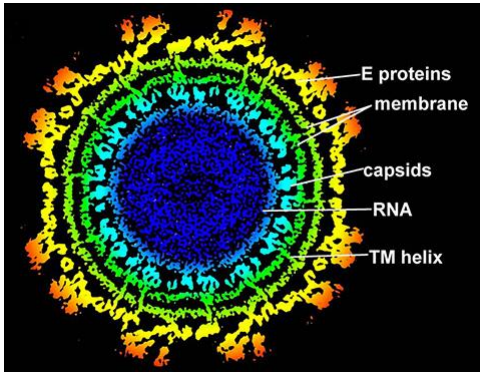
Herramientas diagnósticas moleculares e inmunológicas para el virus Chikungunya



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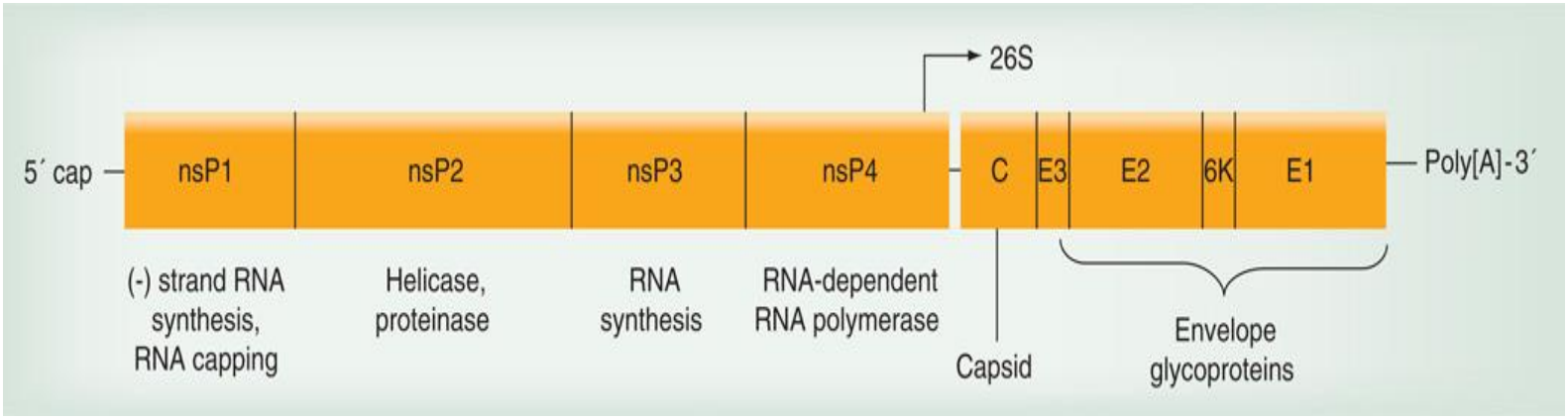
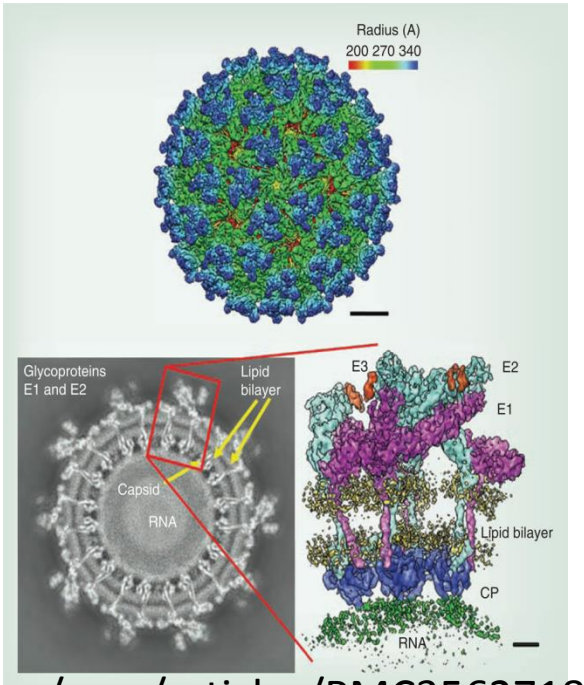
UNIVERSIDAD DEL QUINDÍO

Grupo de inmunología molecular.

Aspectos moleculares del virus

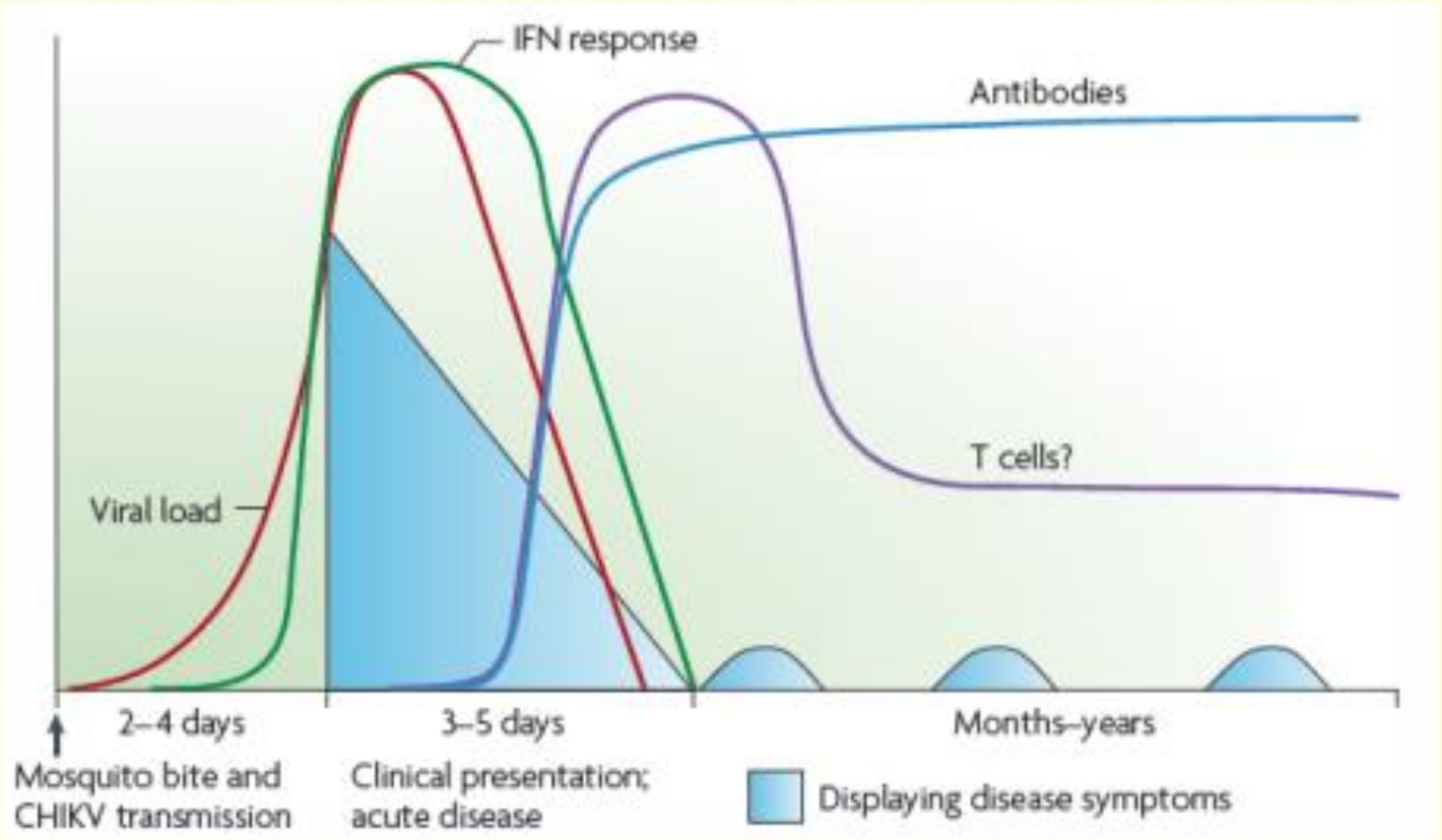
CHIKV es un virus ARN del genero *Alphavirus* y de la familia *Togaviridae*





