

The effects of colostral antibodies from immunized dairy cows on inhibition of absorption into the blood of Verotoxin 2 derived from enterohemorrhagic *Escherichia coli* O157:H7 and on eradication of *Helicobacter pylori* in experimental animals

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実験動物での腸管出血性大腸菌 O157:H7 由来ベロ毒素 2 の吸収抑制

ならびに

Helicobacter pylori の除菌におけるウシ免疫初乳抗体の効果

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要旨

腸管出血性大腸菌 O157:H7 (*E. coli* O157:H7) は、死者の発生を伴う食中毒の原因菌として、*Helicobacter pylori* (*H. pylori*) は、胃潰瘍、胃がんなどを誘発する細菌としてよく知られており、これらの消化器感染症に対する有効な対応策の確立が待たれている。

そこで、乳牛で作製した免疫初乳抗体を用いてこれらの消化器感染症における受動免疫の有効性を動物モデルで明らかにした。腸管感染症モデルでは、*E. coli* O157:H7 の産生するベロ毒素 2 (VT2) に対する免疫初乳抗体を用いてマウスにおける VT2 の吸収阻止効果を明らかにした。胃感染症モデルでは、*H. pylori* に対する免疫初乳抗体及びその抗体と補体（新鮮ウシ血清）とを用いてスナネズミにおける除菌効果を実証した。これらの蛋白質分解酵素に強い抵抗性を有する免疫初乳抗体は、乳牛でそれぞれ作製した。

I. 可溶性 VT2 に対する抗体測定が可能な間接蛍光抗体法 (IFA) の開発

IFA 用の VT2 感作ラテックスの調製及び乳牛への免疫原に用いた VT2 は、マウス抗 VT2 モノクローナル抗体感作 Sepharose4B カラムを用いたアフィニティークロマトグラフィーによって *E. coli* O157:H7 VT2 産生株の培養液から分離した。

VT2 に対する免疫初乳抗体は、分娩 4 ヶ月前の乳牛へ毎週 1 回 VT2 を免疫して作製した。分娩 3 日後までの初乳を採取し、低速遠心による脱脂及びレンネットによる脱カゼインを行って乳清を分離した。これを免疫初乳抗体として供試した。

VT2 感作ラテックスは、粒径 6 μm の 2.5 %ラテックス粒子 0.5 ml へ 30 $\mu\text{g/ml}$ の VT2 1.0ml を感作して調製した。それを 20 %グリセリン、1 % 卵白アルブミン (OVA) を含む食塩加リン酸緩衝液 (PBS) に分散させ、この 5 μl を IFA 用スライドガラスの well に塗抹して IFA スライドガラスを作製した。これに 1:2～1:512 に希釈した免疫初乳抗体 (10 μl / well) を加えて室温で 1 時間反応させた。次に、至適濃度の FITC 標識抗ウシ γ グロブリン、IgG、IgA あるいは IgM 抗体をそれぞれ 10 μl / well 反応させた。反応終了後、スライドガラスを 3.0M の塩化

ナトリウムを含む PBS で洗浄することによって、非特異反応を完全に排除することができた。なお、ラテックス粒子に自家蛍光は認めなかった。一般的な VT2 に対する抗体測定法であるペロ細胞を用いた中和試験では、免疫グロブリンクラス別の抗体測定が不可能であったが、この IFA によってその問題が解決された。この IFA で測定した免疫初乳抗体の抗体価は、免疫に用いた乳牛の血清抗体価の約 4 倍高力価であった。免疫初乳抗体の IFA 価は、分娩直後に採取した初乳が最も高い 1:512 を示し、分娩 3 日後までの初乳も 1:128~1:256 と比較的高力価を示した。

II. マウスにおける免疫初乳抗体による VT2 の血中への吸収阻止作用

マウスの血中に吸収された VT2 の濃度は、0.2 ng/ml まで測定可能な蛍光 ELISA によって測定した。マウス（146 匹）を用いて免疫初乳抗体による VT2 の血中への吸収阻止効果を検討した。

吸収に至適な 477.8 ng/ml の VT2 0.3ml をゾンデを用いて投与し、その 1 時間後から VT2 に対する免疫初乳抗体 0.3ml を 1 時間間隔で計 3 回投与したところ、血中への VT2 の吸収は、わずか 0.3~2.6 ng/ml と微量であった。それに対して、免疫初乳抗体の代わりに VT2 に対する抗体を含まない初乳乳清を投与した対照群では、VT2 投与 12 時間後に 15.4 ± 5.0 ng/ml、16 時間後に 4.3 ± 1.6 ng/ml まで上昇した。免疫初乳抗体を投与したマウスの VT2 濃度は、対照群に比べて有意に低値を示した。これは、腸管内において免疫初乳抗体が VT2 と結合して VT2 の毒素活性部位をブロックするとともに大きな免疫複合体を形成して糞便中へ排泄されたために吸収量が少なかったと推察された。

多量（955.6 ng/ml）の VT2 を投与した場合には、16 時間後にマウスの血中へわずか 8.2 ng/ml しか吸収されなかった。これは多量の VT2 によって腸管粘膜が強く傷害され、吸収機能が低下したためと考えられた。

III. スナネズミにおける免疫初乳抗体による *H. pylori* の除菌効果

H. pylori に対する免疫初乳抗体は、分娩 3 ヶ月前の乳牛へ毎週 1 回 *H. pylori* を免疫して作製した。分娩後 3 日分の初乳を採取し、VT2 に対する免疫初乳抗体と同様の方法で、乳清を分離した。この免疫初乳抗体は、*H. pylori* の菌体及び鞭毛の両方に対する抗体活性を有していることを IFA で確認した上で、本実験に供

試した。

H. pylori の除菌に関する実験には、5~10 週齢のスナネズミ（101 匹）を用いた。スナネズミへの *H. pylori* の接種は、ゾンデを用いて 0.1%重曹 0.3ml を投与後、 5×10^7 CFU の *H. pylori* を 1 日に 1 回、2 日間接種し、2 週間後に ELISA によって *H. pylori* に対する血中の IgM 及び IgG 抗体価の上昇から感染の成立を確認して本実験に用いた。なお、この ELISA での抗体価の上昇が、*H. pylori* 感染成立の指標となることは、別な実験で確認済みである。

除菌処置を施したスナネズミは、除菌処置終了 1 ヶ月後に安楽死させ、胃のホモジネート 10 μ l をウマ血清加 BHI 培地に塗抹して 37°C、微好気環境下で 7 日間培養した後に、*H. pylori* のコロニー形成の有無によって除菌効果を判定した。

H. pylori を感染させたスナネズミへヒトの治療で最も一般的に用いられているオメプラゾール、クラリスロマイシン及びアモキシシリンをヒトにおける用量の約 1.3 倍量に相当する 10mg/kg 及び約 2 倍量に相当する 20mg/kg を 1 日 2 回、7 日間経口投与した。その結果、*H. pylori* の除菌率は 10mg/kg 投与群で 92%（11/12 例）、20 mg/kg 投与群では 100%（12/12 例）であった。

免疫初乳抗体による除菌実験では、スナネズミへ 0.1 %重曹 0.3ml を投与して胃内の pH を中性付近に調整した後に、0.5 ml の免疫初乳抗体を 1 日 2 回、1 ヶ月間または 2 ヶ月間経口投与した。対照群へは、免疫初乳抗体の代わりに、*H. pylori* に対する抗体を含まない初乳乳清を同量投与した。その結果、*H. pylori* の除菌率は、免疫初乳抗体 1 ヶ月間投与群で 83%（10/12 例）、2 ヶ月間投与群では、薬剤 10 mg/kg 投与群と同じ、92%（11/12 例）であった。これらの対照群の除菌率はいずれも 0%（0/6 例）であった。本実験に用いた免疫初乳抗体は、*H. pylori* の菌体と鞭毛の両方に対する抗体活性を有していることから、抗体分子が *H. pylori* の菌体や鞭毛へ結合することによって *H. pylori* の運動性や定着の阻害に加えて *H. pylori* との免疫複合体が形成されて排泄が促進され、除菌効果が発現されたものと考えられた。

免疫初乳抗体と補体とによる除菌実験では、スナネズミへ 0.1 %重曹 0.3 ml 投与後、免疫初乳抗体及び補体をそれぞれ 0.5 ml、1 日 2 回、2~3 日間経口投与した。対照群のスナネズミへは免疫初乳抗体と 56°C、30 分間の加熱によって不活

