RAPID DIAGNOSIS OF FOOT AND MOUTH DISEASE IN ACUTE AND CARRIER STATES

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SUMMARY

Rapid diagnosis of Foot and Mouth Disease (FMD) is of great importance in the prevention and control of FMD. A definitive diagnosis is based on detection of virus in fluids or epithelium from vesical lesions or esophageal-pharyngeal fluid (OPF). In this study, vesical samples were subjected for negative staining electron microscopy (EM) to exclude other vesicular diseases affecting cattle. They were also subjected for FMD virus antigen detection and typing enzyme linked immunosorbent assay (ELISA) kit, Staphylococcus aureus protein A coagglutination test (SpA-COAT) and positive samples were 77.78% and 92.9% respectively. The samples detected negative by ELISA and positive by SpA-COAT were subjected for co-agglutination clumping ultrastructure analysis and they were detected positive. Sensitivity and specificity of ELISA kit and SpA-COAT were conducted using the gold standard, isolation followed by ELISA kit. Results were 77.78% and 100% for ELISA and 96.3% and 100% for SpA-COAT. Hundred OPF samples were subjected for SpA-COAT and isolation / ELISA and the samples detected positive were 8 and 10 respectively. SpA-COAT, because of its simplicity and its low cost, it could be used for detection of FMD virus (alternatively with ELISA) as a rapid, accurate, specific, sensitive, economic and screening test at the site of FMD outbreaks. It can be used for detection of carriers in parallel with tests detecting non structural proteins (NSP) antibodies.

INTRODUCTION

Foot and mouth disease (FMD) is the most contagious disease of animals and has a great potential for causing severe economic loss in susceptible cloven-hoofed
animals (Fenner et al., 1993).

FMD is probably the world's most important animal disease, although its low mortality. Losses occur in many ways. In addition to loss of production, the expense of eradication and the interference with movement of livestock and meat between countries are the most important effects. (Radostits et al., 2007).

FMD is caused by single stranded RNA virus (Huang et al., 2009) of the genus Aphthovirus, family Picornaviridae. There are seven serotypes of FMD virus, namely O, A, C, SAT1, SAT2, SAT3 and Asia 1 (Fenner et al., 1993). There are more than 65 antigenically different subtypes (Donia and Youssef, 2002).

Following recovery from the acute stage of infection, infectious virus disappears from all secretions and excretions with the exception of ruminants, of those of oesophageal-pharyngeal (OP) origin (OIE, 2004).

Rapid diagnosis of FMD is of great importance in the prevention and control of FMD. (Crowther, 1977).

Electron microscopy (EM) can be used for rapid diagnosis. (OIE, 2004), but it is expensive, needs special training and it is not always available.

A presumptive clinical diagnosis associated with laboratory tests such as serology, virus isolation, and antigen detection. Serological tests are not the techniques of first choice to detect an acute infection (Sutmoller et al., 2003). A definitive diagnosis is based on detection of virus in fluids or epithelium from vesicular lesions or esophageal-pharyngeal fluid (OPF) collected with a probang (OIE, 2008).

Complement fixation test (CFT) has been used for diagnosis of FMD in vesicular epithelial samples from suspected animals. (Roeder and Le Blanc Smith, 1987), but it has been replaced in many laboratories by enzyme linked immunosorbent assay (ELISA), as it is more specific and sensitive (OIE, 2004) and it is not affected by pro- or anticomplementary factors.

Virus isolation is the most reliable diagnostic method although it is labor-intensive and time-consuming. ELISA is rapid test to detect viral antigens, but it has low sensitivity, so its primary indication is to confirm and type FMD after isolation in cell culture (Rémond et al., 2002).

Reverse transcriptase polymerase chain reaction (RT-PCR) are now standard methods for virus detection and typing. (Radostits et al., 2007) but great care must be taken to avoid contamination of
samples with extraneous nucleic acids which may lead to false-positive results and mutations in the primer target region may negate the effectiveness of primers. Although real-time RT-PCR is a rapid and sensitive for detection of FMDV (Paixão et al., 2008), it is only capable of identifying the presence of genomic material for previously identified agents. (Hazelton and Gelderblom, 2003). Furthermore, PCR will not identify subviral components such as empty virions, which may be produced late in the infection. Electron microscopy (EM), on the contrary can detect such viral components readily (Green et al., 2002).