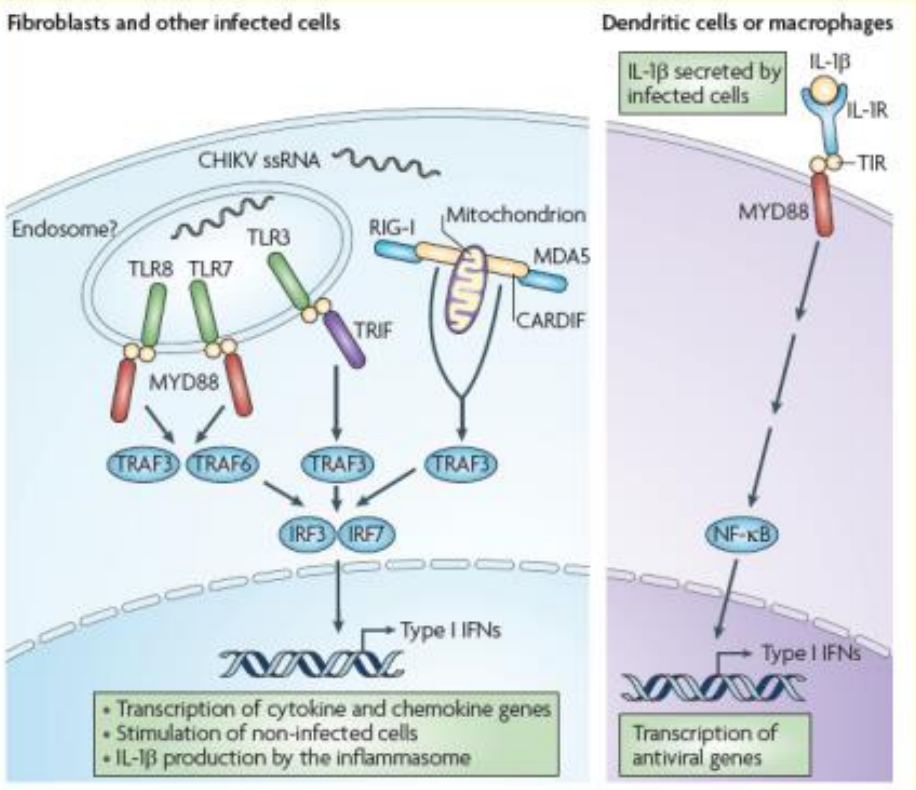
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3562718/#!po=2.63158

Figura No. 2 Patogénesis del Virus Chikungunya



http://spsigss.files.wordpress.com/2010/10/guia-de-bolsillo-para-le-manejo-del-virus-del-chikungunya1.pdf

Figura No. 4 Inmunidad innata contra el Chikungunya

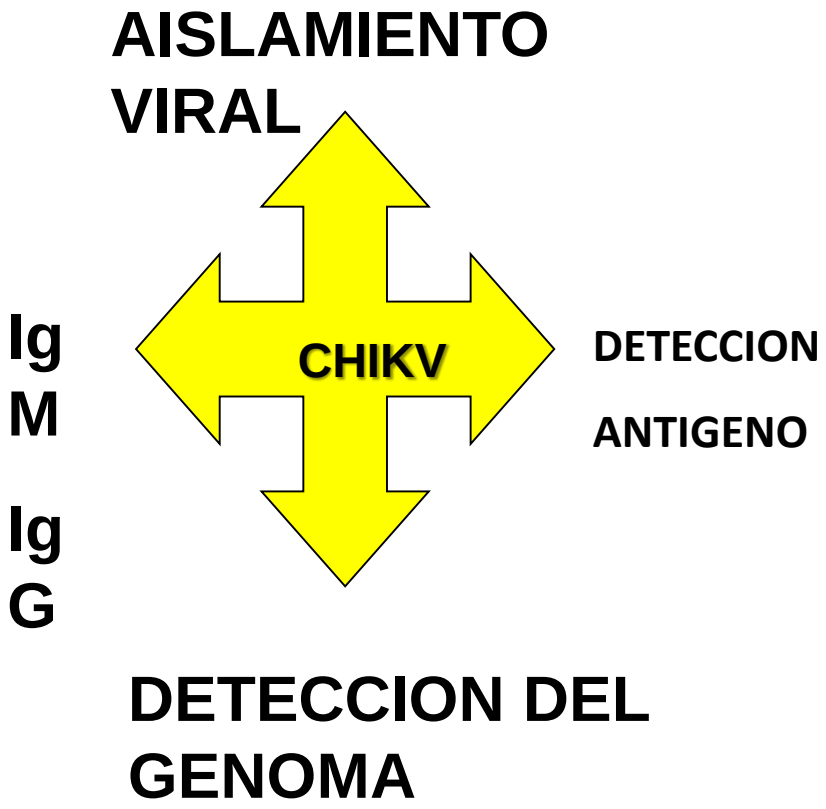


Nota: Chikungunya virus (CHIKV) es un virus ARN de cadena sencilla (ssRNA) y puede generar intermedios de doble cadena de ARN durante la replicación que tienen el potencial para activar el reconocimiento de patógenos de los receptores Toll- like receptor 3 (TLR3) , TLR7 y TLR8 y la retinoico genes - ácido inducible I receptores (RIG- I) - como (RLRs) proteína asociada a la diferenciación de melanoma 5 (MDA5) y RIG- I . Estos receptores activan una cascada de señalización que conduce a la activación de los interferones tipo I (IFN) y la transcripción de citocinas y quimiocinas . La evidencia reciente sugiere que la producción de IFN tipo I por fibroblastos infectados y otros tipos de células está regulada por la proteína adaptadora CARDIF (adaptador TARJETA inducir IFNß, también conocida como MAVS), que actúa encascada de MDA5 y RIG -I.

http://spsigss.files.wordpress.com/2010/10/guia-de-bolsillo-para-le-manejo-del-virus-del-chikungunya1.pdf

Detección y diagnóstico por laboratorio

- Aislamiento del virus : primeros 3 días de la enfermedad
- Amplificación de acido nucleico(RT- PCR) :primeros 3 días de la enfermedad
- Serología: Detección de anticuerpos IgM : Día 5
- IgG : días – meses tomar dos muestras separadas por 14 días a partir del día 7



http://www.slideshare.net/doctorrao/chikungunya-488897

TABLE 2

Strategy for laboratory diagnosis of chikungunya and dengue virus infection, Saint Martin, 2013

Period between start date of clinical symptoms and sample date	Laboratory tests performed
<5 days	Real-time RT-PCR
Between 5 and 7 days	Real-time RT-PCR and serology
>7 days	Serology

http://www.eurosurveillance.org/images/dynamic/EE/V19N13/Leparc_tab2.jpg

Tipos de pruebas de laboratorio disponibles y muestras requeridas

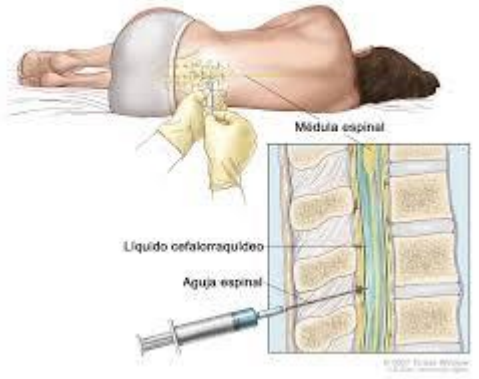
Para el diagnostico de CHIK se utilizan tres tipos principales de pruebas:

- Aislamiento viral,
- Reacción en cadena de la polimerasa con transcriptasa reversa (RT-PCR)
- Serología.

Las muestras tomadas durante la primera semana del inicio de los síntomas deben analizarse por métodos serológicos (ELISA para la detección de inmunoglobulina M [IgM] y G [IgG]) y virológicos (RT-PCR y aislamiento).