化した補体を実験群と同じ条件で投与した。その結果、免疫初乳抗体の2日間投与群では83% (10/12例)、3日間投与群では100% (12/12例)の除菌効果が認められた。*in vitro*で、*H. pylori*へ免疫初乳抗体と補体とを作用させた場合に、*H. pylori*が強く傷害(溶菌)される現象が確認されたことから、胃内においても、*in vitro*と同様に、活性化された補体によって*H. pylori*の菌体が傷害された結果、短期間で強い除菌効果が発現したと考えられた。これらの対照群では、いずれも8% (1/12例)の除菌率を示した。これは各除菌処置日における初回投与前に不活化した補体の機能が時間の経過に伴って復活し、2回目の抗体・不活化補体投与時に補体が活性化されて*H. pylori*を傷害したためと考えられた。

ヒトの*H. pylori*の除菌治療においては、薬剤耐性菌の出現及び薬剤に対する過敏症患者への投与が問題となっている。それに対して、免疫初乳抗体あるいは免疫初乳抗体と補体とを用いる方法は、牛乳アレルギーを有するヒト以外の患者へ、耐性菌の問題が全くなく、反復投与ができる有用な*H. pylori*の感染予防法あるいは除菌法として応用が可能と考えられた。ことに、胃内で補体を活性化させる方法は、きわめて短期間で除菌が可能な画期的な手法になりうると考えられた。

Original articles

New application of indirect fluorescent antibody (IFA) technique using latex particles coupled with verotoxin 2 from *Escherichia coli* O157:H7 in order to determine colostral antibody titers in immunized dairy cows

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Running title: New application of IFA technique using latex particles

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Abstract

A simple and novel assay method for determining colostral and serum against soluble verotoxin 2 (VT2) titers by indirect fluorescent antibody (IFA) assay using latex sensitized with VT2 was devised. The latex particles did not auto-fluoresce, and nonspecific reactions disappeared after washing with phosphate buffered saline containing 3 M NaCl. The highest titer measured by neutralizing test was observed at 1 day after delivery. The highest titer for each immunoglobulin class measured by enzyme linked immunosorbent assay (ELISA) or IFA using latex sensitized with VT2 was also observed at 1 day after delivery. The changes in titer measured by each method showed similar patterns. Furthermore, the titers for IgG antibody were higher than those for IgM or IgA antibodies. Thus, the titers

of bovine immune colostral antibody and each immunoglobulin class could be measured by IFA using latex sensitized with VT2.

Introduction

We have employed a neutralizing test using vero cells^[1] to determine the neutralizing antibody titers of colostral and serum antibodies against vero toxin 2 (VT2) obtained from cows immunized with VT2.^[2-4] However, this neutralizing test has some unresolved issues. For example, it is necessary to maintain vero cells for the neutralizing test, and the test itself requires at least 3 days to obtain the results. In addition, it is very difficult and/or impossible to determine the neutralizing antibody titers of each immunoglobulin class in the neutralizing test. Therefore, the protease resistance activity of each immunoglobulin class of colostral antibody recovered from the intestinal tract of beagle dogs was observed by enzyme-linked immunosorbent assay (ELISA).^[4]

The authors have already reported a simple and novel indirect fluorescent antibody (IFA) technique using latex particles (latex) as a carrier for free soluble antigen to determine antibody titers.^[4] This technique was considered to for application to various measurement methods. The aim of this study was to develop a novel assay method for determining colostral and serum antibodies against soluble VT2 and its immunoglobulin class titers by IFA using latex sensitized with VT2.

Materials and Methods

Escherichia coli 0157:H7

A strain of *Escherichia coli* 0157:H7 producing only VT2 was used.

[2]

Quantification of proteins

Purified VT2 was quantified by the Bradford method ^[23] using Coomassie brilliant blue G-250. Concentrations of colostral whey and each immunoglobulin class isolated from bovine colostrum were quantified using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Preparation of bovine colostral anti-VT2 antibody

Three dairy cows were immunized with purified or crude VT2 according to the method of Kuribayashi *et al.* ^[2]

Latex particles

Polybead polystyrene microspheres (2.5% (w/v)) (Polyscience,

Inc., Warrington, PA) having grain sizes of 6.0 μm were used.

[4]

Isolation of VT2 by affinity chromatography

Mouse anti-VT2 monoclonal antibody (Capricorn Products, LLC, Portland, ME, USA) was coupled to CNBr-activated Sepharose 4B (GE Healthcare UK Ltd., Little Chalfont, UK) as an immunoadsorbent and was then packed into column for affinity chromatography^[21, 22]. Culture medium containing VT2 from *E. coli* O157:H7 was applied to the affinity column, which was washed with 0.01 M phosphate buffered saline (PBS) containing 0.14 M NaCl. After washing, VT2 was dissociated from the immunoadsorbent using 0.14 M glycine-HCl buffer (pH 2.3) and was immediately adjusted to pH 7.0 with Tris-HCl buffer (pH8.9).

Neutralizing test

Neutralizing test of colostral antibody titer was conducted according to the methods of Konowalchuk *et al.*^[24]

Preparation of VT2-sensitized latex for IFA

The preparative procedure of VT2 sensitized latex was carried out according to the method of Kuribayashi *et al.*^[4] Briefly, VT2 (30 µg/ml) was dialyzed against 0.1 M borate buffer (pH 8.5) for 48 h and was used for sensitization to 0.5 ml of 2.5% latex. Latex was spread in PBS containing 20% glycerin, 1% ovalbumin (OVA) and 0.01% NaN₃. Five microliters of latex coupled with VT2 was placed onto the each well on the slide glass for fixation onto the slide glass for 12 h at room temperature.

IFA technique

IFA technique for bovine colostral antibody and each immunoglobulin class of colostral antibody was performed using a modification of the method reported by Killinger *et al.*^[25] FITC-conjugated anti-bovine γ -globulin, IgG (Rockland Immunochemicals Inc., Gilbertsville, PA, USA), IgM (Bethyl Laboratories Inc., Montgomery, TX, USA) and IgA (Bethyl Laboratories Inc.) antibodies were diluted 2~512 times and then placed into each well to determine optimal dilutions. Slide glasses were washed with PBS containing 3 M NaCl and/or 0.14 M NaCl for 15 min after the reaction. The IFA patterns were examined under a fluorescence microscope and the strength of fluorescence of VT2 sensitized latex was classified as 3+ ~ -.

IFA titers of antibodies were read as the maximum dilution at which a positive reaction was observed.

Enzyme linked immunosorbent assay (ELISA)

VT2 diluted with carbonate buffer (pH 9.6) was fixed to immunoplates for ELISA (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 1 h and 1% ovalbumin dissolved in carbonate buffer (pH 9.6) was used to block unabsorbed sites. One hundred microliters of horseradish peroxide-labeled anti-bovine IgG, IgA or IgM was then added, and after 1 h, 2-fold diluted bovine colostral antibodies were added. Absorption was measured using a microplate reader equipped with 415 nm and 492 nm filters (Corona Electric Co., Ltd., Ibaragi, Japan).

Results

IFA patterns

The VT2-sensitized latex particles fixed onto the slide glass remained fixed during immersion, as described by Kuribayashi *et al.*^[4] The latex particles did not show auto-fluorescence, and nonspecific reactions disappeared after washing with PBS containing 3 M NaCl (Figure 1). IFA patterns are shown in Figure 2. Titers of bovine colostral antibodies obtained from three cows measured by neutralizing test and IFA using VT2-sensitized latex particles and ELISA are shown Figure 3.

The highest titer measured by neutralizing test was observed at first milking after delivery. The titers of each immunoglobulin class measured by ELISA or IFA using

VT2-sensitized latex particles also showed the highest titer(s) in colostrum at first milking after delivery. The titers measured by each method decreased gradually with time. Furthermore, the decrease in titers of bovine colostrum antibodies measured by each method was similar. The titers for IgG antibody measured by ELISA and IFA using VT2-sensitized latex particles were higher than those for IgM and IgA antibodies.

Discussion

We believe that it is possible to use IFA assay to determine soluble antibody titers using latex particles as a carrier of soluble antigen.^[4] We therefore investigated whether antibody titers could be measured by IFA using VT2-sensitized latex particles. When washing with PBS, some antibody observed showed

weak nonspecific reactions. However, these nonspecific reactions disappeared after washing with PBS containing 3 M NaCl. This is likely due to the F/P ratio, which is 3.0~5.0 for FITC-conjugated antibodies

The titers of bovine colostrum antibodies measured by each method showed similar changes. Furthermore, the titers of each immunoglobulin class could be measured by IFA using VT2-sensitized latex particles. This technique was very useful, as it allows the titers of free soluble antigens to be easily and more rapidly determined. Furthermore, this technique can measure titers for each immunoglobulin class.

