Development and characterization of monoclonal antibodies against avian reovirus σC protein and their application in detection of avian reovirus isolates

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Avian reovirus (ARV) is a non-enveloped virus with a segmented double-stranded RNA genome surrounded by a double icosahedral capsid shell. ARVs are associated with viral arthritis, immunosuppression, and enteric diseases in poultry. The σC protein was involved in induction of apoptosis and neutralization antibody. In the present study, σC-His protein was expressed in Sf9 insect cells and purified by immobilized metal affinity chromatography. Eight monoclonal antibodies (mAbs) against σC-His and three mAbs against His were screened from hybridoma cells produced by fusion of splenocytes from immunized mice with NS1 myeloma cells. Among the eight mAbs against σC protein, all belonged to the IgG isotype except three for IgM. It was discovered that all anti-His mAbs were mixtures of IgG and IgM isotypes. mAbs reacted with σC-His protein in a conformation-independent manner based on dot blot and western blotting assays. The competitive binding assay indicated that all mAbs recognized the same epitope on σC protein that was conserved in different isolates. Compared with the commercial anti-ARV S1133 polyclonal antibody, mAb (D15) had universal reactivity to all serotypes or genotypes of ARVs tested. This monoclonal antibody may therefore be useful for the development of an antigen-capture enzyme-linked immunosorbent assay for rapid detection of field isolates.

Introduction

Avian reovirus (ARV) is one of the most important infectious agents to avian species. ARVs have been associated with viral arthritis, immunosuppression, and enteric diseases in poultry (Glass et al., 1973; Heironymus et al., 1983; Rosenberger et al., 1985). Similar to mammalian reovirus, ARV is enclosed by a double-shell capsid with a genome consisting of 10 segments of double-stranded RNA (Gouvea & Schnitzer, 1982; Wickramasinghe et al., 1993). ARV-encoded proteins including at least 10 structural proteins (λA, λB, λC, μA, μB, μBC, μBN, σA, σB, and σC) and four non-structural proteins (μNS, σNS, p10, and p17) have been demonstrated (Bodelon et al., 2001; Varela & Benavente, 1994). Protein σC, structurally similar to protein σ1 of mammalian reovirus, is a minor outer-capsid protein of ARV that is encoded by the largest open reading frame of the S1 segment (Kant et al., 2003). It is not only an attachment protein (Martínez-Costas et al., 1997), but also an apoptosis inducer (Shih et al., 2004). Some studies have suggested that σC protein is the target for type-specific neutralizing antibodies while σB is target for group-specific antibodies (Wickramasinghe et al., 1993). The neutralization activity by antibodies in various cell types showed the tissue tropism of σC protein (Meanger et al., 1999; Antczak & Joklik, 1992; Yin et al., 2000).

Although virus isolation is a reliable way for detection of ARV infection, this procedure is laborious and time-consuming (Van der Heide et al., 1976; Wood et al., 1986; Meanger et al., 1995). Several diagnostic techniques based on antigen and antibody reactions have been developed (Slaght et al., 1978; Islam & Jones, 1988; Lee et al., 1994; Li et al., 1996; Liu et al., 1999a,b, 2000, 2002, 2004; Liu & Giambonne, 1997; Chen et al., 2004; Ke et al., 2006). In this study, eight monoclonal antibodies (mAbs) against σC and three mAbs against His were produced. The competitive binding assay showed that all mAbs were against the same epitope. The binding of mAbs to the epitope was conformation independent. When all ARV isolates were tested with the developed mAbs and the commercial anti-ARV S1133 polyclonal antibody, our mAbs demonstrated universal reactivity. Due to its capability to react with various serotypes or
The pellet was resuspended in cold lysis buffer (10 mM Tris/C1 scattered proteins of S1133 strain expressed in insect cells. Sf9 cells were infected with an adenovirus vaccine strain (SA133) and seven field isolates (750505, 919, 601G, R2/TW, and 1017-1, and 919) were used in this study. Based on a cross-neutralization test for serotyping, S1133, 601G, and R2/TW have been grouped into genotype I, while strains 918 and 1017-1 were grouped into genotype III. R2/TW and 601G belong to genotype II and others were grouped as genotype IV (Liu et al., 1992). As far as genotypes are concerned, S1133, 750505, and 919 have been grouped into genotype I, while strains 918 and 1017-1 were grouped into genotype III. R2/TW and 601G belong to genotype II and others were grouped into genotype IV (Liu et al., 2005). All viruses were propagated in vero cells at 37°C for 36 h and then subjected to three freeze–thaw cycles. The supernatant obtained by centrifugation of these lysates was treated with 1% Triton X-100 and used as a crude antigen for the antigen-capture ELISA.