Típo de muestra

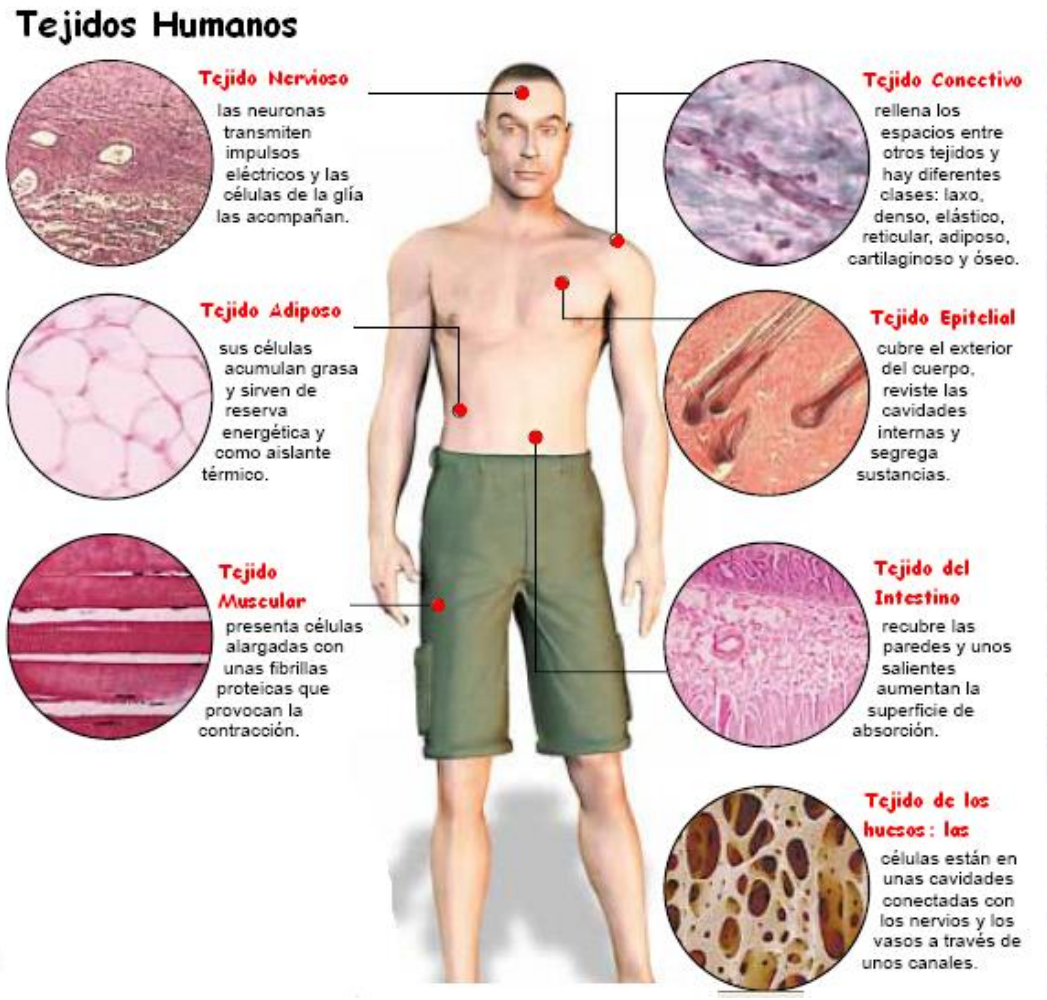
Las muestras generalmente son sangre o suero, pero en casos neurológicos con características meningoencefalicas también se puede obtener líquido cefalorraquídeo (LCR).

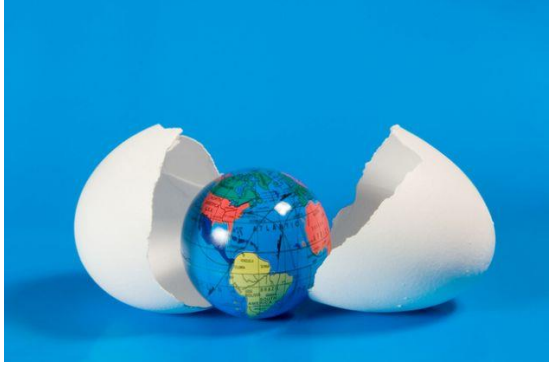


Se dispone de poca información sobre la

detección del virus por aislamiento o RT-PCR a partir de tejidos u órganos.

Ante la sospecha, en casos fatales, se puede intentar la detección del virus en las muestras disponibles.





La elección de la prueba de laboratorio apropiada se basa en el origen de la muestra

(humano o mosquitos recogidos en campo) y en el momento de recolección de

la muestra con relación al comienzo de los síntomas (en el caso de muestras de

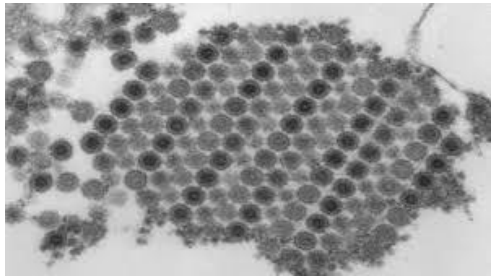
origen humano).

Aislamiento Viral

- CHIKV producira los efectos citopaticos tipicos (ECP) dentro de los tres dias posteriores a su inoculacion en una variedad de lineas celulares, que incluyen celulas Vero, BHK-21 y HeLa.

Aislamiento v i r a l

- El aislamiento del virus puede realizarse a partir de mosquitos recogidos en campo o muestras de suero de la fase aguda (≤8 días)