We believed that it was possible to use IFA assay to measure free soluble antigen based on a preliminary study.^[4] Titers for bovine colostrum antibody against VT2 could be measured using

this method, but the most useful characteristic of this method is that the titer of each immunoglobulin class can be determined. In contrast, neutralizing tests using vero cells are unable to determine the titer of each immunoglobulin class. This method is thus considered to be very useful, and we plan to apply it to other free soluble antigens.

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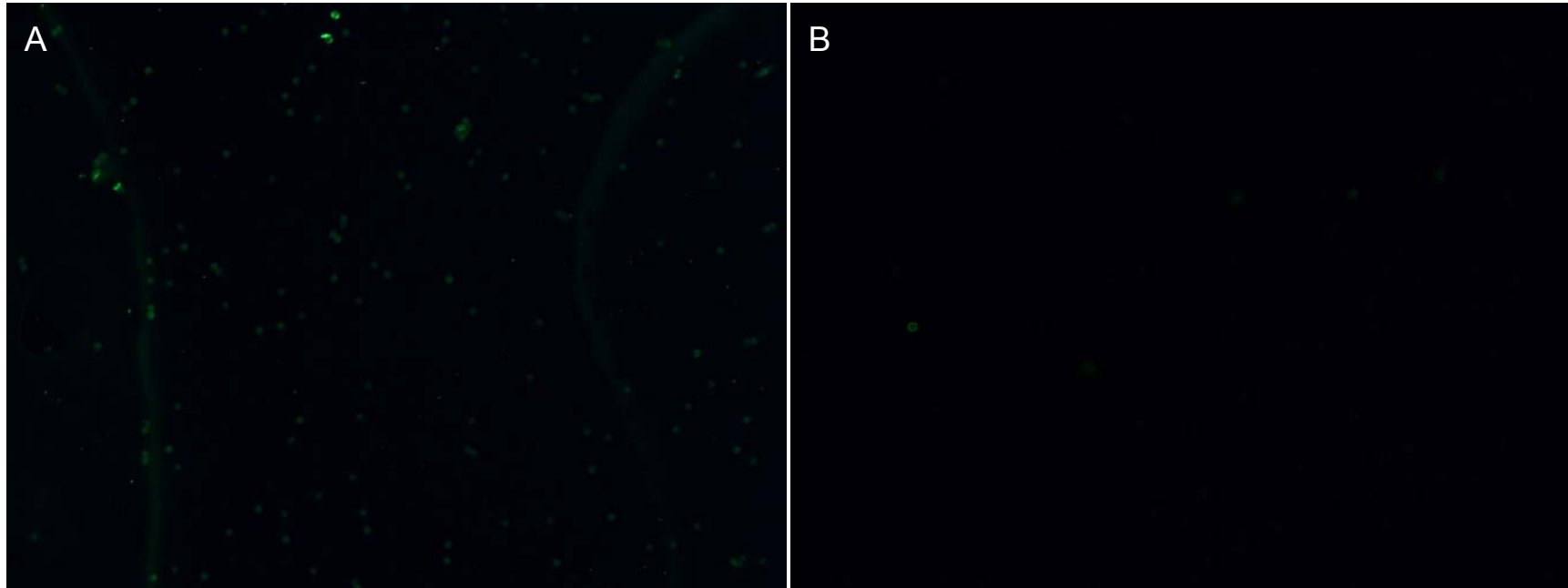


Figure 1: Nonspecific reactions of IFA disappeared after washing with 3 M NaCl.

A, Washing by phosphate buffer saline containing 0.14M NaCl; B, Washing by phosphate buffer saline containing 3M NaCl.

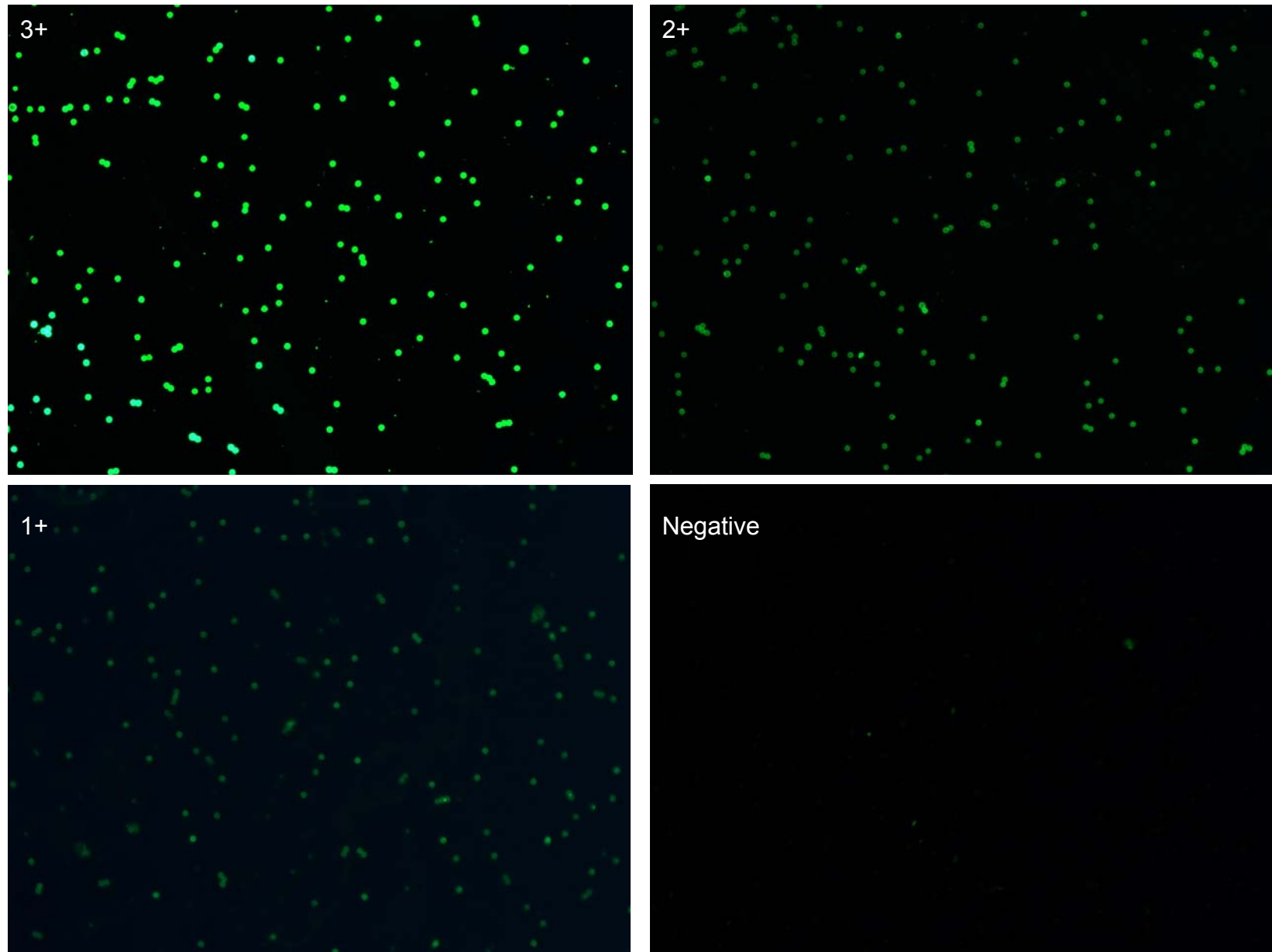


Figure 2: Appearance of latex under fluorescence microscope.