Antigen preparation. Antigens used for the production and characterization of anti-σC-His mAbs were prepared from avian reovirus σC-His proteins of S1133 strain expressed in insect cells. Sf9 cells were infected with Autographa californica (AcNPV)-σC-His at 10 plaque-forming units/cell (Hu et al., 2002) and harvested after 72 h for centrifugation. The pellet was resuspended in cold lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM MgCl2, 200 mM NaCl, 0.95% Triton X-100) and further incubated for 10 min at 4°C. The supernatant containing proteins was collected after centrifugation at 10,000 rpm after centrifugation for 10 min. The proteins were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by western blot using mouse anti-σC polyclonal antibody from our laboratory. The preparation of σNS-His proteins was described previously (Huang et al., 2005). The recombinant plasmid pET32a containing the full-length σNS-encoding gene with His tag was used to transform Escherichia coli BL21 (DE3). The fusion protein was expressed after induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the soluble fractions were purified using a His-Band Resin column (Novagen) according to the manufacturer’s instruction manual. After verification using an anti-σNS monoclonal antibody (1F9) (Hou et al., 2001; Huang et al., 2005) in a western blot, purified σNS-His proteins were used as control groups in dot blotting assays.

Monoclonal antibody production. Anti-σC-His mAbs were produced by immunization of BALB/c mice with the antigens prepared earlier. The mice were immunized intraperitoneally with 35 μg antigen in complete Freund’s adjuvant, followed by two boosts with the same amount of antigen at 2-week intervals. A third and final boost was carried out with 20 μg antigen 6 weeks after the initial immunization. The spleens were removed and splenocytes were fused with NS1 myeloma cells. Hybridoma cell lines secreting anti-σC-His antibodies were screened and subcloned at least three times using the limiting dilution method. The cloned hybridomas were used for production of ascites fluids in mice. Hybridoma culture fluids and mouse ascitic fluids were used for subsequent analyses.

Isotype determination. The Zymed MAb kit (Zymed Ltd) was used for determination of the immunoglobulin class of the hybridoma antibodies from ascitic fluids of mice. Rabbit antiserum to mouse IgM, IgG1, IgG2a, IgG2b, and IgA and goat anti-rabbit IgG serum conjugated with horseradish peroxidase (HRP) were used in ELISA according to the manufacturer’s instruction manual.

Western blot and dot blot assays. Western blotting was used to examine the binding ability of mAbs to denatured σC-His proteins. Purified σC-His protein was subjected to 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were probed with different mAbs followed by a secondary HRP-conjugated goat anti-mouse antibody. The reaction was detected by ECL reagent (Amersham Bioscience) according to the manufacturer’s instruction.

The same σC-His and σNS-His proteins were used for dot blotting assays. σNS-His proteins and anti-σNS monoclonal antibody (1F9) (Hou et al., 2001; Huang et al., 2005) were used as control groups. Approximately 1 μg antigen was diluted with TNE buffer (0.01 M Tris–HCl, 0.1 M NaCl, 0.001 M ethylenediamine tetraacetic acid, pH 7.4) and spotted onto nitrocellulose membrane. The membranes were probed with the same mAbs as for western blot.

Immunofluorescence assay. Vero cells were infected with ARV S1133 strain (10 M.O.I.) and incubated at 37°C for 24 h. The cells were fixed with a mixture of 50% acetone and 50% methanol for 10 min, and then probed with different anti-σC-His mAbs. Bound antibodies were visualized using fluorescein isothiocyanate (FITC)-conjugated antibodies against mouse IgG under a fluorescence microscope (Olympus IX70, Japan).

