its structure (Fig.3).

(2)Formalin fixation → t-butylalcohol dehydration group
After removal of mucus, the ultrastructure could be demonstrated clearly compared with specimens fixed only in formalin, (Fig.4).

(3)Formalin fixation → platinum blue staining → t-butylalcohol dehydration group
Due to platinum blue staining, an increase in BSE signal was demonstrated and the distribution of *Helicobacter* was easily detected (Fig.5).

(4)Formalin fixation → platinum blue staining → t-butylalcohol dehydration group
Compared with the other group, *Helicobacter* was easily observed and the ultrastructure most clearly revealed (Fig.6).
Based on the numbers of spirals and no pericytoplasmic fibers, the bacteria found in the dog's stomach was determined as H. heilmannii (Fig.6b).

Gastric Helicobactors in a cheetah

Two kinds of *Helicobacter* (*H. heilmannii* and *H.felis)* with different forms were observed in the gastric lumen and on the surface of the gastric mucosa (Fig.7,Fig.8). *H. felis* had pericytoplasmic fibers on its body surface, and these fibers could be simply and quickly observed by use of the technique (Formalin fixation → platinum blue staining → t-butylalcohol dehydration) and the VP-SEM with the cooling stage.

Corti's organ of guinea pig

Fig.9 shows the appearance of the tectorial membrane when observed by ordinary SEM after chemical fixation. Fig.10 shows the appearance of the untreated tectorial membrane when observed using the VP-SEM with the cooling stage. Differences between the two observation techniques were as follows:

1) In observation by ordinary SEM after chemical treatment, numerous crater-like hollows were revealed at the limbal zone (Fig.9).In contrast, when observed using the VP-SEM with the cooling stage, the untreated tectorial membrane was seen to have a mesh-like appearance in the limbal zone (limbal net) (Fig.10).In findings of ordinary SEM, there was no level difference in the boundary of limbal zone and middle zone of tectorial membrane. The crater-like hollows existed on the same plane (Fig.9) (Fig.11).However, observation of the untreated tectorial membrane by the VP-SEM with the cooling stage limbal zone (limbal net) with pillar structures under it was in the position higher than the middle zone (Fig.10) (Fig.12).
2) In the chemically prepared specimen, a covering net consisting of fibers at the middle zone was observed by ordinary SEM (Fig.11). However, observation of the untreated tectorial membrane by the VP-SEM with the cooling stage revealed no fibrous structure at the middle

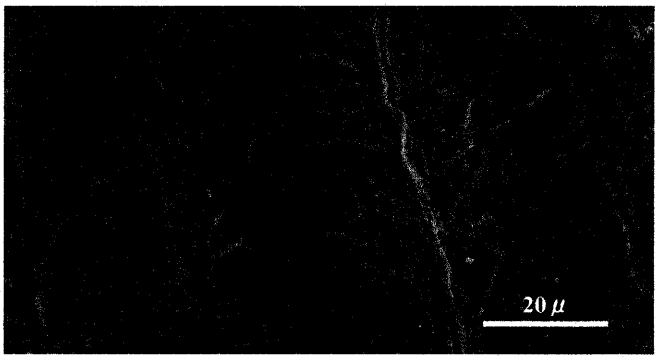

Fig.3a. Only formalin fixation (×1,000)

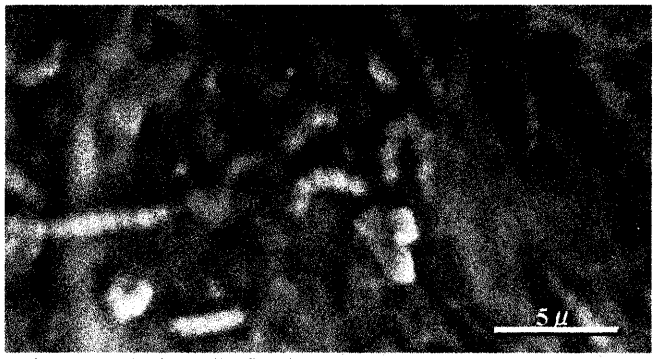

Fig. 3b. Only formalin fixation (×5,000)