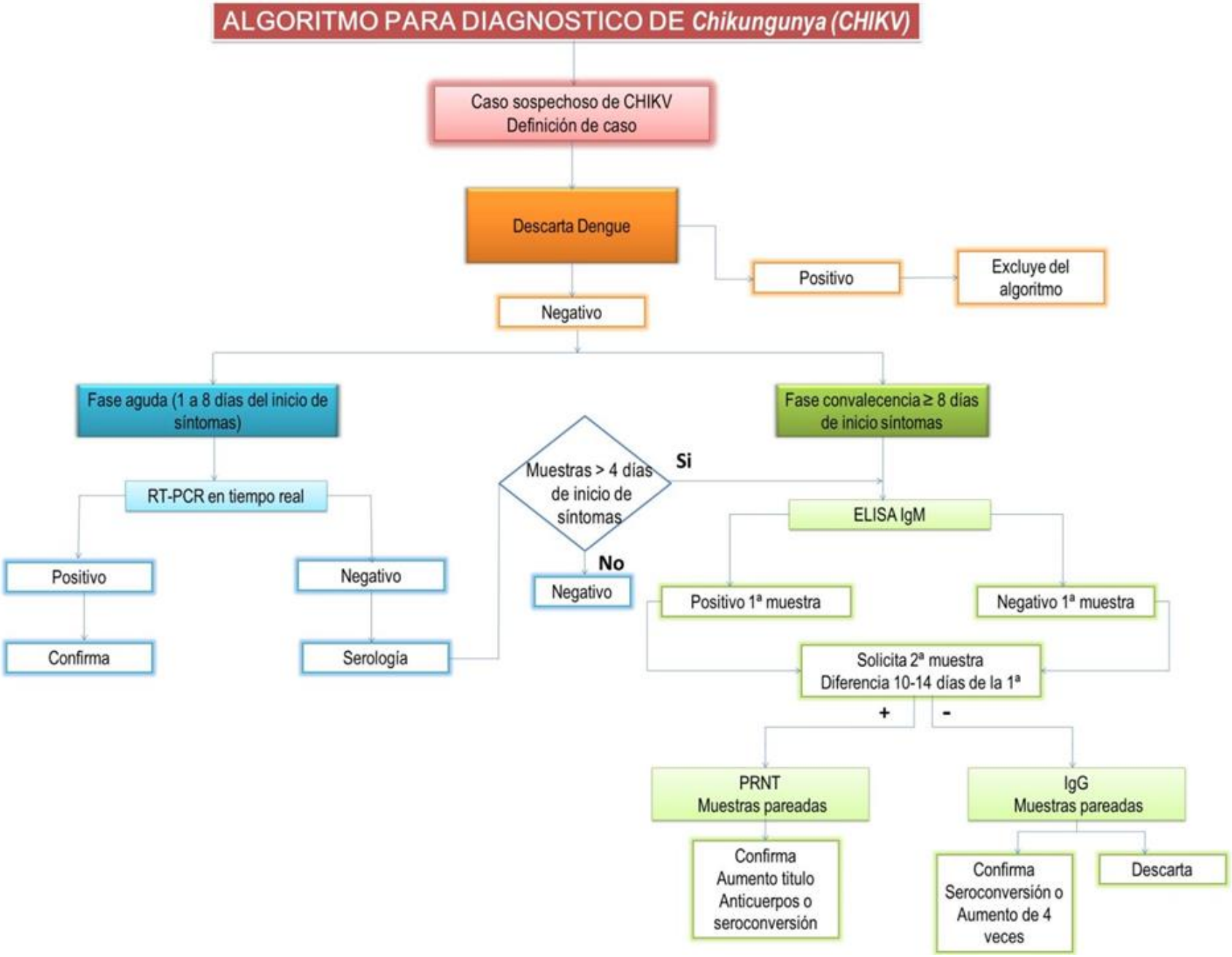


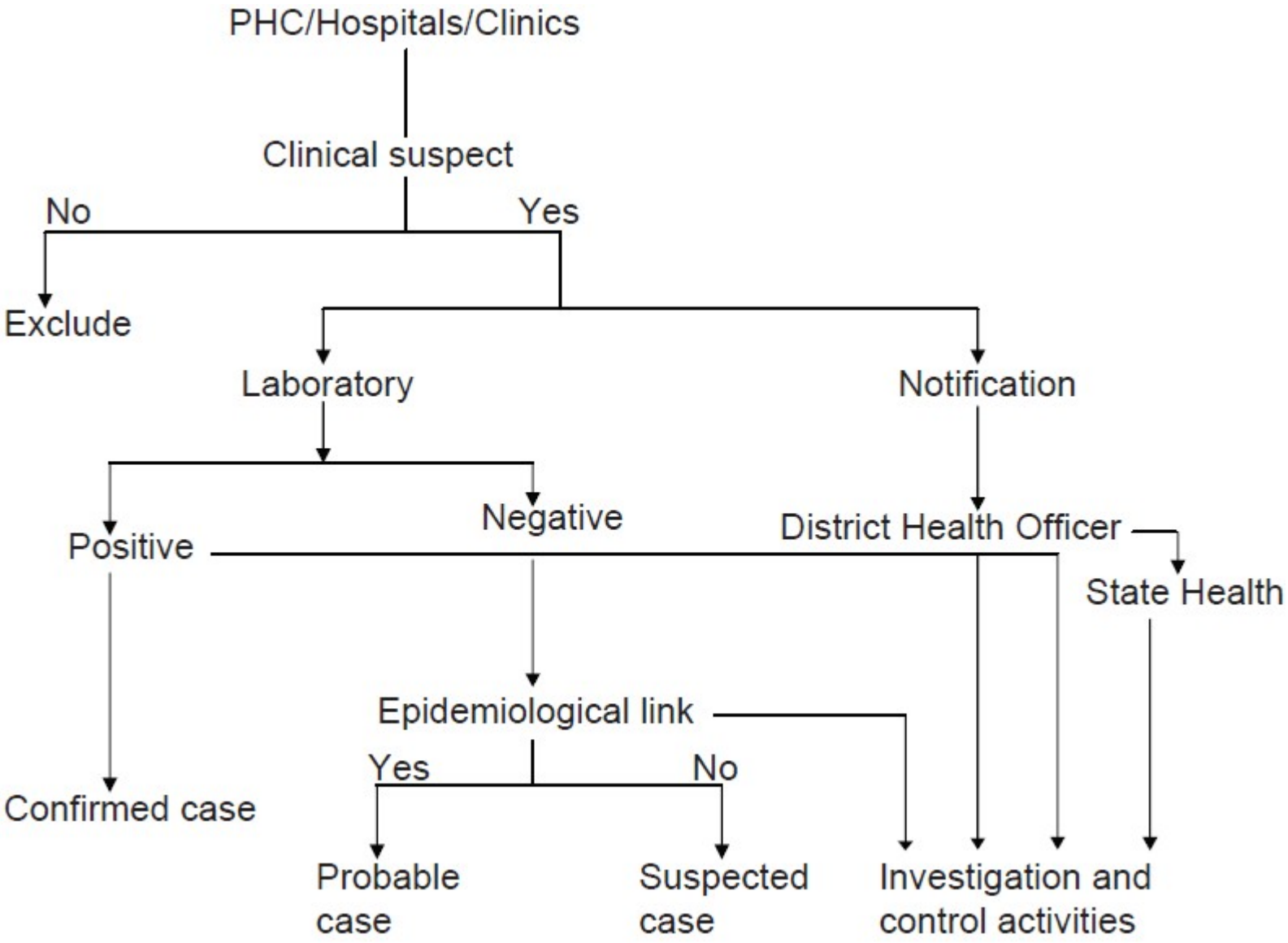
Diagnóstico

- Clinical criteria: Acute onset of fever > 38°C and severe arthralgia/arthritis not explained by other medical conditions.
- Epidemiological criteria: Residing or having visited epidemic areas, having reported transmission within 15 days prior to the onset of symptoms.
- Laboratory criteria: At least one of the following tests in the acute phase
  - Virus culture, isolation.
  - Presence of viral RNA by RT-PCR.
  - Presence of virus-specific IgM antibodies in single serum sample collected in acute or convalescent stage.
  - Four-fold increase in IgG antibody values in samples collected at least three weeks apart.
- On this basis, cases are to be categorized as:
- Possible case: A patient meeting clinical criteria.
- Probable case: A patient meeting both clinical and epidemiological criteria.
- Confirmed case: A patient meeting the laboratory criteria, irrespective of the clinical presentation.

Muktikesh Dash, Indrani Mohanty, Sanghamitra Padhi. Laboratory diagnosis of chikungunya virus: Do we really need it?. Indianmmedsci.2011;65(3):83-91.

http://www.indianjmedsci.org/viewimage.asp?img=IndianJMedSci_2011_65_3_83_104781_b1.jpg


ALGORITMO PARA DIAGNOSTICO DE Chikungunya (CHIKV)
Caso sospechoso de CHIKV
Definición de caso
Descarta Dengue
Positivo
Excluye del algoritmo
Negativo
Fase aguda (1 a 8 días del inicio de síntomas)
RT-PCR en tiempo real
Positivo
Confirma
Negativo
Serología
Muestras > 4 días de inicio de síntomas
Si
No
Negativo
Fase convalecencia ≥ 8 días de inicio síntomas
ELISA IgM
Positivo 1ª muestra
Negativo 1ª muestra
Solicita 2ª muestra
Diferencia 10-14 días de la 1ª
+
-
PRNT
Muestras pareadas
Confirma
Aumento titulo
Anticuerpos o
seroconversión
IgG
Muestras pareadas
Confirma
Seroconversión o
Aumento de 4
veces
Descarta



Muktikesh Dash, Indrani Mohanty, Sanghamitra Padhi. Laboratory diagnosis of chikungunya virus: Do we really need it?. Indianmmedsci.2011;65(3):83-91.