The cell wall of Staphylococcus aureus containing protein A binds the Fc fragment of IgG from most mammalian species. This bacterium has been used as solid phase immunosorbent assay for the detection of bacterial and viral antigens in a form of agglutination assay called coagglutination test (COA test). (Herbert et al., 1981 and Durigon et al., 1991). SPA-COAT is based on the ability of SpA to bind Fc fragments of immunoglobulin G (IgG) of different mammalian species. When specific antibodies are added to a stabilized suspension of Staphylococci, the antibodies bind to the SpA located on the cell wall, thus orientating the Fab-located IgG-combining sites outwards and, after mixing with homologous antigens, coagglutination occurs after 5 minutes (Kronvall et al., 1970).

The COAT has proved to be an efficient serological tool for the detection of FMD viruses in infected animal cells as a fast, simple and inexpensive alternative method for the direct diagnosis of these viruses. (Montassier et al., 1994).

Detection of carriers based on the detection of FMD in OPF (OIE, 2004) or detection of antibody to the non structural proteins (NSP) which results from viral replication (Bergman et al., 1993) but false results may develop if tested animals were repeatedly vaccinated with not properly purified vaccine which contain traces of NSPs (Mackay, 1998, OIE, 2004 and Iman et al., 2005).

In this study, samples were subjected for negative staining EM, isolation in BHK-21 cells, SpA-COAT and antigen detection and typing ELISA Kit. Comparison was made between antigen detection and typing ELISA Kit and SpA-COAT for the direct diagnosis of FMD in vesical samples with the characterization of sensitivity and specificity for both techniques. SPA-COAT was used also for detection of carriers for FMDV in OPF samples and comparing its results with virus isolation fol-
ollowed by antigen detection and typing ELISA.

**MATERIAL AND METHODS:**

**Animals:**
A total number of 128 Holstein breed cattle of both sexes and of ages ranged between 3 to 8 years old belonging to private farms in Fayoum and El-Beheira governorates were subjected to this study. Twenty-eight animals have been suffered from fever (41°C), lameness, vesicles in the mucosa of the mouth, tongue and lips and on the coronary band between claws (group 1). The other 100 animals with history of FMD 2 to 3 months ago (group 2).

**Samples:**
Twenty-eight epithelial vesicular samples (intact vesicles or tags of epithelium from the edges of ruptured vesicles) were taken from the tested animals (group 1) and maintained in glycerol phosphate buffer (50% glycerol, v/v), pH 7.6. A 20% suspension of sample (w/v) was prepared in Phosphate Buffer Saline (PBS), pH 7.4, by grinding in a mortar with sand and centrifuged, and the supernatant was used for testing purposes. 100 oesopharyngeal fluid (OPF) samples obtained with probang cup from cattle, 2 to 3 months following FMD outbreak (group 2) were subjected also, for this study.

**Viruses:**
The FMDV strains O and A of a titer 10⁷ TCID₅₀/ml previously isolated from Egypt, 2006 obtained from virology department, Animal Health Research Institute, Dokki, Cairo were used as positive control in the tests.

**Antisera:**
Positive antiserum against FMD virus of both types O and A (bivalent antiserum) were prepared in guinea pigs according to the procedures described by Montassier et al. (1994) using bivalent FMD vaccine containing 50% of both of FMD virus type O and type A which was obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo.

**Tissue Culture:**
Baby hamster kidney (BHK-21) cell culture obtained from virology department, Animal Health Research Institute, Dokki, Cairo was used.

**Staphylococcus aureus suspension:**
It was used for COAT and is locally prepared according to (Kessler, 1975) was supplied by Reproductive Research Institute El-Haram, Giza.