Coupling of HRP to mAbs. Ascitic fluids were precipitated with an equal volume of saturated ammonium sulfate at 4°C. Isolated fractions were dialysed against phosphate-buffered saline overnight with several changes, and then purified using a protein G-agarose affinity column. Antibodies were coupled to HRP and stored at −20°C.

Determination of antibody titres. The titres of mAbs were determined using an ELISA. Expressed σC-His proteins were coated into each well of plates with 20 μg at 37°C for 2 h. The plates were washed three times with washing buffer (0.01 M phosphate-buffered saline, pH 7.2, 0.05% Tween 20) and blocked with 100 μl TNE buffer containing 2.5% bovine serum albumin. After washing, two-fold serial dilutions of 1 μg/ml uncoupled or HRP-coupled mAbs were added and incubated for 1 h. For uncoupled mAbs, an additional 50 μl HRP-coupled goat anti-mouse antibodies were added and incubated for 1 h. One hundred microlitres of substrate solution (500 mM citric acid, pH 4.0, 2.3 mM H2O2, 40 mM 2-2’-azino-bis-3-ethylbenzthiazoline sulfonic acid) was added and reaction was stopped by adding 50 μl of 2.5 M H2SO4 after 20 min. The absorbance value was read at 405 nm with a Dynex ELISA reader (Dynex Technologies, Inc., Chantilly, Virginia, USA). The level of binding for the relative activity was measured from the resulting dose–response curve.

Competitive binding assay for epitope mapping. Similar procedures to antibody titrations were used in the competitive binding assay except for a mixture of the HRP-conjugated mAbs at twice the concentration.

Figure 1. Expression and identification of protein σC-His. Sf9 cells were infected with recombinant baculovirus AcNPV-σC-His and lysed at 72 hour post-infection (h.p.i.). The cell extracts were fractionated on a 12% SDS-PAGE gel (1a) or were transferred to PVDF membranes and probed with a mouse anti-σC polyclonal antibody (1b). Lane 1, protein marker; lane 2, expressed σC-His protein. Arrow indicates the band of expressed σC-His protein.
Results

Expression of σC-His and σNS-His proteins. A recombinant baculovirus (AcNPV-σC-His) was constructed to produce sufficient quantity of the ARV σC-His. To monitor the expression of the recombinant σC-His protein in insect cells, soluble extracts from infected Sf9 cells were subjected to SDS-PAGE analysis and extracts contained a 39-kDa polypeptide (Figure 1a). The nature of this polypeptide was verified using western blot and it reacted specifically with a mouse anti-σC polyclonal antibody (Figure 1b). These results indicated that σC-His protein was expressed in soluble form in insect cells.

After IPTG induction of E. coli containing the pET32-σC-His recombinant at 37°C for 3 h, cell extracts were analysed by SDS-PAGE. A 60-kDa polypeptide with the correct size of σNS-His protein was obtained, and western blot probed with an anti-σNS

Table 1. Properties of monoclonal antibodies against the σC-His proteins of the ARV S1133 strain

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+, positive results; −, negative results; ND, not done.
monoclonal antibody further confirmed authenticity of this polypeptide (data not shown).

**Production and characterization of mAbs.** Eleven mAbs were selected for subcloning using the limiting dilution method. The isotypes of mAbs against σC (D15, J621, D11, J2122, and J161N1) were of IgG class; three were IgG1 and two were IgG2b. Concentrations of uncoupled antibody ranged from 1.5 to 12.2 μg/ml, whereas concentrations increased to 97.6 to 781.2 μg/ml after HRP coupling. Another three mAbs against σC (C421, D1423, and C143) were all IgM class. However, mAbs against His including J62, J44, and J13 were all mixed types of IgG2a and IgM.

![Figure 3](image1.png)

**Figure 3.** Western blots of σC-His proteins using anti-σC mAbs. Lane M, protein markers; lane 1, a mouse anti-σC polyclonal antibody; lane 2, normal mouse serum as a negative control; lane 3, mAb D15; lane 4, mAb J621; lane 5, mAb D11; lane 6, mAb J2122; lane 7, mAb J161N1.