ELISA


SD
Chikungunya IgM


biocan
C
C
2
ca105730505.trustpass.alibaba.com


© www.medindia.net


CHIKUNGUNYA

- Collection of samples for culture, isolation and molecular diagnosis
- Sample: Serum, plasma or whole blood (in heparinized tube)
- Time of collection: Within first five days of illness
- To collect serum:
  - Aseptically collect 4ml to 5ml of venous blood in a tube or vial.
  - Allow blood to clot at room temperature, centrifuge at 2000 rpm to separate serum. Collect serum in clean dry vial.
  - All clinical samples should be labeled with patient's name, identification number and date of collection.
  - All clinical samples should accompany the clinical information as per proforma.
- Collection of samples for serology
- Sample: Blood in plain vial/serum.
- Time of collection:
  - 1st sample: Five days after onset of illness for IgM antibody as it appear at this time.
  - 2nd sample: At least 7 to 14 days after the first sample or, in event of a fatality, at the time of death.
- Transportation of samples
  - Transport specimens to the laboratory at 2°C- 8°C (ice box) as soon as possible.
  - Do not freeze whole blood, as hemolysis may interfere with serology test results.
  - If more than 24-hour delay is expected before specimen can be submitted to the laboratory, the sample should be separated and stored at refrigerated temperature.
  - Samples for virus culture, isolation and molecular diagnosis should always be stored and transported frozen.

Muktikesh Dash, Indrani Mohanty, Sanghamitra Padhi. Laboratory diagnosis of chikungunya virus: Do we really need it?. Indianmmedsci.2011;65(3):83-91.

Tests for CHIKV	*Pros*	*Cons*
Virus culture and Isolation	Gold standard for diagnosis of CHIKV	Requires facilities and Skills Also requires biosafety level 3 containment
Nucleic acid detection by RT-PCR and RT-LAMP	Highly sensitive and specific	Reagents and equipmentare very costly for widespread use.
Antigen detection tests	Not yet available commercially	Not widely available CHIKV antigen commercial assays Performance characteristics are not clearly defined. Also requires biosafety level 3 containment during preparation
Serological tests for detection of antibodies (IgM or IgG) by ELISA and ICTs	Widely available	Cross-reactivity with other alphaviruses.
	Easier to perform	Single raised IgM may indicate recent past infection rather than acute infection.
	Relatively cheaper	Sensitivities vary widely,>80%after 1 week of clinical presentation, less useful for clinicians

Muktikesh Dash, Indrani Mohanty, Sanghamitra Padhi. Laboratory diagnosis of chikungunya virus: Do we really need it?. Indianmmedsci.2011;65(3):83-91.

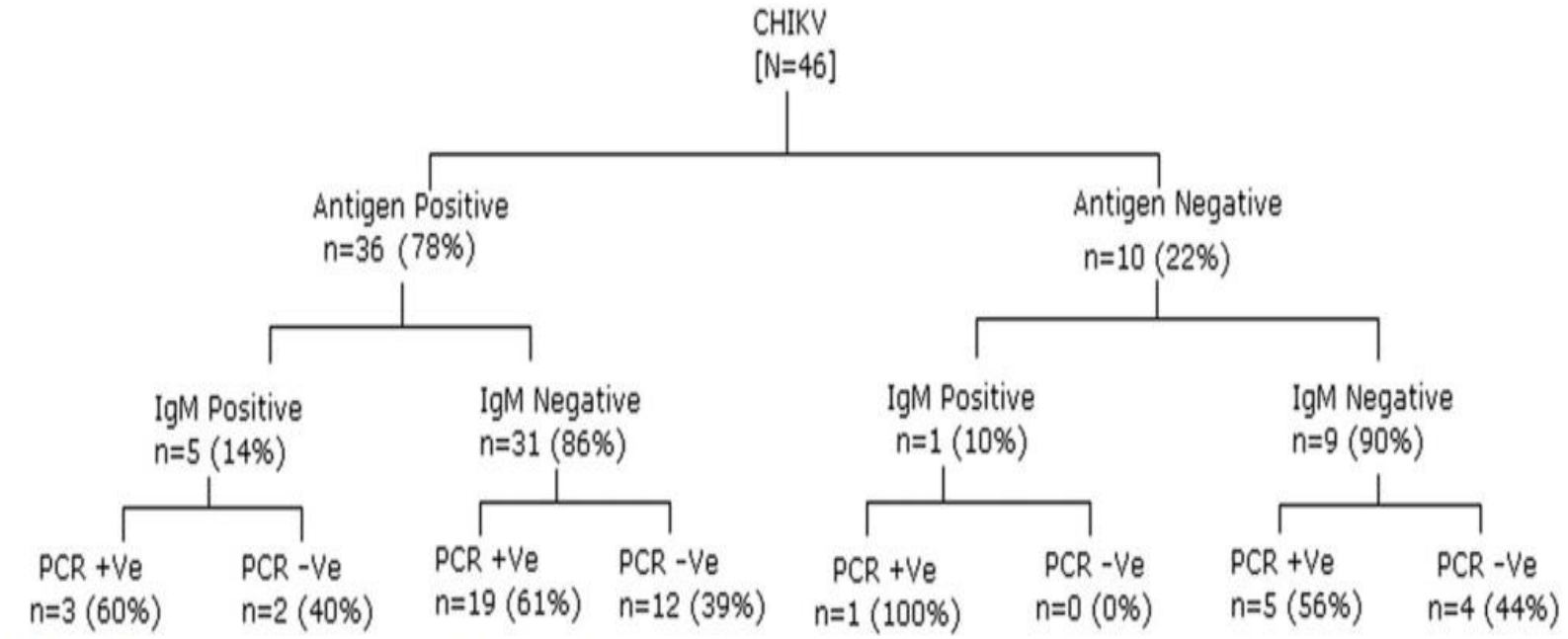


FIG. 3. Flowchart showing a comparison of the results between the antigen and IgM assays for patients with CHIKV infection.

Overall, the findings of our study suggest that the diagnostic sensitivity of CHIKV antigen detection by use of an ELISA-based system is higher than that of IgM and IgG detection and the ELISA-based system is helpful for the detection of antigen throughout the infection, even in the earlier stages of infection. Therefore, we conclude that CHIKV antigen detection can be very effective for diagnosis not only in the early stage of the disease but possibly also in the prodromal or subclinical stage.

Kashyap et al. CLINICAL AND VACCINE IMMUNOLOGY, Feb. 2010;17(2): 291–97.