**Antibody coating of Staphylococcus:**
The CoA-anti-FMD virus conjugate was prepared according to Montassier et al. (1994).
Diagnostic Methods:

Virological examination:

A) Negative Staining electron microscopy (TEM):

Negative staining of epithelial vesical samples was conducted according to Payment and Trudel (1993). Samples were minced and then ground in a sterile mortar with a pestle. A small volume of PBS (pH 7.4) was added and the aqueous material was collected and centrifuged again for 15 min. at 5000 rpm. The supernatant was collected and 4 were pooled as one sample. A droplet of 3% phosphotungstic acid (PTA) was mixed with a droplet of the sample and a copper grid coated with carbon formvar was dipped into the mixture. After drying, the grid was examined with the EM.

Coagglutination test:

The test was performed according to Montassier et al., (1994). Twenty-five ml of the clarified vesical sample suspension were mixed with an equal amount of COA anti-FMD conjugate (prepared by mixing staphylococci suspension with guinea pig FMDV bivalent antiserum against FMD virus types O and A and control COA mixture was also prepared with normal inactivated guinea pig serum) on a glass surface and the mixture was gently rocked for up to 5-10 min. at room temperature. Each sample was tested in duplicated with the control and the anti-FMD type O and A conjugates. The agglutination pattern was read macroscopically.

FMDV co-agglutination clumping ultra-structure analysis:

A drop obtained 5 min. after mixing 20 ml of the 1:10 dilution of anti FMDV type O and A COA conjugate with an equal amount of sample homogenate supernatant was examined by negative staining EM using 1% potassium phosphotungstate by the procedure of Payment and Trudel (1993).

Antigen detection and typing ELISA Kit:

Antigen detection and typing ELISA Kit (Pirbright laboratory) described by Roeder and Le Blanc Smith (1987) and OIE (2004) for the detection and typing of FMD viruses. Rabbit antisera specific for the seven serotypes of FMDV are passively adsorbed to polystyrene microwells. With the addition of test sample, antigen (if present) is trapped by the immobilized antibodies. Specific guinea pig anti-FMD detecting antibodies are then added which react with the trapped antigen. The bounded guinea pig antibodies are detected by means of the rabbit anti-guinea pig Ig conjugated to horse radish peroxidase. With the addition of substrate / chromogen solution, a colour product develops which may be measured and interpreted with respect to the antigen content.
Virus isolation:
Samples were subjected for inoculation onto BHK-21 cell culture according to (OIE, 2004). The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE to another 48 hours. When CPE appeared in the cultures, the fluids were subjected for confirmation and typing by FMDV antigen detection and typing ELISA Kit.

Calculation of sensitivity and specificity:
The performed COAT in this study was compared with the ELISA Kit for detection and typing of FMDV and measuring its sensitivity and specificity with virus isolation followed by FMDV antigen detection ELISA (gold standard) according to Vanrompay et al., (1994) and the following formula were used.

Sensitivity = \[\frac{TP}{TP + FN}\] x 100, where TP is a true – positive result as determined by the gold standard and FN is a false – negative result.

Specificity = \[\frac{TN}{TN + FP}\] x 100, where TN is a true-negative result as determined by the gold standard and FP is a false-positive result.

RESULTS
Animals were showing ropy salivation (Fig.1). Some of the animals showed vesicles in the mucosa of the mouth, tongue and lips, gum and on the coronary band. Others showed ruptured vesicles (erosions). (Fig. 2 and 3).

Results of virological examination:

Results of TEM:
All the 7 pools obtained from the 28 vesical samples (group 1) were positive for picornaviral particles. (Fig. 4).

Results of SPA-COAT:
Typical macroscopic patterns of positive and negative coagglutination reactions obtained from the interaction between the homogenate supernatants of the 28 vesical samples (group 1) and 100 OPF samples (group 2) with positive (bivalent) and negative sera of guinea pigs against FMDV type O and A conjugated with SPA. 26 vesical samples and 8 OPF samples have been reacted with guinea pig serum against FMDV type O and A conjugated with SPA (Fig. 5, Table 1, 2 and 4).