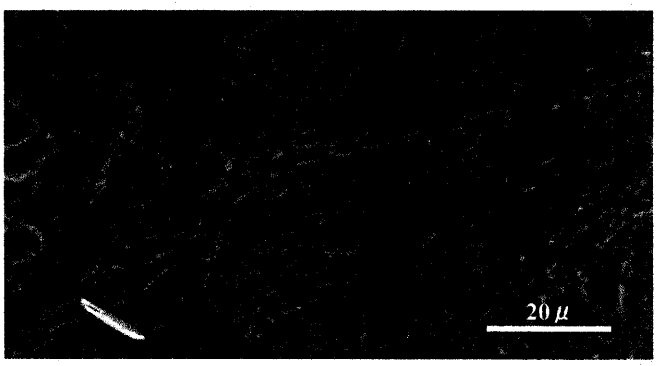

Fig. 4a. Formalin fixation → t-butyl alcohol dehydration (×1,000)

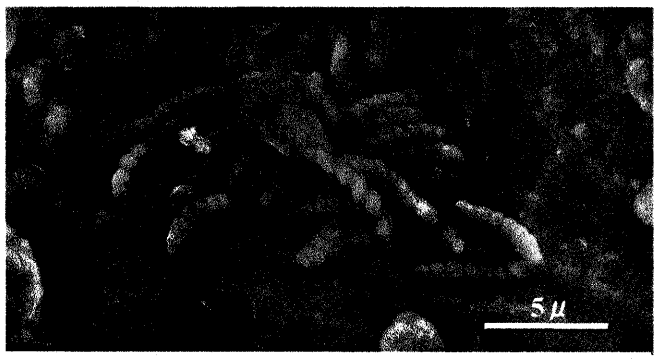

Fig. 4b. Formalin fixation → t-butyl alcohol dehydration (×5,000)

Fig. 5a. Formalin fixation → platinum blue staining → DMSO (×1,000)

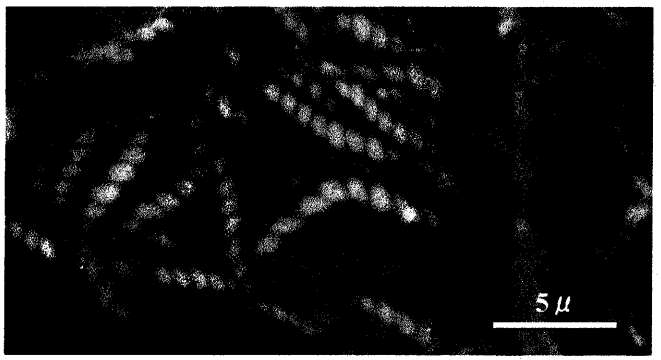

Fig. 5b. Formalin fixation → platinum blue staining → DMSO (×5,000)

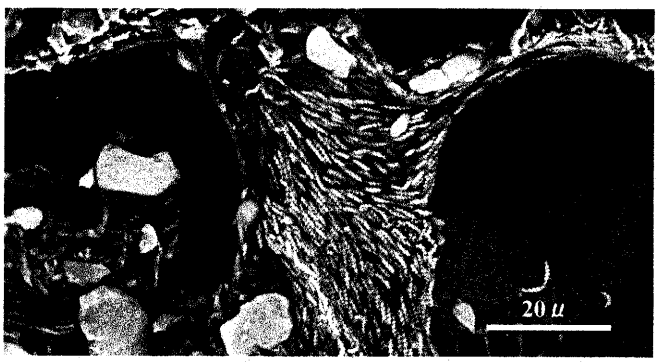

Fig. 6a. Formalin fixation → platinum blue staining → t-butylalcohol dehydration (×1,000)