![Figure 4](image2.png)

**Figure 4.** avidity assays of mAbs to σC-His proteins. Serial 10-fold dilutions of different mAbs from 100 μg/ml were incubated with σC-His proteins absorbed on the wells of microtitre plates. After washing, bound mAbs were detected by the HRP-labelled goat anti-mouse IgG (4a) or directly incubated with serial 10-fold dilutions of different HRP-labelled mAbs (4b). The resulting OD405 was read and used to rate avidity.
The need for native conformation for antibody binding. A dot blotting assay was used to determine the need for native conformation for antibody binding. mAbs D15, J621, D11, J2122, J161N1, C4211, D1423, and C143 only recognized the native structure of sC-His in TNE buffer. mAbs J62, J44, and J13 could react with both sC-His and sNS-His. They were anti-His mAbs (Figure 2 and Table 1). The expressed sC-His proteins were denatured by boiling in SDS and 2-mercaptoethanol, and subjected to western blotting; mAbs D15, J621, D11, J2122, and J161N1 still recognized them (Figure 3 and Table 1). No neutralization activity was demonstrated in IgG-class mAbs. The immunofluorescence assay test was performed in ARV S1133-infected Vero cells, and positive signals were detected in cytoplasm of infected cells using anti-sC mAbs (D15, J621, D11, J2122, and J161N1) (Table 1) but not in the mock-infected cells. We may therefore conclude that these mAbs are specific for sC of ARV based on western blotting and immunofluorescence assay test results.

Avidity of mAbs to σC-His. The numbers of mAbs bound to the proteins can be quantified within the linear range of absorbances. This offers an estimation of the relative avidity of mAbs for their binding proteins. Binding degrees of mAbs to σC-His using ELISA titration indicated that all mAbs saturated at dilutions from $10^{-1}$ and $10^2$, except J2122 and J161N1 at $10^{0.5}$ to $10^2$ (Figure 4a). All mAbs retained their binding capacity after coupling to HRP, and the dilution range of saturation was $10^{1.5}$ to $10^2$ for D15, J621, and D11 mAbs. No apparent saturation appeared in the remaining HRP mAbs (Figure 4b).

Mapping of the epitopes. HRP-labelled mAbs were used in the competitive binding assay. The proper concentrations for the competitive binding assay were determined using dose–response curves plotted for unconjugated and HRP-conjugated mAbs. One mAb was used as a competitor as well as a HRP-coupled probe. Results showed that all anti-σC mAbs recognized a single epitope based on the strong reciprocal binding competition (>75%) (Figure 5).

Antigen-capture ELISA using mAb D15 for clinical samples. mAb D15 was used to prepare an antigen-capture ELISA since it can react with all ARV isolates. The commercial polyclonal antibody against ARV S1133 from IDEXX, Inc. on the other hand, only recognized four strains (S1133, 750505, 918, and 919) (Figure 6). As expected, negative results were obtained using the IDEXX negative polyclonal antibody. These results indicated that mAb D15 could recognize all ARV
recombinant baculovirus AcNPV-particles are used as antigens. Infection of Sf9 cells with obtain anti-
infected cells because it is a minor capsid protein
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C protein can bind specifically to avian cells and the
attachment of ARV to cells can be blocked using expressed σC protein (Grande et al., 2000). It plays an
important role in ARV pathogenesis due to its capability to elicit a neutralization antibody reaction in
virus infection (Wickramasinghe et al., 1993). Both classical and molecular-based techniques have been
developed for detection of ARV (Li et al., 1996; Liu & Giambrone, 1997; Liu et al., 1999a,b, 2000, 2004; Ke
et al., 2006). Virus isolation is a reliable diagnostic method but not suitable for rapid diagnosis. Recently,
the antigen-capture ELISA using anti-ARV antibodies has been becoming an ideal choice for large screening
because of its high sensitivity, reproducibility, and automation. Several ELISAs using antibodies gener-
ated from whole viruses or recombinant ARV proteins as antigens have been reported. The antigen-capture
ELISA using monoclonal antibodies against a single protein is usually less sensitive than others; however,
non-specific reactions could be significantly reduced (Liu et al., 2002).