Results of FMDV antigen detection ELISA Kit:
Seventeen vesical samples were detected FMDV type O and 4 vesical samples were detected FMDV type A (Table 2) and after inoculation on BHK-21 cell cultures, the result was 22 FMDV
type O and 5 FMDV type A.

**Results of SPA-TEM:**
Vesical samples detected positive by SPA COAT and negative by antigen detection and typing ELISA Kit was confirmed electron microscopically and results with negative staining EM showed aggregate of picorna viral particles coated with FMDV antiserum closely attached to surface of staphylococcus aureus. (Fig. 6).

**Results of isolation in BHK-21 cell culture:**
Twenty seven vesical and 10 OPF samples were positive for virus isolation as indicated by CPE in BHK-21 cells in the form of cell destruction (Fig. 7 and table 2 & 4).

Comparison between results of SPA COAT and ELISA Kit for detection of FMDV in the tested samples using the gold standard (isolation in BHK-21 cells and antigen detection ELISA kit) was shown in Fig. 8.

**Sensitivity and specificity of the ELISA kit and SPA-COAT for detection of FMDV in vesical and OPF samples:**
The sensitivity and specificity of SPA-COAT were calculated against isolation in BHK-21 / ELISA and they were 96.3% and 100% respectively. Sensitivity and specificity of ELISA Kit were 77.78% and 100% respectively (Table 3).

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Fig. (1): Cattle showed ropy salivation
Fig. (2): Ruptured vesicle (erosion) developed on the gum.

Fig. (3): Ruptured vesicle (erosion) developed on the coronary band.
Fig. (4): The virion is roughly spherical where the capsid appeared smooth and round in outline. It consists of core of tightly coiled RNA surrounded by a protein shell. (70000 x)

Fig. (5): Agglutination pattern obtained from the mixture of CoA-anti FMDV conjugate with vesical sample extract (right) and pattern of the negative reaction with the same CoA-conjugate and the non-infected BHK-21 cell culture extract (left).
Fig. (6): Electron micrograph of the mixture of CoA-anti FMDV conjugate with its homologous antigen. The arrows show viral particles closely attached to the S. aureus surface.
A: (14000 x)  B: (70000 x)

Fig. (7): Inoculated BHK-21 cells showed complete destruction of the cells (CPE)
Fig. (8): Percentage of FMDV positive samples detected by ELISA Kit, SPA COAT and isolation in BHK-21 cells followed by identification and typing by ELISA.

Table (1): Percentage of positive vesical samples detected by SPA-COAT, ELISA kit and isolation in BHK-21 cells/ELISA kit.

<table>
<thead>
<tr>
<th>The test</th>
<th>Positive samples</th>
<th>Negatives samples</th>
<th>% of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation / ELISA kit</td>
<td>27</td>
<td>1</td>
<td>96.4</td>
</tr>
<tr>
<td>ELISA Kit</td>
<td>21</td>
<td>7</td>
<td>77.78</td>
</tr>
<tr>
<td>SpA COAT</td>
<td>26</td>
<td>2</td>
<td>92.9</td>
</tr>
</tbody>
</table>
Table (2): Results of SPA-COAT in comparison with the ELISA kit for detection and typing of FMDV in vesical samples using the gold standard.

<table>
<thead>
<tr>
<th>Number of vesical samples</th>
<th>Result of test</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isolation / ELISA</td>
<td>ELISA kit</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table (3): Sensitivity and specificity of SpA COAT in comparison with the ELISA Kit using isolation in BHK-21 cells/ELISA kit (The gold standard method).