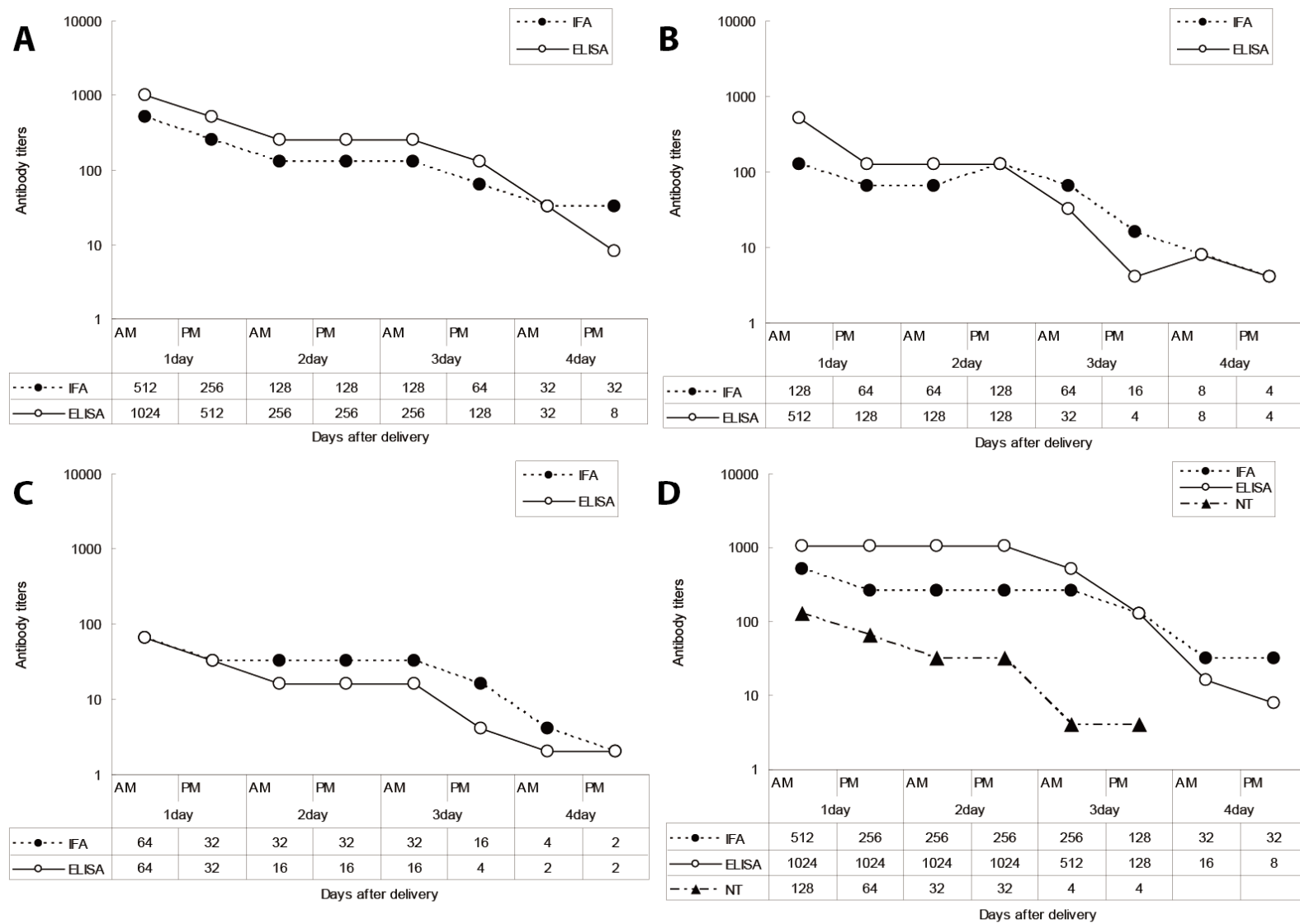


Figure 3. Changes in titers of each immunoglobulin class separated from bovine colostrum antibody against verotoxin 2 (Cow No.1). Days indicate time after delivery. A, IgG; B, IgM; C, IgA; D, γ -globulin. IFA: Antibody titers measured by IFA; EIA: Antibody titers measured by EIA; NT: Neutralization antibody titers measured using Vero cells.

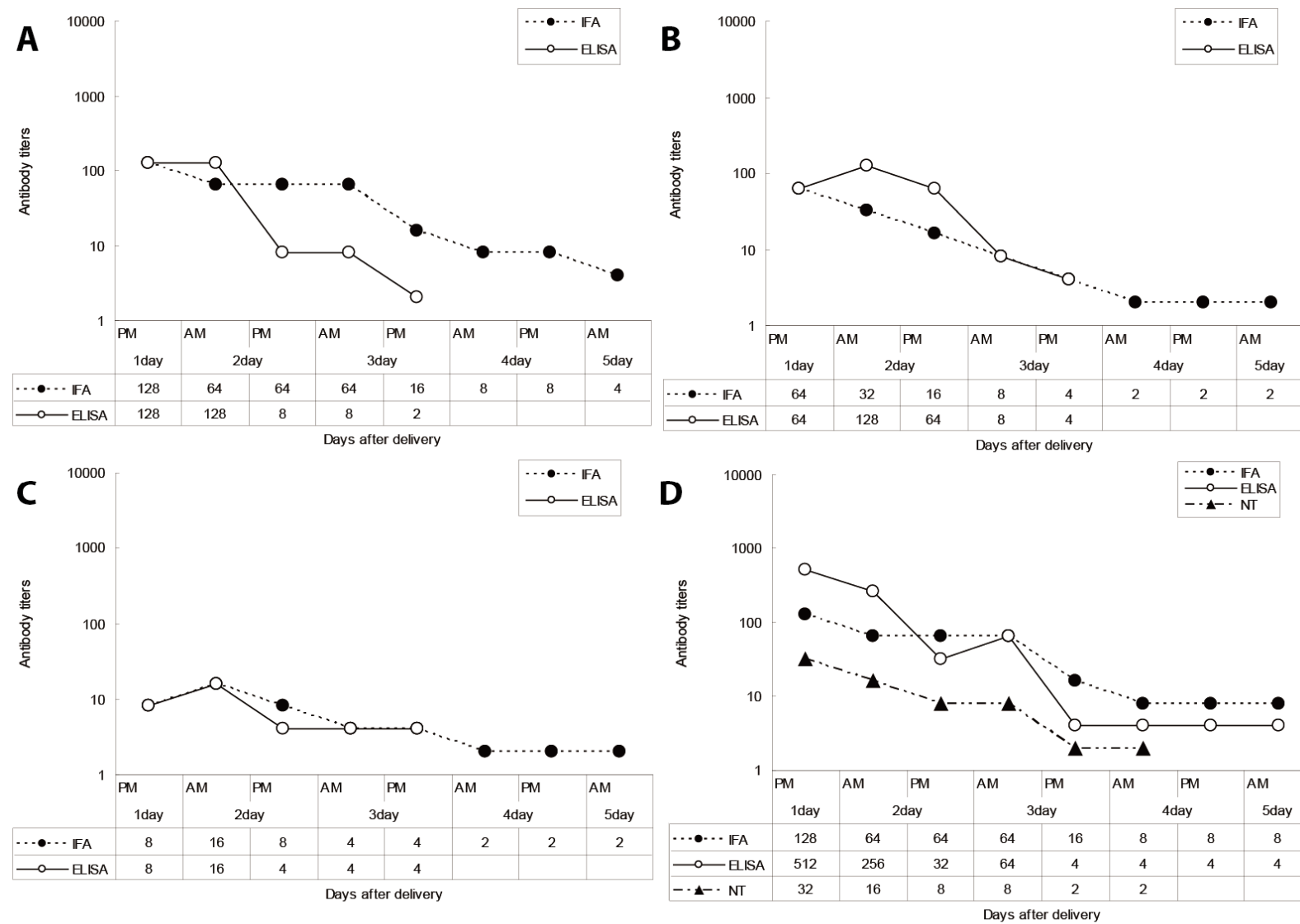


Figure 3. Changes in titers of each immunoglobulin class separated from bovine colostrum antibody against verotoxin 2 (Cow No.2). Days indicate time after delivery. A, IgG; B, IgM; C, IgA; D, γ -globulin. IFA: Antibody titers measured by IFA; EIA: Antibody titers measured by EIA; NT: Neutralization antibody titers measured using Vero cells.