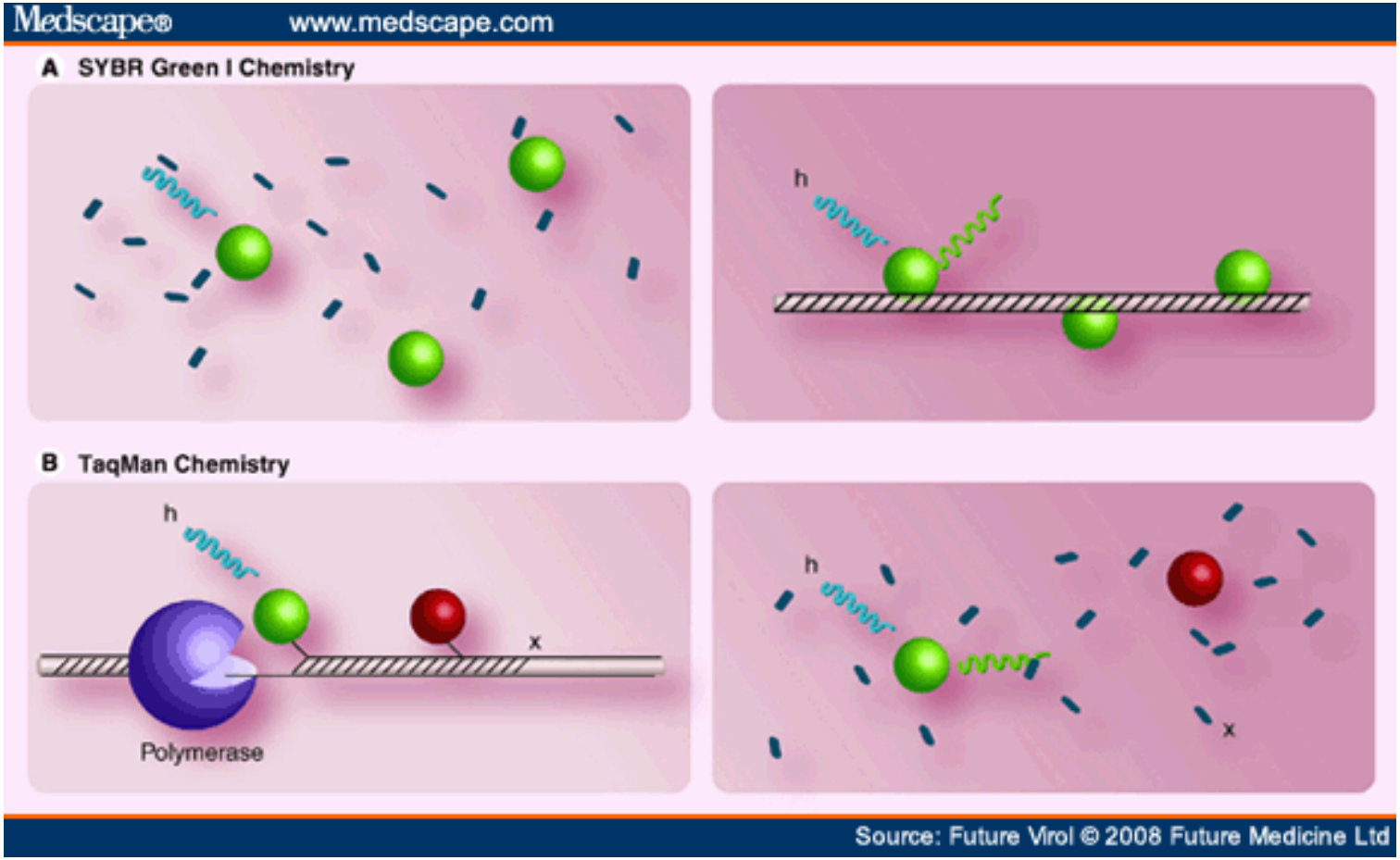
Advantages & Disadvantages of Molecular Approach-based Reverse Transcription-PCR Technologies

Virus isolation	Serology	Conventional PCR	Real-time PCR	LAMP
Advantage				
• Gold standard • Confirmatory • Fulfills Koch"s postulate	• Widely adapted as rapid screening test in routine diagnostic laboratories • Cost effective	• Alternate gold standard for virus isolation in the absence of live virus • Early confirmatory diagnosis • Widely used molecular diagnostic format	• Simultaneous amplification and detection during exponential amplification • Real-time monitoring of amplification as it happens • Quantitative, thus useful for monitoring the virus load • Lower carry-over contamination due to closed tube operation • Increased sensitivity due to fluorescent chemistry • High-throughput analysis due to	• Isothermal field-based gene amplification without requirment for thermal cycler • Amplification can be accomplished with waterbath/ heating block • Real-time as well as quantitative • Higher amplification efficiency and sensitivity • Naked eye visual monitoring either through turbidity or color change by fluorescent intercalating dye (SYBR Green I)

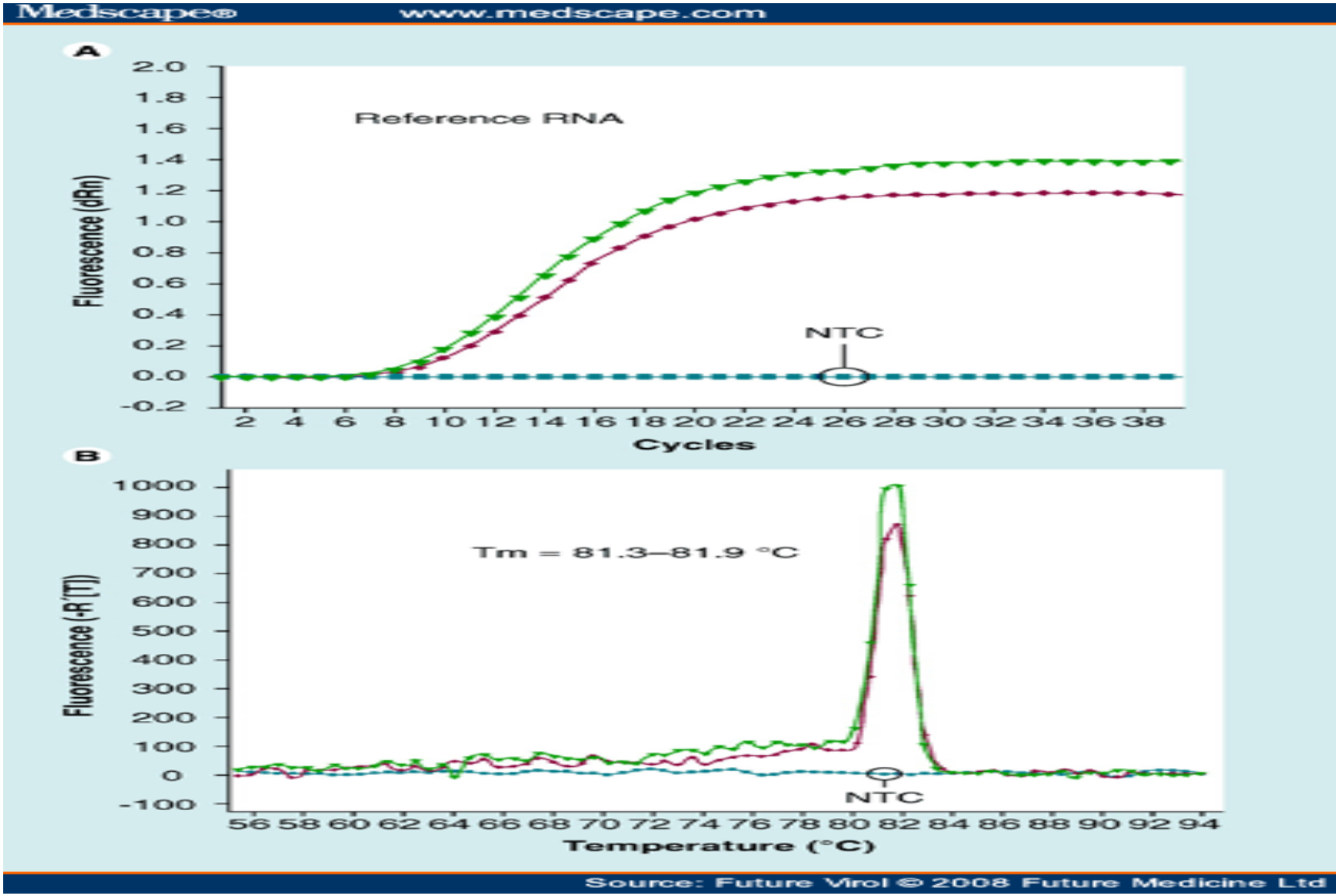
Advantages & Disadvantages of Molecular Approach-based Reverse Transcription-PCR Technologies

Virus isolation	Serology	Conventional PCR	Real-time PCR	LAMP
Disadvantage				
• Time consuming • Tedious • Requirement for susceptible cell line • Requirement of viable virus • Requirement for containment facility for processing high-risk pathogens	• Not confirmatory, always considered as probable diagnosis • Paired sera analysis • Time lag for appearance of antibodies • Not suitable for early diagnosis • Cross-reaction among closely related species	• Qualitative (yes or no format) • End-point detection in plateau phase, with nonspurious amplification • Post-PCR handling leading to carry-over contaminations • Less sensitive, thereby missing border-line cases with low gene copies • Laboratory-based Time consuming (3-4 h) • Requirement of thermal cycler and gel documentation system	• Expensive detection equipment and consumables • Requirement for fluorescent probe • Restricted to referral laboratory with good financial support	• Complicated primer designing (requirement of six primers) • Two long primers of HPLC grade purity • Restricted availability of reagents and equipments in international scenario