<table>
<thead>
<tr>
<th>Gold Standard</th>
<th>Test</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation in BHK-21 cells followed by ELISA kit for antigen detection</td>
<td>ELISA Kit</td>
<td>21/21+6 x 100 = 77.78 %</td>
<td>6/6+0 x 100 = 100 %</td>
</tr>
<tr>
<td></td>
<td>SPA-COAT</td>
<td>26/26+1 x 100 = 96.3 %</td>
<td>1/1+0 x100 = 100 %</td>
</tr>
</tbody>
</table>

Sensitivity = [TP/(TP + FN)] x 100
Specificity = [TN/(TN + FP)] x 100

Table (4): Results of OPF samples detected with SPA-COAT compared to virus isolation followed by antigen detection ELISA kit (gold standard)

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPA-COAT</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>Virus isolation / ELISA</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>
DISCUSSION

FMD is feared by farmers and Veterinary authorities because of its highly contagious nature and the drastic measures required to eradicate the infection. Also, FMD is the major constraint to international trade in livestock and animal products. (Alexandersen et al., 2002).

The need to identify FMD is of paramount importance in all countries even in those countries where it occurs enzootically. (Radostits et al., 2007).

Due to the economic and political significance of FMD and its similarity to other vesicular diseases, a rapid definitive diagnosis is essential. In countries where general vaccination is practiced every year, outbreaks are usually caused by the importation of carrier animals or infected meat (Radostits et al., 2007). Animals which harbor FMDV in the OP for more than 28 days after infection are referred to as carriers. (OIE, 2004).

Vesicular stomatitis (VS) and bluetongue (BT) may present a problem in differentiation with FMD in cattle (Radostits et al., 2007). So EM examination of lesion material is useful to differentiate FMD from disease caused by other viruses (OIE, 2004).

The current study revealed picorna virus particles in the pooled vesical samples examined by EM. These results exclude the other vesicular diseases caused by viruses in cattle (Radostits et al., 2007). Therefore, negative staining EM may be concentrated on infectious disease emergencies. The "open view" of electron microscopic testing allows rapid detection of viruses and other agents if sufficiently high particle concentration exist. Because of this capability, EM must be a frontline method (Green et al., 2002).

The use of negative staining EM has the advantages of ease of sample preparation and rapid analysis (same day results) and high specificity. Furthermore, it can detect antibody-bound viruses which are failed to be detected in the immunological methods on late samples (Reynolds et al., 1984).

Vesical samples were subjected for examination and typing with ELISA kit due to ELISA is the preferred method for the detection of FMD viral antigen and identification of viral serotype (OIE, 2004). 21 (77.78%) only were detected FMDV types O and A. ELISA, detecting all seven serotypes of FMDV in parallel, and it is able to produce a result within a few hours if sufficient antigen is present (Rémond et al., 2002). Also, ELISA can be carried out using inactivated antigens, less restrictive biocontaminant facilities can be used compared to virus
neutralization, and these results are in agreement with those obtained by Shaw et al. (2007) who stated that ELISA of a suspension of vesicular epithelium detects about 70-80% of FMDV-positive samples.

When samples were subjected for isolation before testing with the ELISA Kit, 27 (96.4%) were detected FMDV types O and A. These results may be due to some of the viral particles were bound to neutralizing antibody (Ireland and Binepal, 1998) and the free particles were inadequate and when subjected for isolation were amplified to achieve levels that allow detection by the ELISA Kit (Al-Yousif et al., 2001 and OIE, 2004). So our results are in agreement with those obtained by Rémond et al. (2002) who found that ELISA has low sensitivity, so its primary indication is to confirm and type the FMDV after isolation in cell culture.

However, ELISA can not be used directly on OPF samples and this must be passaged in cell culture before testing, adding a delay (OIE, 2004). It also has some drawbacks such as requirements for relatively sophisticated instrumentation and technical training that are not always avialble (Hamblin et al., 1984).

In this study these vesical samples were subjected for SpA COAT for identification of FMD virus only and not serotyping to avoid cross-reactions and results were 26(92.9%) for FMDV. Also, SPA-COAT detects FMDV in 8 (80%) of positive OPF samples detected by isolation / ELISA. These results were in agreement with those obtained by FAO (2000).

SPA-COAT compared to ELISA Kit was more sensitive and specificity of SPA COAT was detected with electron microscopy for the ELISA negative and positive SPA COAT. These results were in agreement with those obtained by Peshev et al. (1996).