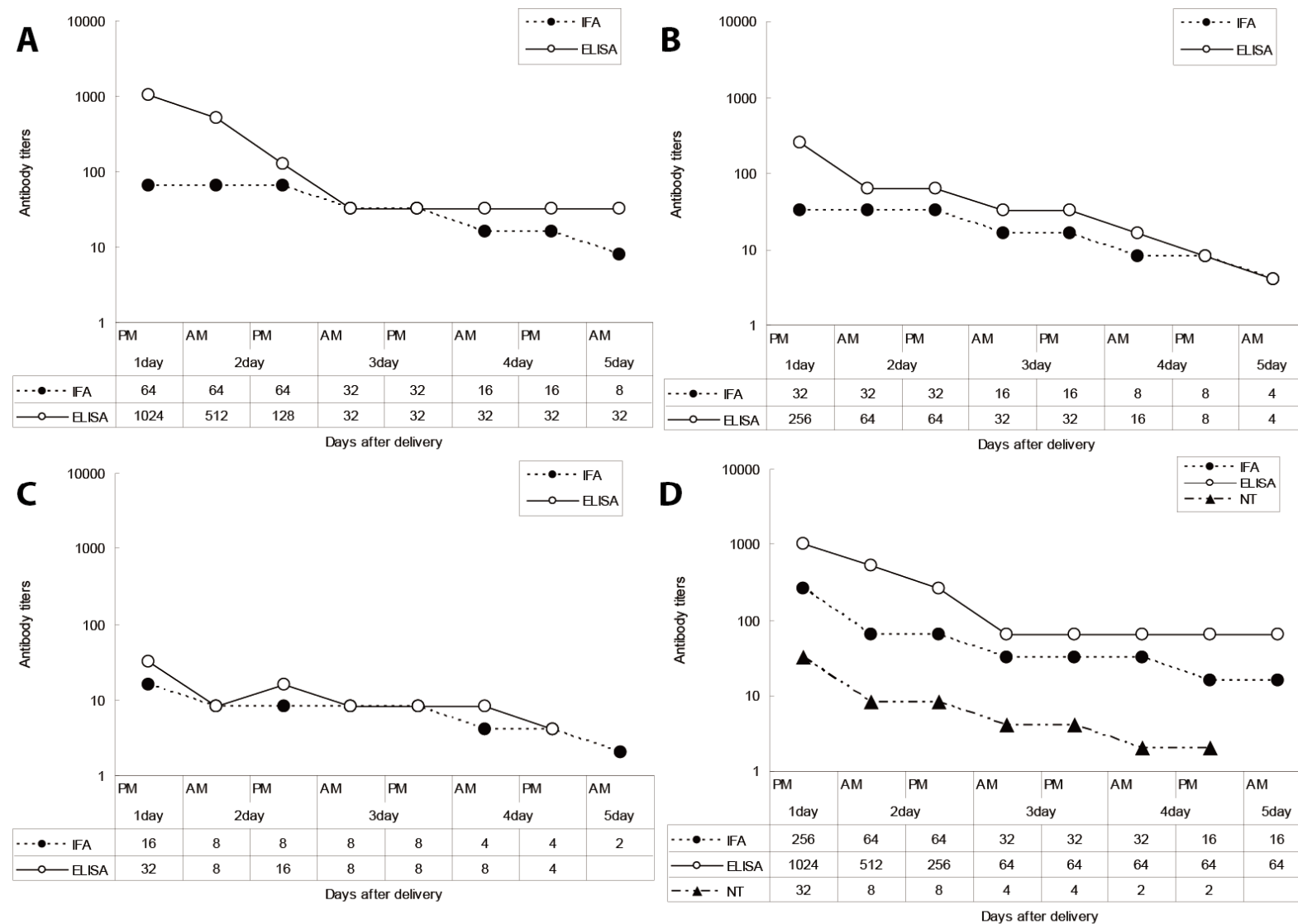


Figure 3. Changes in titers of each immunoglobulin class separated from bovine colostrum antibody against verotoxin 2 (Cow No.3). Days indicate time after delivery. A, IgG; B, IgM; C, IgA; D, γ -globulin. IFA: Antibody titers measured by IFA; EIA: Antibody titers measured by EIA; NT: Neutralization antibody titers measured using Vero cells.

Inhibition of the absorption to systemic blood of verotoxin (VT)
2 from intestine by repeatedly administration of bovine immune
colostral antibody against VT 2 in mice

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Abstract

Whether absorption of verotoxin (VT) 2 from the intestine in mice is inhibited by administration bovine immune colostral antibody to VT2 was investigated. Three-week-old mice were administered VT2 solution at 477.8 ng/ml or 955.6 ng/ml, and bovine immune colostral antibody against VT2 was then administered three times. Whey without antibody against VT2 was administered to control mice. Serum levels of VT2 were measured by fluorescence enzyme immunoassay. Serum levels of VT2 in mice administered VT2 solution at 477.8 ng/ml and bovine immune colostral antibody against VT2 scarcely changed. On the other hand, serum levels of VT2 in control mice increased and peaked at 12 hours after administration. Peak values were 15.4 ± 5.04 ng/ml. Furthermore, serum levels of VT2 at 12 and 16 hours in

control mice were significantly higher than in mice administered bovine colostral antibody against VT2. Serum levels of VT2 in mice administered antibody at 955.6 ng/ml showed no significant differences between repeated administration of bovine immune colostral antibody and controls. These results suggest that absorption of VT2 from the intestine was inhibited by repeated administration of bovine immune colostral antibody against VT2 at early stages of *E. coli* O157:H7 infection, while VT2 in the intestine remained at low levels.

Key words: mice, absorption, intestine, VT2, bovine colostral antibody

Introduction

Food poisoning caused by *Escherichia coli* (*E. coli*) O157:H7 continues to occur in Japan (Koseki *et al.*, 2011; Asano *et al.*, 2013). Treatment for this type of infection generally does not involve antibiotics (Carter *et al.*, 1987; Karch, *et al.*, 2005), as verotoxin 2 (VT2) released from *E. coli* O157:H7 killed by antibiotics induces serious complications, such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TPP) and brain damage (Ito *et al.*, 1997; Wong *et al.*, 2000; Baum *et al.*, 2005). The authors have reported the neutralizing efficacy of bovine immune colostral antibody against VT2 in mice and beagle dogs (Kuribayashi *et al.* 2006 and 2009; Seita *et al.*, 2013). We compared serum levels of VT2 between co-administration of immune colostral antibody against VT2 and saline in mice

administered VT2 (Seita *et al.*, 2013). Serum levels of VT2 were lower than in control mice after single administration of immune bovine colostrum antibody. In particular, serum levels of VT at 8 and 12 hours after administration of VT2 were significantly lower than in control mice (Seita *et al.*, 2013). However, the absorption of VT2 was not completely inhibited in this experiment. Thus, several administrations of bovine immune colostrum antibody are necessary to inhibit the absorption of VT2 from the intestine. This study therefore investigated serum levels of VT2 in mice administered immune bovine colostrum antibody repeatedly after administration of VT2.

Materials and Methods

Verotoxin 2

VT 2 was used supernatant of culture the *E. coli* O157:H7 produced VT2 isolated from human.

Mice

Male SPF ICR mice (age, 3 weeks) were purchased from Charles River Inc. (Yokohama, Japan). Mice were kept in cages at a temperature of $23 \pm 2^{\circ}\text{C}$, and a relative humidity of $55\% \pm 10\%$, on a 12/12 dark (18:00–6:00)/light (6:00–18:00) cycle with the air exchanged 12 times or more per hour. Mongolian gerbils were fed MF (Oriental Yeast Co., Ltd., Tokyo, Japan), and were allowed free access to water. All experiments were approved by the Institutional Review Board of Azabu University and were conducted in accordance with the institute's Animal Experimentation guidelines (Japanese Association for

Laboratory Animal Science, JALAS, 1987).

Animal experiments

First, the toxicity of VT2 administration was estimated. Four VT2 concentrations (955.6, 477.8, 318.5 or 238.9 ng/ml) were assessed, and four mice were administered VT2 at these concentrations. Mice were sacrificed at 16 hours after administration. Serum VT2 concentrations, hemoglobin and red blood cell counts were measured. Hemoglobin and red blood cell counts were measured by Celltac α (Nihon Kohden Corporation, Tokyo, Japan).

Mice were orally administered VT2 solution at 477.8 ng/ml or 955.6 ng/ml. Bovine colostral antibody against VT2 was given at 1 hour after administration, 3 times at 1-hour intervals

(bovine immune colostral antibody group). The control group was administered whey without antibody against VT2 instead of bovine colostral antibody against VT2. Blood was collected before and at 4, 8, 12, 16, 24, 36 and 48 hours after administration. Three mice were sacrificed for blood collection blood at each time point. Sera were obtained by centrifugation of blood at 1,610 × g for 10 min. Sera were stored at -80°C until measurement.