Insolubility of recombinant protein in inclusion bodies can be overcome by expressing the protein under the
control of different promoters and hosts, truncating the protein, and changing the growing temperature for E.
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Antigen-capture ELISAs are more suitable when using an antibody against a single protein. This type of ELISA
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tively small amounts of protein σC were present in infected cells because it is a minor capsid protein
(Martinez-Costas et al., 1997). It is also difficult to obtain anti-σC monoclonal antibodies if whole virus
particles are used as antigens. Infection of Sf9 cells with recombinant baculovirus AcNPV-σC-His resulted in
expression of a large amount of σC protein for production of mAbs.

The competitive binding assays were used to determine epitopes of mAbs based on the notion that a mAb
binding to a specific site can block the attachment of another mAb to the same site. All anti-σC mAbs
recognized the same epitope. As for the conformation of σC-His protein in antibody binding, all mAbs bound
to the σC-His in its native conformation. When strong reducing agents such as SDS and 2-mercaptoethanol
were used to denature the σC-His protein, this binding still remained, indicating that the recognized epitopes
were not affected by breaking of disulfide bonds. This led us to suggest that the mAb binding was conformation-
independent. mAb D15 could recognize serotype I, serotype II, and some unclassified ARVs, confirming
the suggestion that the epitope of σC-His protein against mAb D15 was conserved in all ARV strains. Interestingly,
all ARV isolates that were classified as four genotypes could react with mAb D15 but only genotypes I and III
could be recognized by the commercial polyclonal antibody. Our results suggest that mAb D15 may be a group-
specific antibody and has higher affinity for σC proteins than the commercial polyclonal antibody.

Discussion

Protein σC can bind specifically to avian cells and the
attachment of ARV to cells can be blocked using expressed σC protein (Grande et al., 2000). It plays an
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The avian reovirus genome segment S1 is a functionally tricistronic
gene that expresses one structural and two nonstructural proteins in

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experimentally virus-infected chickens monitored by a monoclonal
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characterization of a virus associated with arthritis of chickens. Avian
Disease, 17, 415–424.


Non-English Abstracts

Development and characterization of monoclonal antibodies against avian reovirus σC protein and their application in detection of avian reovirus isolates

Chien Hsu1,2, Chi Wang3, Long Lee4, Wen Shih5, Chi Chang2, Hsueh Cheng2, Julius L.C. Chulu1,6, Wen Ji1 and Hung Liu1,2*

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Développement et caractérisation des anticorps monoclonaux contre la protéine σC du réovirus aviaire et leur application dans la détection des souches d’ARV

Les réovirus aviaires sont des virus non enveloppés avec un génome ARN segmenté, double brin (dsRNA), entouré d’une capsule icosahédrique double. Les réovirus aviaires sont associés à l’arthrite virale, l’immunodépression et les maladies entéritiques chez les volailles. La protéine σC est impliquée dans l’induction de l’apoptose et des anticorps neutralisants. Dans cette étude, la protéine σC-His a été exprimée dans les cellules d’insecte Sf9 et a été purifiée par chromatographie d’affinité IMAC. Huit anticorps monoclonaux (MAbs) contre σC-His et trois MAbs contre HIS ont été sélectionnés à partir des cellules d’hybridome produites par la fusion de splénocytes de souris immunisées avec des cellules de myelome NS1. Parmi les huit MAbs anti protéine σC, tous appartiennent à l’isotype IgG à l’exception de trois qui sont des IgM. Il a été mis en évidence que tous les MAbs anti His étaient des mélanges d’isotypes IgG et IgM. Les tests dot blot et Western blot ont montré que les MAbs réagissaient avec la protéine σC-His de façon indépendante de sa conformation. Le test de fixation compétitif a montré que tous les MAbs reconnaissaient le même épitope sur la protéine σC qui était conservé sur les différentes souches. Le MAb D15 a présenté une réactivité universelle à tous les sérotypes ou génotypes de ARVs testés, comparé à l’anticorps polyclonal anti-ARV S1133 du commerce. Cet anticorps monoclonal peut, par conséquent, être utilisé pour le développement d’un test ELISA de capture antigénique pour la détection rapide des souches du terrain.