Although guinea pig anti-FMDV sera might have antibodies to bovine Ig and BHK-21 cell proteins (Roeder and Le Blanc Smith, 1987), these antibodies did not react in the COA-test, probably due to the co-agglutination is a secondary binding immunological test. Thus, before co-agglutination takes place, an ideal number of antibody coated S. aureus particles must combine with an ideal concentration of specific soluble antigens, cross-linking them resulting in their clumping. On the other hand, ELISA, a primary serological test, detects directly the interaction between antibody and antigen, even when they are at very low concentrations (Tizard, 1982).

Sensitivity and specificity of SPA COAT (compared to ELISA Kit before isolation) for detection of FMDV in vesical samples were
achieved with the results of isolation / ELISA which is the gold standard method (Al-Yousif et al., 2001). SPA COAT was highly sensitive and specific on identification FMDV under laboratory and field conditions and these results were in agreement with those reported by Montassier et al. (1994). SPA COAT can be carried out in 5-10 min. and does not require special equipment. These results are in agreement with those stated by other investigators, using SPA COAT for the diagnosis of various diseases (Kronval, 1973; Zhang et al.; 1989; Durigon et al., 1991 and Peshev et al., 1996).

In conclusion, SPA-COAT could be used for detection and identification of FMDV (alternatively with ELISA) as a simple rapid, accurate, specific, sensitive, economic and screening test at the site of FMDV outbreaks. Also it can be used for detection of carriers in parallel with tests detecting NSP antibodies. Furthermore study must be made for preparing (polyvalent) antiserum against the 7 serotypes of FMD virus to identify the disease in field or laboratory.

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التشخيص السريع لمرض الحمى القلاعية في الحالات الحادة والحاملة للمرض

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الملخص العربي

مرض الحمى القلاعية أكثر أمراض الحيوان انتشاراً وتأثيراً على الاقتصاد حيث يصيب الحيوانات مشقوقة الظلف، والتشخيص السريع لهذا المرض ضروري للسيطرة على المرض ومنع انتشاره. يعتمد التشخيص على اكتشاف الفيروس في الخلايا الطائفية للنَّباتات أو في سائل كل من البلعوم والمريء. وتم التشخيص لمرض الحمى القلاعية في هذه الدراسة لعدد ٢٨ حيواناً، على أعراض المرض بالفحص بواسطة الميكروسكوب الإلكتروني واختبار الإلِيزة التجاري واختبار التجمع باستخدام بروتين A الذي يوجد بجدران المكور الذبيحي الحمضودي وهذه الاختبارات للكشف عن أنتيجين الفيروس. وأيضاً تم الفحص لاختبار التجمع للعينات الإيجابية بالميكروسكوب الإلكتروني. كما تم حساب كل من الحساسية والتنوع لاختبار الإلِيزة واختبار التجمع باستخدام العزل وتكرره باختبار الإلِيزة وكانت النتيجة ٧٧.٨٦٪، ١٠٠٪ لاختبار الإلِيزة و٦٩.٢٥٪ ١٠٠٪ لاختبار التجمع. وتم أيضا استخدام اختبار التجمع والعزل وتكرره باختبار الإلِيزة للكشف عن فيروس الحمى القلاعية في مادة عينية من سائل البلعوم والمريء، لحيوانات كانت مصابة بالحمى القلاعية منذ ٢ إلى ٣ شهور وكانت عد العينات الإيجابية ٨ و١٠ على التوالي. لذا يمكن استخدام اختبار التجمع كاختبار اقتسادي، بسيط، سريع ومنوعي لتشخيص فيروس الحمى القلاعية في حالات تفشي المرض بالتبادل مع اختبار الإلِيزة، كما يمكن استخدامه لتشخيص الحيوانات التي تم شراءها وظل حاملة الفيروس بالتوازي مع اختبارات الكشف عن الأجسام المضادة للبروتينات الغير تركيبية للفيروس.

المحكمون:

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