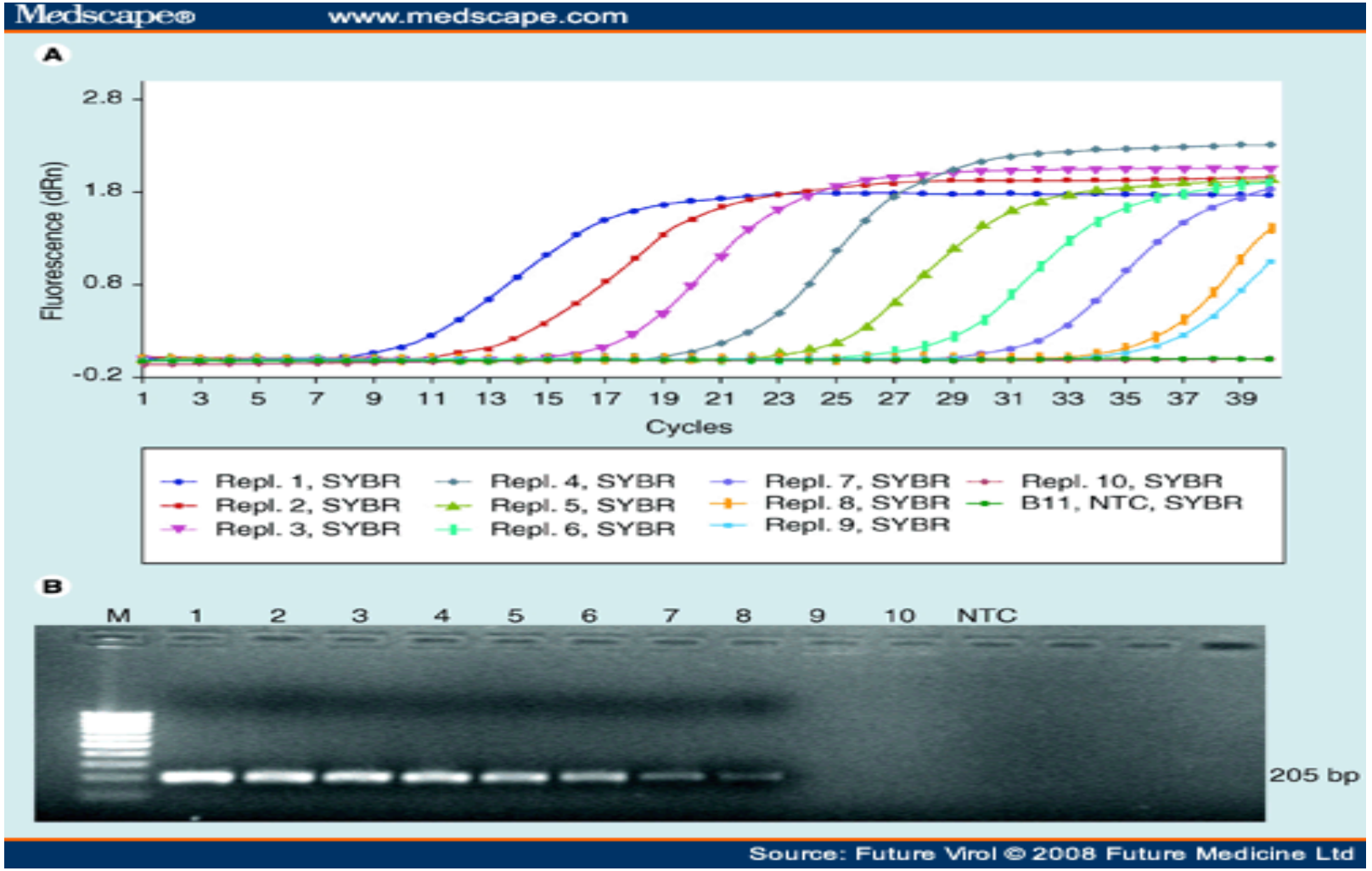
HPLC: High performance liquid chromatography; LAMP: Loop-mediated amplification.



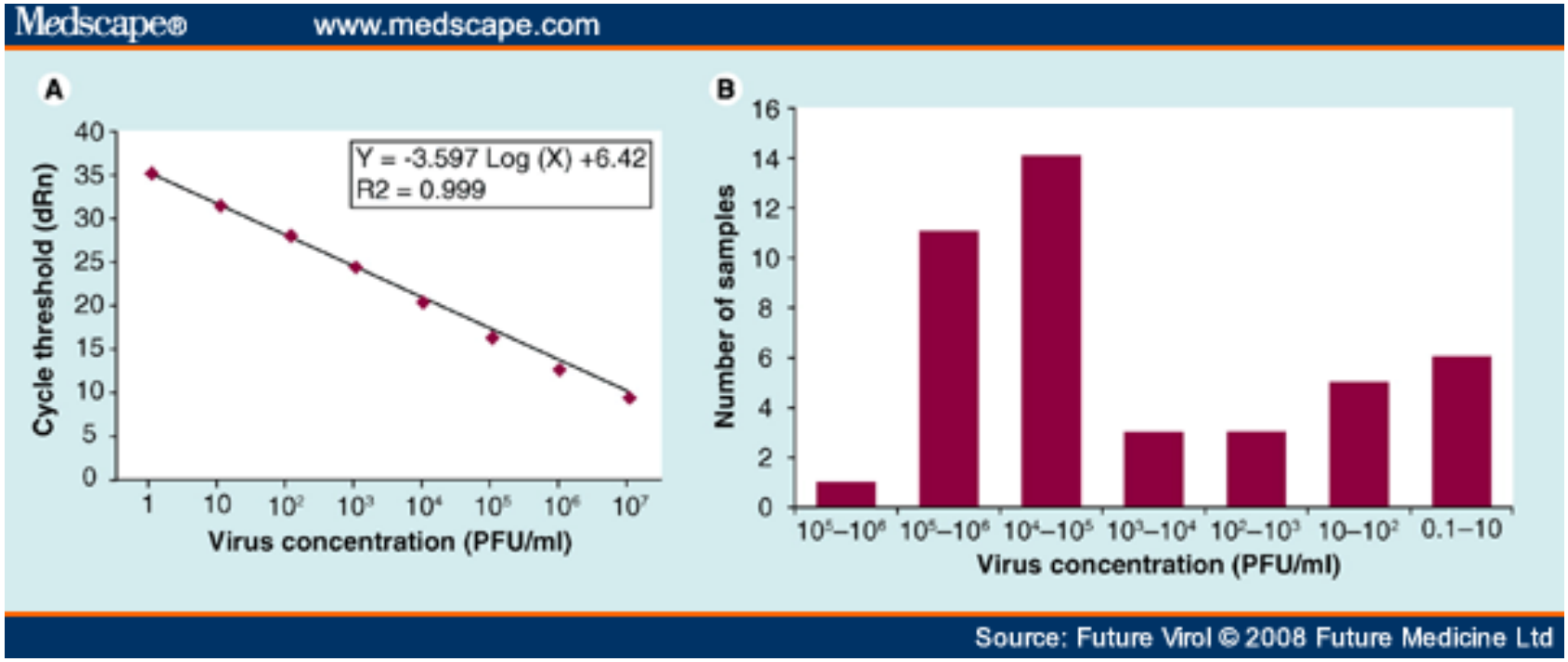
Principles and chemistry of SYBR® Green I- and TaqMan-based real-time assays. (A) SYBR Green I chemistry is a sequence-independent cost-effective method that relies on the intercalation of dsDNA-binding flurophores. (B) TaqMan chemistry is a sequence-specific reliable approach that utilizes fluorescent probes labeled with high energy dye on the 5' base, and a low energy quenching dye on the 3' base. h: With fluorescence; x: No fluorescence.