Entwicklung und Charakterisierung von monoklonalen Antikörpern (Mabs) gegen das σC-Protein des aviären Reovirus (ARV) und ihre Anwendung beim Nachweis von ARV-Isolaten

Das aviäre Reovirus (ARV) ist ein unbewehrtes Virus mit segmentiertem, doppelsträngigem RNS (dsRNS)-Genom mit doppelter icosahedrischer Kapsidhülle. ARVs werden beim Geflügel mit viraler Arthritis, Immunsuppression und Darmerkrankungen assoziiert. Das σC-Protein ist in die Induktion von Apoptose und neutralisierenden Antikörpern involviert. In der vorliegenden Studie wurde das σC-His-Protein in Sf9-Insektenzellen exprimiert und mittels immobilisierter Metallionen-Affinitätschromatographie (IMAC) gereinigt. Aus Hybridomzellen, die durch Fusion von Milzellen aus immunisierten Mäusen mit NS1-Myelomzellen produziert wurden, konnten acht MAbs gegen das σC-His-Protein und drei MAbs gegen His gewonnen werden. Bis auf drei der MAbs gegen das σC-Protein, die vom IgM-Isotyp waren, gehörten alle zum IgG-Isotyp. Bei den anti-His-MAbs wurde festgestellt, dass es sich um Mischungen der IgG- und IgM-Isotypen handelte. Die MAbs reagierten mit dem σC-His-Protein konformationsunabhängig in Dot Blot- und Western Blotting-Tests. Der kompetitive Bindungstest zeigte, dass alle MAbs das gleiche Epitop auf dem σC-Protein, das in verschiedenen Isolaten konserviert ist, erkannten. Verglichen mit den kommerziellen, polyklonalen anti-ARV-S1133-Antikörper besitzt der MAB D15 eine universelle Reaktionsvermögen mit allen Sero- und Genotypen der gesteteten ARVs. Dieser monoklonale Antikörper kann deshalb für die Entwicklung eines Antigen-capture-ELISA zum schnellen Nachweis von Feldisolaten geeignet sein.

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Desarrollo y Caracterización de anticuerpos monoclonales frente a la proteína σC del reovirus aviar y su aplicación en la detección de aislamientos de ARV

El reovirus aviar es un virus sin envuelta con un genoma RNA segmentado de doble cadena (dsRNA) rodeado por una doble cápside icosaédrica. Los reovirus aviares se asocian con artritis viral, inmunosupresión, y enfermedades entéricas en aves. La proteína σC está involucrada en la inducción de apoptosis y de anticuerpos neutralizantes. En este estudio, se expresó proteína σC-His en células de insecto Sf9 y se purificó mediante cromatografía de afinidad con ión metálico inmovilizado (IMAC). Se testaron ocho MAbs frente a σC-his y tres frente a His creados a partir de células de híbrido obtenidas a través de la fusión de esplenocitos de ratones inmunizados y de células de mieloma NS1. Los ocho MAbs frente a la proteína σC pertenecían al isotipo IgG excepto tres que pertenecían al IgM. Se descubrió que todos los MAbs anti-His eran mezclas de los isotipos IgG y IgM. Los MAbs reaccionaron con la proteína σC-His de modo conformación-independiente en base a las técnicas de dot-blot y Western blot. El ensayo de unión competitiva indicó que todos los MAbs reconocían el mismo epitopo en la proteína σC, un epitopo que estaba conservado en los distintos aislamientos. En comparación con el anticuerpo policlonal anti-ARV S113 comercial, MAb (D15) mostró una reactividad universal frente a todos los serotipos o genotipos de ARVs testados. Por lo tanto, este anticuerpo monoclonal podría ser útil para el desarrollo de un ELISA de captura de antígeno para la detección rápida de aislamientos de campo.