Measurement method for serum concentration of VT2

Serum concentrations of VT2 were measured by fluorescence enzyme immunoassay according to the procedure of Seita *et al.* (Seita *et al.*, 2013)

Statistical analysis

Data are presented as means \pm standard deviation for three mice at each time point. Statistical analysis of serum concentrations of VT2, hemoglobin and red blood cell count were performed by unpaired Student-*t* test. Differences were considered to be significant at $p < 0.05$.

Results

Determination of VT2 doses

Serum levels of VT2 were 8.2 ng/ml, 40.5 ng/ml, 2.9 ng/ml and 2.3 ng/ml at 16 hours after administration of the various test concentrations (Figure 1). Mean hemoglobin and red blood cell counts are shown in Table 1. Hemoglobin in mice administered VT2 solution at 955.6 ng/ml was significantly lower when compared to mice administered other VT2 concentrations. Red blood cell

counts in mice administered VT2 solution at 955.6 ng/ml were also significantly lower when compared to mice administered VT2 solutions at 477.8 or 318.5 ng/ml.

Changes in VT2 levels in mice

Serum levels of VT2 in mice administered VT2 solution at 955.6 ng/ml and 477.8 ng/ml are shown in Figures 2 and 3, respectively.

Serum levels of VT2 in mice administered VT2 solution at 955.6 ng/ml did not show significant differences between repeated administration of bovine immune colostral antibody and controls.

On the other hand, serum levels of VT2 in mice administered VT2 solution at 477.8 ng/ml showed little changes in the repeated bovine immune colostral antibody administration group. Serum levels in control mice increased after administration of VT2

and peak levels were observed at 12 hours after administration (Figure 2). Peak levels were 15.4 ± 5.04 ng/ml. Serum levels in control mice at 12 and 16 hours were significantly higher than those in mice repeatedly administered bovine immune colostral antibody.

Discussion

VT2 derived from *E. coli* O157:H7 in the intestine is known to induced serious complications, including HUS and brain damage, in patients infected with *E. coli* O157:H7 (Pavia, *et al.*, 1990; Clary *et al.*, 2004; Phillips *et al.* 2005; Tarr *et al.*, 2005). In infection models, mice showing intestinal bleeding died, but those not showing intestinal bleeding did not die (Kuribayashi *et al.*, 2006; Seita *et al.*, 2013). The cause of death was presumed

toxicity of VT2 absorbed from the intestine. The authors have reported that serum levels of VT2 continue to increase for 24 hours in mice administered VT2 (Seita *et al.*, 2013). Serum levels of VT2 in mice administered bovine immune colostrum antibody against VT2 were lower than those in control mice (Seita *et al.*, 2013). However, absorption of VT2 was not fully inhibited by single administration of bovine immune colostrum antibody. We presumed that serious complications were prevented by inhibiting absorption of VT2 from the intestine. Bovine immune colostrum antibodies were thus administered repeatedly in this study. Serum levels at 16 hours after administration of VT2 were highest in mice administered VT2 solution at 477.8 ng/ml. On the other hand, hemoglobin and red blood cell counts in mice administered the VT2 solution at 955.6 ng/ml were significantly lower than

in mice administered VT2 solution at other concentrations. These results suggest that severe intestinal bleeding occurred and this interfered with intestinal function. Thus, the doses of VT2 used were 955.6 ng/ml and 477.8 ng/ml in order to evaluate the inhibition of VT2 absorption in mice.

Serum concentrations of VT2 peaked at 12 hours after administration of VT2 and decreased in control mice administered VT2 solution at 477.8 ng/ml. In particular, serum levels of VT2 at 12 and 16 hours in control mice were significantly higher than in mice administered bovine immune colostral antibody repeatedly. These results suggest that absorption of VT2 from the intestine was inhibited by three-time administration of bovine immune colostral antibody. However, serum levels of VT2 were not significantly different between repeated

administration in the bovine colostral antibody group and the control group with VT2 administered at 955.6 ng/ml. It was assumed that VT2 was unable to be absorbed by the intestine to systemic circulation due to severe intestinal damage. The cause of death in mice with severe intestinal damage was considered to be bleeding.

Kita *et al.* estimated the serum levels of Shiga toxin (Stx) 1 in mice after inoculation with *E. coli* O157:H7 producing Stx 1 and 2. Peak levels of 34.8 ± 4.6 pg/ml were observed at 4 days after inoculation (Kita *et al.* 2000). Higher levels of VT2 were inhibited by repeated administration of bovine immune colostral antibody in this study. Furthermore, treatment of *E. coli* O157:H7 infection with fosfomycin at early stages prevents progression to serious symptoms (Sawamura, *et al.* 1999; Takada *et al.* 2003).

Thus, adsorption of VT2 appeared to be inhibited by administration of bovine immune colostral antibody at early stages after infection with *E. coli* O157:H7, despite VT2 levels in intestine increasing from disruption of *E. coli* O157:H7 by antibiotics. Furthermore, serious complications such as HUS or encephalopathy caused by VT2 were prevented.

Unfortunately, the mechanism of inhibition for absorption of VT2 by administration of bovine immune colostral antibody was not clarified in this study. Further study will therefore be necessary.

In conclusion, the absorption of VT2 was inhibited by repeated administration of bovine immune colostral antibody against VT2 in mice.

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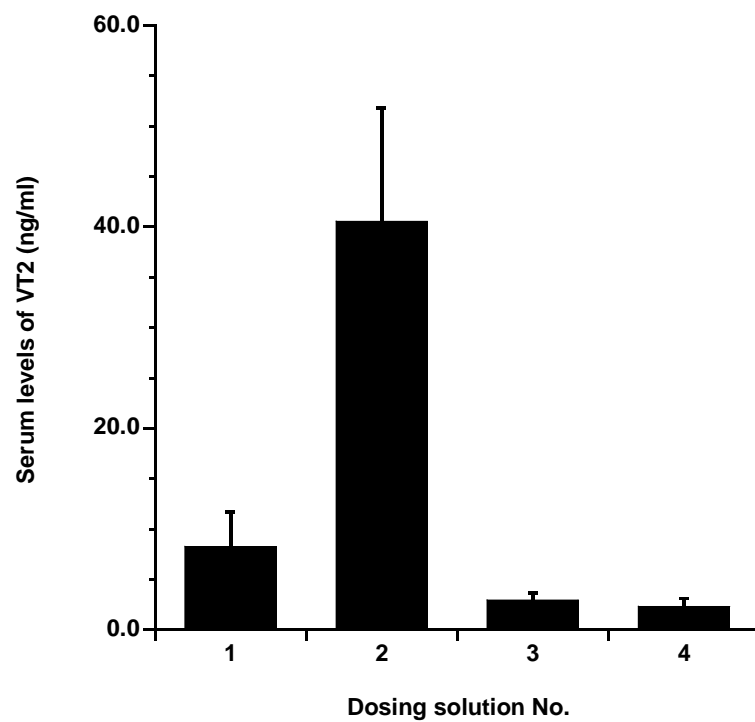


Figure 1 The serum levels at 16 hours after administered with different VT2 concentrations dosing solution to mice. Dosing solutions indicated VT2 concentration were following; 1:955.6 ng/ml, 2:477.8 ng/ml, 3:318.5 ng/ml, 4:238.9 ng/ml.

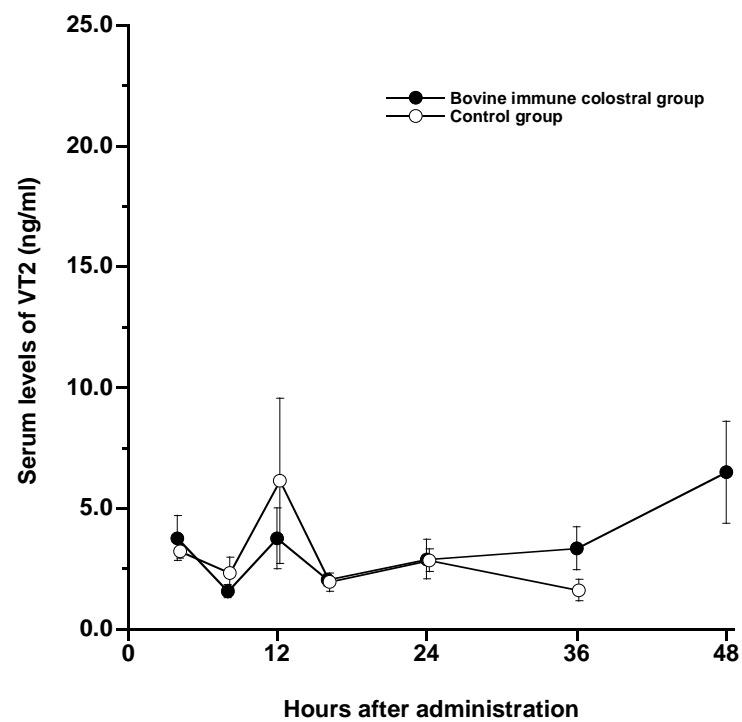


Figure 2 The serum levels of VT2 in mice administered VT2. The values at each time points represented mean \pm standard deviation (n=5).