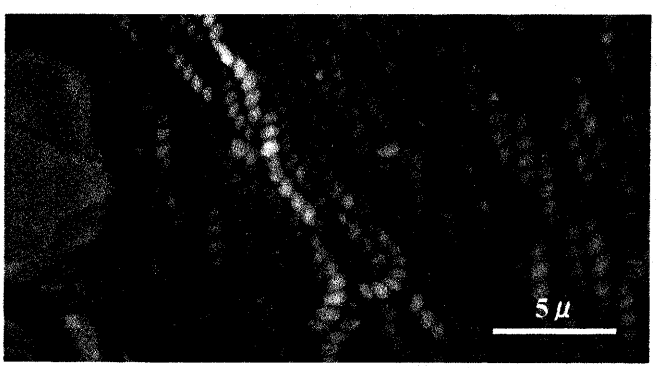

Fig. 6b. Formalin fixation → platinum blue staining → t-butylalcohol dehydration (×5,000)

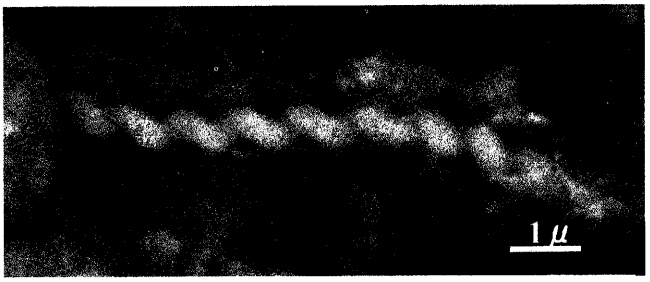

Fig.7 Gastric *H.heillmann* in a cheetah

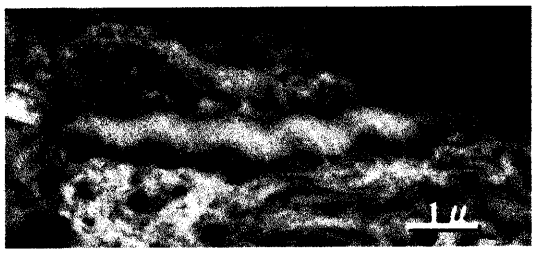

Fig.8 Gastric *H.felis* in a cheetah

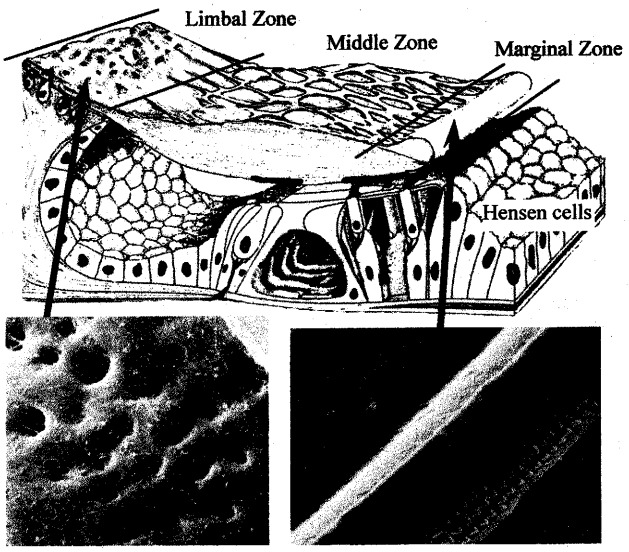

Fig. 9 Illustration of Corti's organ (chemical preparation performed)