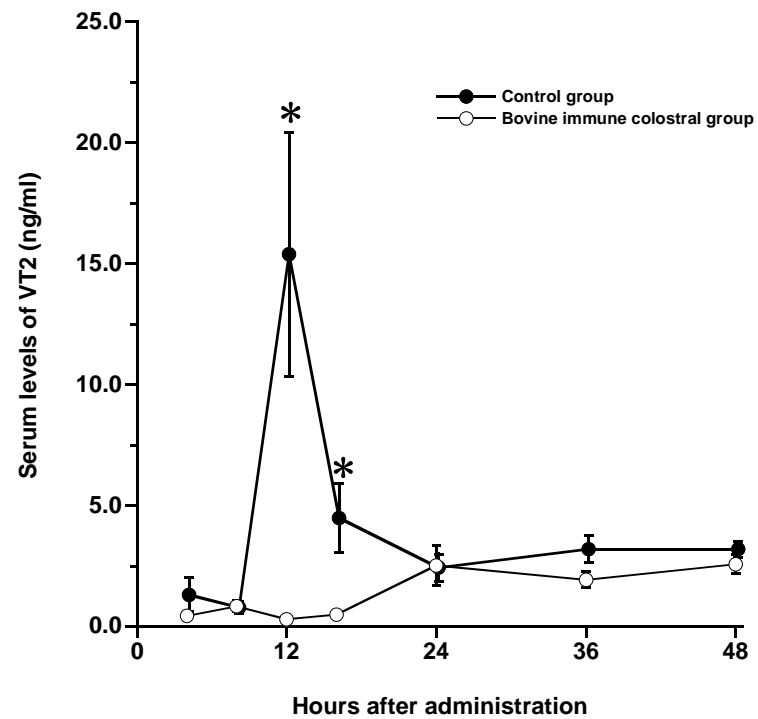


Figure 3 The serum levels of VT2 in mice administered VT2. The values at each time points represented mean \pm standard deviation (n=5). *Value differs significantly from that of 3-week-old rats ($p < 0.05$).

Table 1 Hemoglobin and Red blood cell count at 16 hours after administered with different VT2 concentrations dosing solution to mice. Each value represented mean \pm standard deviation (n=4). *Value differs significantly from mice administered with 477.8 of VT2 ($p<0.05$).

Dosing levels of VT2 (ng/ml)	Hemoglobin (g/dL)	Red blood cell count ($\times 10^6/\text{L}$)
955.6	7.2 \pm 1.8*	386.3 \pm 102.3*
477.8	10.3 \pm 2.1	549.8 \pm 82.7
318.5	11.6 \pm 2.7	609.3 \pm 124.6
238.9	9.8 \pm 1.2	502.3 \pm 69.1

Eradication effects of *Helicobacter pylori* in Mongolian gerbils by colostral antibody obtained from dairy cows immunized with *H. pylori* and its antibody with (bovine) complement compared with antibiotics

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Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative helical bacillus that was first isolated from the gastric mucosa in human patients by Warren and Marshall ¹. *H. pylori* produces urease and sustains infection in the strongly acidic stomach environment by disassembling urea into ammonia and CO₂ ^{2, 3}. Approximately half the population of Japan is considered to be infected with *H. pylori* ^{4, 5}. As such, *H. pylori* infection is called the Japanese national disease. Chronic inflammation of gastric mucosa caused by *H. pylori* infection induces gastrointestinal diseases, such as gastric or duodenal ulcer ^{6, 7}, gastric cancer ^{8, 9}, and gastric MALT lymphoma ¹⁰. Other non-gastrointestinal diseases such as idiopathic thrombocytopenic purpura have also been reported. ^{11,}

¹².

Eradication therapy for patients with known *H. pylori* infection involves several antibiotics ^{13, 14}, but three antibiotics, Omeprazole, Amoxicillin and Clarithromycin, tend to be used for treatment of patients infected with *H. pylori* ¹⁵. Treatment failure can occur with this antibiotic therapy, but the rate of bacterial eradication is 90% ^{16, 17}. The risk of drug allergy and/or increased resistance of *H. pylori* to these antibiotics have also been reported ¹⁸. Therapies for bacterial eradication in experimental animals using *Lactobacillus* ¹⁹, green tea ^{20, 21}, and cladosiphon fucoidan ²² has been reported. However, these therapies are not likely to completely eradicate *H. pylori* infection.

The efficacy of bovine immune colostral antibody has already been investigated in human diseases. For example, bovine colostral

antibody targeting rotavirus gastroenteritis is known to be particularly effective^{23, 24}. The authors have also reported that bovine immune colostral antibody has neutralizing efficacy against to verotoxin 2 derived from *Escherichia coli* O157:H7 in mice and beagle dogs²⁵. We prepared a bovine immune colostral antibody with activity against *H. pylori* by immunizing dairy cows before delivery.

Mongolian gerbils are an experimental model of *H. pylori* infection²⁶. The effectiveness of immune colostral antibody and its antibody with bovine complement to eradicate *H. pylori* infection was thus investigated in Mongolian gerbils in this study.

Materials and methods

H. pylori

An *H. pylori* strain isolated from human patients was donated by Professor Yasuhiro Koga, Tokai University School of Medicine, and was used in this study. *H. pylori* was cultured according to the procedure of Watanabe *et al.*²⁷

Bovine immune colostral antibody

A pregnant dairy cow (age, 8 years), 3 months prior to delivery, was used for preparation of immune colostral antibody against *H. pylori*. Colostrum was collected for 3-5 days after delivery. Skim milk was obtained by centrifuging colostrum in order to remove the fat. One hundred milligrams of RENNET (ICN Biomedicals, Aurora, OH) was added to 1 l of this skim milk. Colostrum was

obtained from skim milk by centrifugation.²⁸

Experimental animals

One hundred and one Specific Pathogen Free (SPF) Mongolian gerbils (Japan SLC Inc., Shizuoka, Japan) aged 5-10 weeks were housed in an air-conditioned room with a 12-h light-dark cycle. They were fed normal diet and had free access to water until the start of experiment. All Mongolian gerbils were deprived of food beginning at 18 hours before experiments. All experiments conformed to Japanese regulations concerning animal care and use, as laid out in the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987), and were approved by the Institutional Animal Care and Use Committee of Azabu University.

Animal experiments

Inoculation of *H. pylori*

Mongolian gerbils were orally inoculated with 1 ml of BHI culture fluid suspension of 5.0×10^7 *H. pylori* after administration of 0.1% sodium carbonate in order to raise internal stomach pH. This was performed once per day over a period of two days. Sera were collected after 2 weeks of inoculation and IgG and IgM titers against *H. pylori* were measured by enzyme linked immunosorbent assay (ELISA). Mongolian gerbils confirmed to be infected with *H. pylori* by increased antibody titers were used for subsequent *H. pylori* eradication experiments.

Administration of antibody, complement and medications

Mongolian gerbils were orally administered with 0.3 ml of 0.1% sodium bicarbonate solution to increase the pH in the stomach at 10 minutes before administration of colostral antibody. Subsequently, Mongolian gerbils were orally administered with 0.5 ml of colostral antibody against *H. pylori*. Whey not including antibody against *H. pylori* was administered to control Mongolian gerbils instead of colostral antibody against *H. pylori*. Colostral antibody against *H. pylori* or whey was administered to Mongolian gerbils twice a day for one month or two months. Mongolian gerbils were orally administered Omeprazole, a proton pump inhibitor, and the antibiotics Clarithromycin and Amoxicillin at a dose of 10 mg/kg or 20 mg/kg body weight twice a day for 7 days in order to compare the efficacies between standard therapies for humans. These drug substances were

dissolved in 0.5% methylcellulose 400 (Wako Pure Chemical Inc., Osaka, Japan).

In order to study the bactericidal effects of complement, Mongolian gerbils were orally administered 0.5 ml of colostral antibody and complement twice a day for 2-3 days. Bovine serum was incubated at 56°C for 30 minutes in order to inactivate complement. Then, inactivated complement was administered to control Mongolian gerbils.

Culture from the stomach

One month after finishing administration experiments, stomachs from euthanized gerbils were extracted and homogenized with a 9-fold volume of PBS. Homogenates were diluted and 10 µl was cultured. *H. pylori* was cultured according to the procedure of

Result

IFA using colostral antibody, fluorescence was seen on both the membrane and flagella of *H. pylori* (Fig. 1). Animal experiments were performed using this colostral antibody.

Bacterial eradication rates in the groups administered drug at 10 mg/kg and 20 mg/kg were 92% (11/12) and 100% (12/12), respectively. Eradication rates in the groups administered colostral antibody for one month and two months were 83% (10/12) and 92% (11/12), respectively. On the other hand, the eradication rate was 0% (0/6) in the group administered whey without colostral antibody, and *H. pylori* was detected in the stomach. In Mongolian gerbils administered complement and colostral antibody, in

groups administered medication twice a day or for three days, the eradication rates were 100% (12/12) and *H. pylori* was not detected in the stomach. On the other hand, in groups administered medication twice a day for two days, the eradication rate was 83% (10/12) and, the rate was 17% (1/6) in the control group given inactivated complement.

Colonies grown from the gastric specimens were subjected to urease test, and the results confirmed that all colonies were *H. pylori*. These results were subsequently confirmed by CLO test.

Discussion

H. pylori induces gastritis, ulcers and gastric cancer ^{8, 9} and it is recommended to eliminate the bacterium. Treatment with antibiotics is the currently standard therapy to eliminate

bacteria in various countries, including Japan ³⁰. However, treatment with antibiotics involves risks such as appearance of side effects, drug allergy and bacterial resistance. Moreover, some cases of difficult to eliminate bacteria have been reported. The number of patients infected *H. pylori* is estimated to be thirty million in Japan; thus, *H. pylori* infection in Japan cannot be overlooked.

It is known that colostral whey contains a rich S-IgA, which is resistant to protease enzymes and is often present at levels similar to IgG. The authors have reported improved survival rates in mice treated with verotoxin or inoculated with *Escherichia coli* O157:H7 after administration of bovine immune colostral antibodies against verotoxin ^{25, 28}. We estimated the eradication of *H. pylori* in Mongolian gerbils by administration of bovine

immune colostral antibody in this study. Fluorescence was observed on both the membrane and flagella using IFA, and colostral antibody obstructed adhesion.

Disinfection rates were evaluated at one month after completing administration of bovine immune colostral antibody or both antibiotics and PPI in this study. Disinfection of *H. pylori* in humans was evaluated at one month after the end of treatment with drug therapy in order to confirm that *H. pylori* did not increase after the end of drug therapy. Elimination of *H. pylori* in Mongolian gerbils was evaluated after the administration period.

The elimination rates by administration of antibiotics and PPI were similar to those in previous reports. These elimination rates were similar to those with administration of bovine colostral antibody for 1 or 2 months. Thus, *H. pylori* disinfection

by bovine colostral antibody without antibiotics was satisfactory. However, the treatment periods are longer than with standard drug therapy. Therefore, we evaluated the synergistic efficacy of bovine immune colostral antibody and complement.

Administration of bovine immune colostral antibody and complement for three days resulted in complete eradication of *H. pylori* in Mongolian gerbils. The bovine immune colostral antibody was also able to avoid allergic responses and induction of drug resistance. The disinfection efficacy for *H. pylori* by bovine immune colostral antibody indicates its utility as a novel treatment for *H. pylori* infection.

Sterilization with continuous oral treatment for 1 and 2 months with immunized colostral antibodies can give a successful

eradication rate of 83% and 92%, respectively, and antibody alone has an eradication rate of 92% with 2 months of continuous administration. However, when complement and colostral antibodies are administered both at the same time, twice a day for 3 days, 100% eradication was achieved. It is known that complement has a strong bactericidal action, but bacterial growth is not stopped with antibody alone or inactive complement. Sterilization with bovine immunized colostral antibodies and complement showed a high eradication rate in experimental animals. In contrast to antibiotics, there is a lower risk of creating resistant strains, and it is expected to be effective against previously reported resistant strains. The appearance of resistant bacteria is causing problems in the eradication treatment of *H. Pylori*. Bovine immune colostral antibody or

complement with immune colostral antibody can be utilized on a daily basis, except in those who are allergic to milk. Furthermore, the method of combining complement and immune colostral antibody is useful because it can eradicate *H. pylori* in a very short period of time. Therefore, this combination treatment is a good alternative to antibiotics.

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Table 1 Eradication rate of *H. pylori* infected Mongolian gerbils treated with three medications

Medications		Duration of administration	Eradication rate
Agent	Dose(s) (mg/kg)		
OPZ, CAM, AMPC	10, 10, 10	7 days	92% (11/12)
OPZ, CAM, AMPC	20, 20, 20		100% (12/12)

Table 2 Eradication rate of *H. pylori* infected Mongolian gerbils treated with bovine immune colostral antibodies

	Duration of administration	Eradication rate
Bovine immune colostral antibody administration group	2 months	92% (11/12)
	1 months	83% (10/12)
Control colostral administration group	2 months	0% (0/6)
	1 months	0% (0/6)

Table 3 Eradication rate of *H. pylori* infected Mongolian gerbils treated with bovine immune colostral antibodies

	Duration of administration	Eradication rate
Bovine immune colostral antibody and complement administration group	3 days	100% (12/12)
	2 days	83% (10/12)
	1 day	40% (2/5)
Bovine immune colostral antibody and inhibited complement administration group	3 days	17% (1/6)
	2 days	17% (1/6)

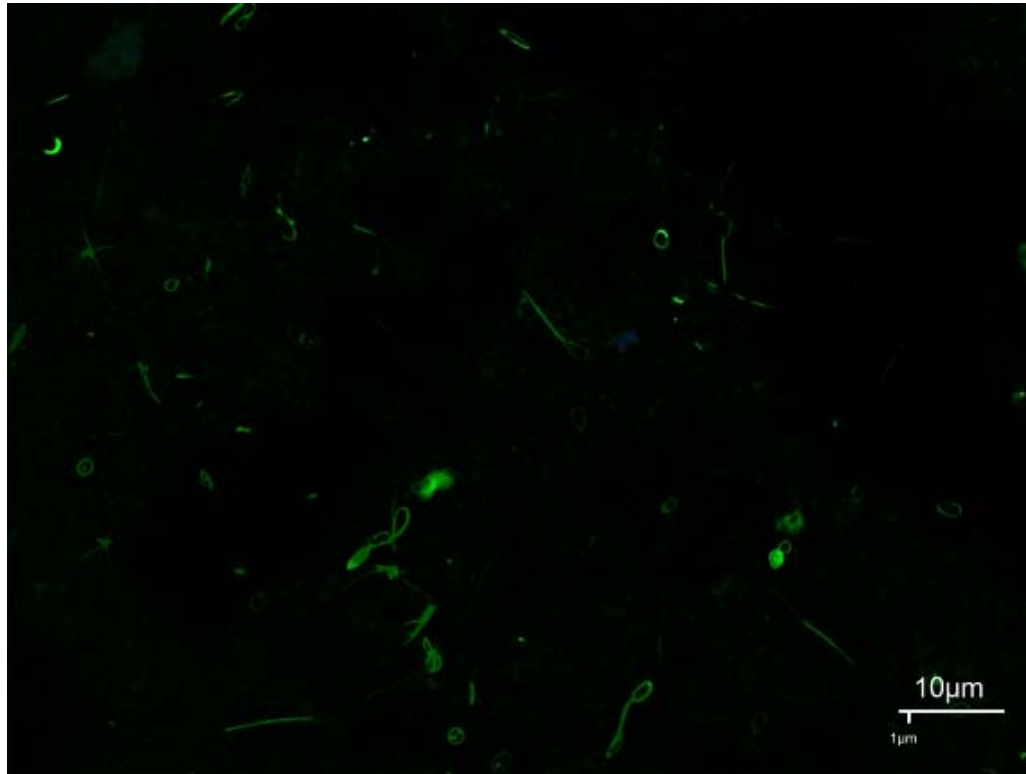


Figure 1 Specificity of bovine immune colostral antibody against *H. pylori* using the IFA method. Flagellum and somatic shows fluorescence

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