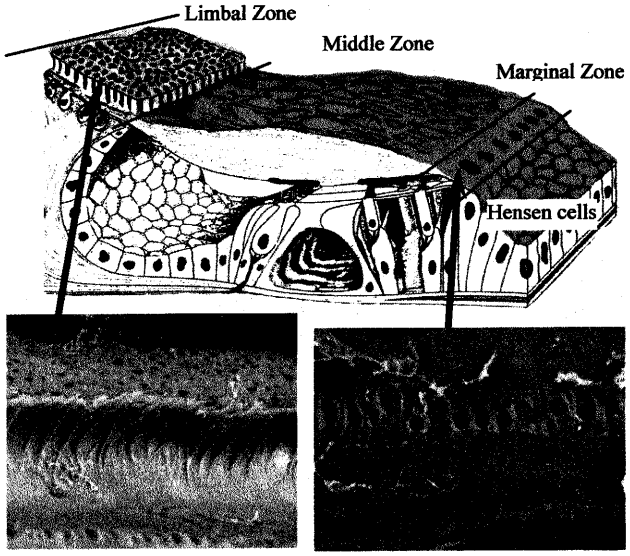

Fig. 10. Illustration of Corti's organ (untreated specimen)

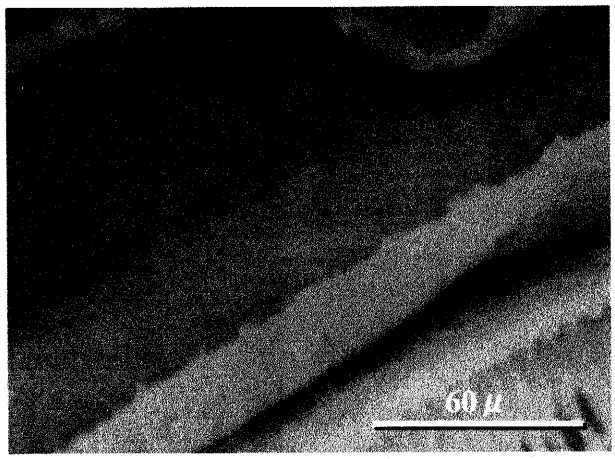

Fig.11. Cochlea which chemical preparation performed

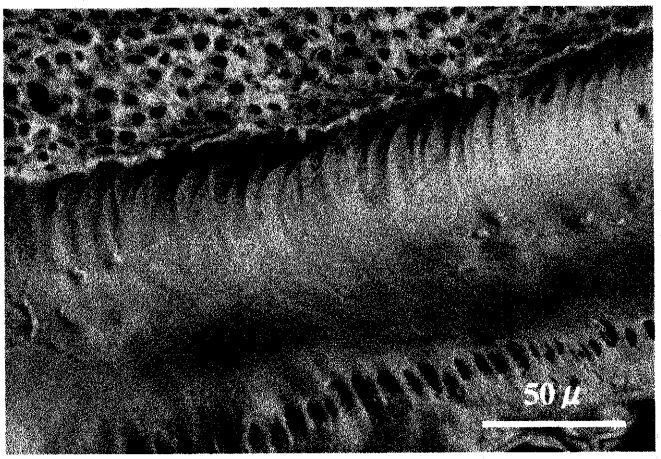

Fig.12. Untreated cochlea (VP-SEM with the cooling stage).

zone (Fig.12).

3) Observation by ordinary SEM after chemical fixation showed the marginal zone as a free end band (Fig.9) (Fig.11) , however,when observed by the VP-SEM with the cooling stage, the untreated tectorial membrane showed a pillar-like structure attaching to the surface of Hensen cells. Between pillars and pillars, marginal holes were identified and the strand-like structures (or funicular or trabecular structures) were present in each marginal hole (Fig.8) (Fig. 11).

IV. Discussion

To observe dehydrations of surface of the specimen by ordinary SEM, usually secondary electron is used under the high vacuum conditions. However this method requires preparation of the biological specimen, namely by chemical fixation, successive dehydration in alcohol and critical point drying.This procedure is energy- and time-consuming, and often induce contraction and alteration of the tissue in some cases.

In this study, the author employed VP-SEM with the cooling stage technique which permits prompt observation without pre-treatment. This system permits pressure of its specimen chamber to range from 1 to 270 Pa during operation, and allows temperatures of the stage from +10℃ to -20℃.Therefore it allowed suppressing vaporization of water and decreasing the damage from electron beam. Furthermore, the artifacts can be obstructed to the minimum since chemical pre-treatment is not needed in this system.

Observation by the VP-SEM with the cooling stage relies on backscattered electrons instead of secondary electrons.Therefore BSE carry information from deeper area (from tens to 100 μ m) of the tissue(SE carry information from within 10nm). Namely the information of internal structure of the tissue is detectable. Additionally the BSE reflects atomic number contrast (which represents the element compositions),so that the part where it is stained by heavy metal would demonstrate a difference in contrast,as the intensity of heavy metal is high.

Using these peculiarities of VP-SEM, the authors were able to identify gastric *Helicobacter* in the dog, cheetah and Corti's organ of the guinea pig.

Gastric *Helicobactor* of dog

Helicobactor species is classified into over 23 types and is considered to be related in human not only to gastritis, gastric ulcers, but also cancer. *Helicobacter* is found to exist in a free state in the mucus of the gastric surface or in the mucus of the gastric lumen and incultivatable types are known. Therefore, in high vacuumed condition as under the observation of ordinary SEM, the existence of the mucus covering the bacteria makes the observation difficult.

To observe the distribution of *Helicobacter* in the stomach or the ultrastructure of the bacteria, the preparation which removes mucus is required. If the removal of mucus were attempted, *Helicobacter* also would be removed and be more inconvenient. Therefore the authors applied the technique VP-SEM with the cooling stage and performed the method (Formalin fixation,platinum blue staining and t-butylalcohol dehydration). The tissue preparation by this method requires only 1 h after formalin fixation,while conventional procedures including conductive staining, dehydration,critical point drying,and metal coating, need about 1 day or more.

Helicobacter are classified according to the form of bacteria, biological and molecular biological characteristics of cultured bacteria. However a certain kind of bacteria cannot grow in artifactual medium.For this reason, chilled VP-SEM which can observe *Helicobacter* also in stomach with much mucus is useful.

Corti's organ of guinea pig

The function of inner ear is distributed to the part that concerns to "balance" and the part that concerns to auditory function which is called "cochlea". Cochlea is a spiral membranous canal that is subdivided into scala vestibuli, scala media, and scala tympani. The Corti's organ which perceives auditory stimulation is situated in the scala media. And the tectorial membrane that easily sustains damage during the chemical fixation is located in the Corti's organ. Tectorial membrane is reported to expand under osmium tetroxide and to contract in the series of dehydration or the critical point drying[5]. Therefore the authors performed the technique VP-SEM with the cooling stage to avoid any artifact to contaminate.

The limbal zone was observed as a meshed like structure and pillars were demonstrated under it. And the marginal zone was not revealed as a free end band but a pillar structure.

V. Summary and Conclusions

1. The group after the formalin fixation → platinum blue staining → t-butyl alcohol dehydration was the most convenient procedure to observe by VP-SEM with the cooling stage and revealed the free swimming condition of *Helicobacter* in the surface of mucosa of dog's gastric and the mucus of gastric ductus.
2. VP-SEM with the cooling stage observation basically requires no preparation of the specimen and employing this technique, the realization of immediate observation, the distribution and the type of the bacteria had been revealed.
3. The limbal zone of the tectorial membrane was observed as a meshed like structure "limbal net" and pillar structure lying under it by VP-SEM with the cooling stage.

   Covering net in the middle zone was not confirmed by VP-SEM with the cooling stage.

   Marginal zone of the tectorial membrane was observed a pillar structure and to attach to the surface of the Hensen cell. Strand like substance was observed through the marginal hole.
4. Employing the VP-SEM with the cooling stage, the cochlea would be possible to observe without any chemical treatment, therefore, the contamination of artifact considered to be as minimum..
5. VP-SEM with the cooling stage avoid slight changes in the specimen tissue and make observation easy, compared with ordinary SEM in the high vacuum condition. Furthermore the tissue preparation is simple and shorten.This will be found useful for biological studies in the future.

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