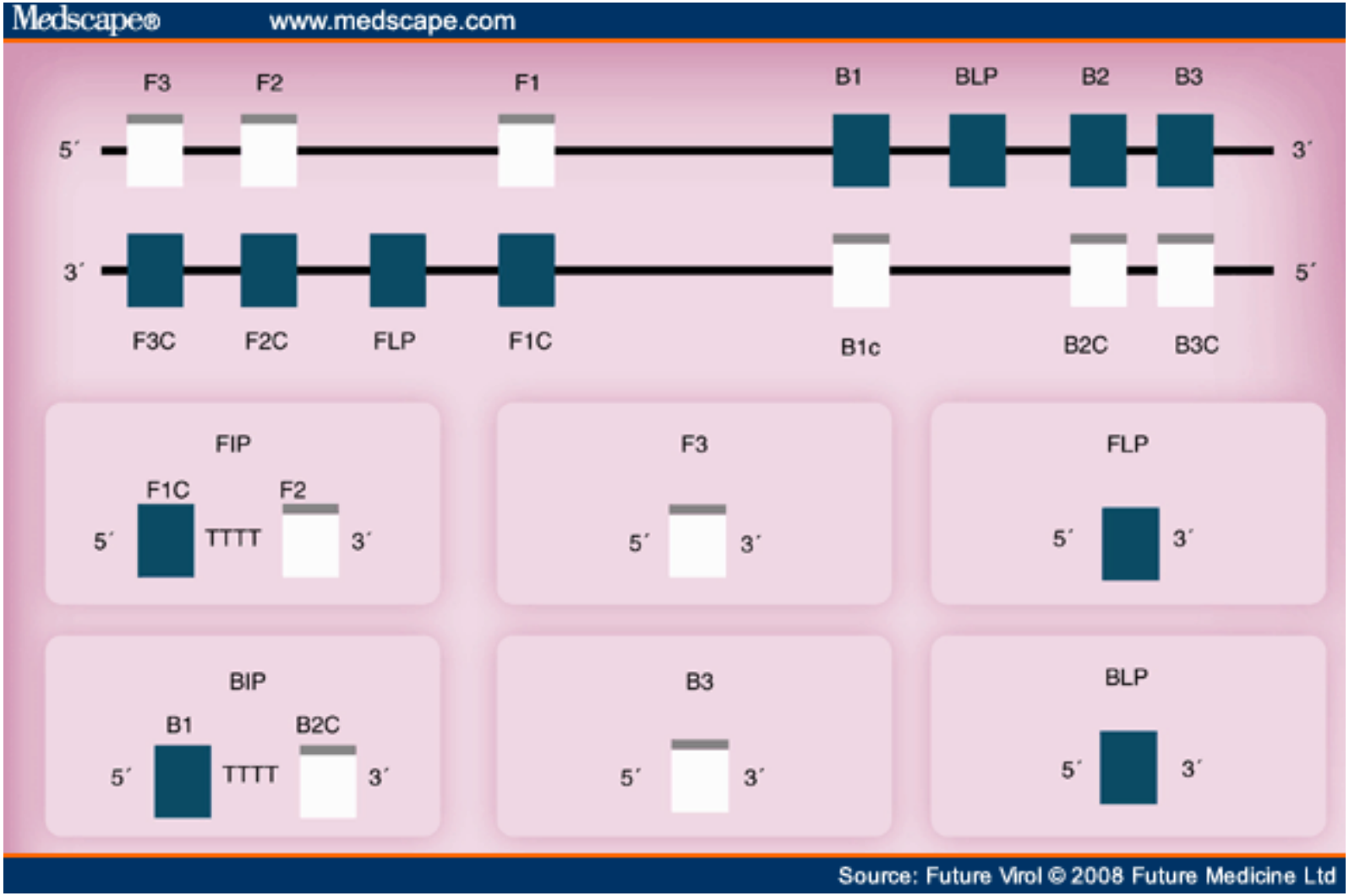
Real-time kinetics of CHIKV E1 gene-specific SYBR® Green I-based real-time RT-PCR. Figure demonstrates the amplification and dissociation curve for the reference RNA from two isolates. (A) Amplification plot. (B) Melting curve analysis depicting dissociation plot. CHIKV: Chikungunya virus; NTC: No template control; RT: Reverse transcription



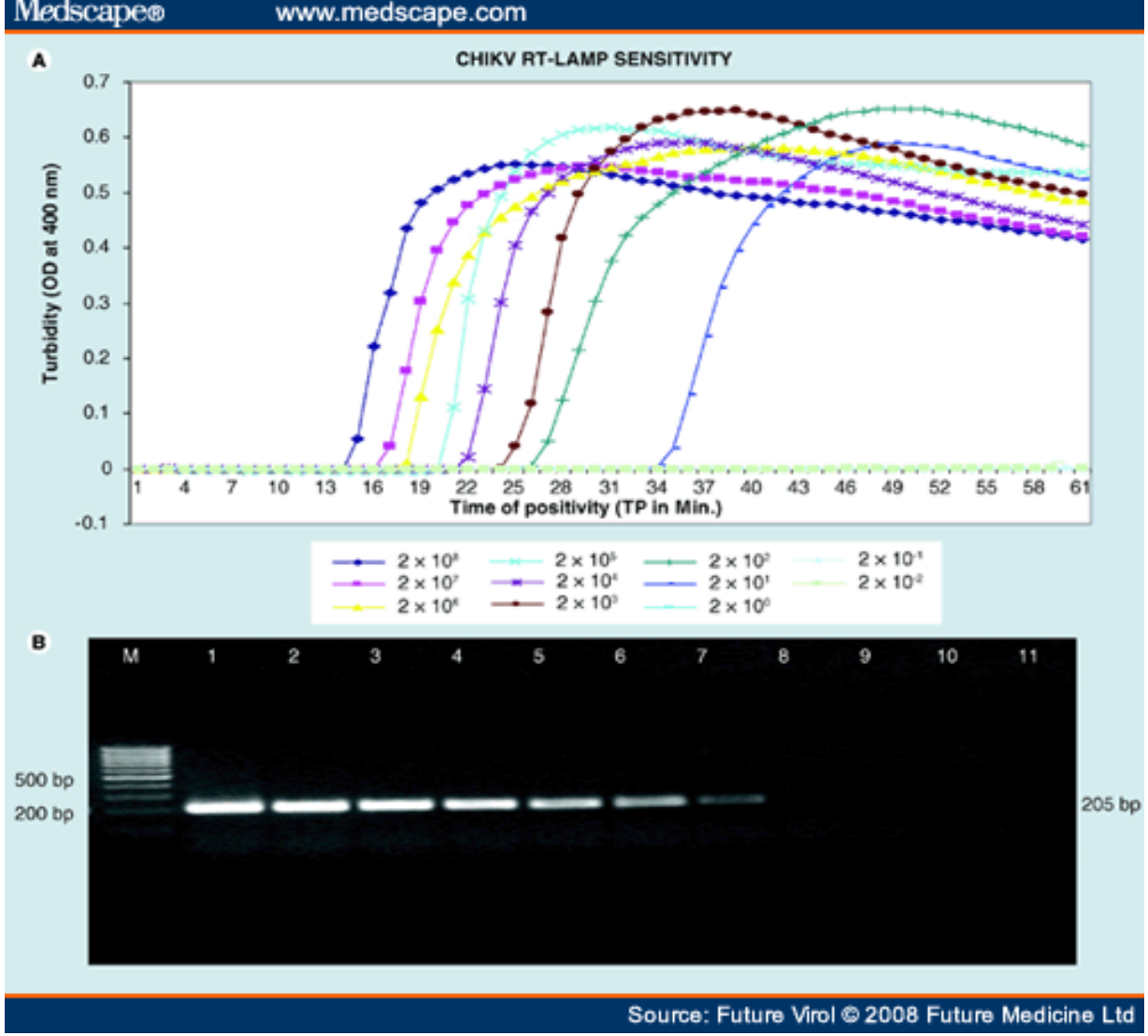
Comparative sensitivity of SYBR® Green I real-time RT-PCR assay with conventional RT-PCR for detection of the CHIKV E1 gene. (A) Sensitivity of real-time assay as shown in the amplification plot from left to right (Repl.1 to Repl.10 as shown in figure) are the curves of decreasing concentration of virus from 107 to 0.01 plaque-forming unit (PFU)/ml in serial tenfold dilution. The detection limit for the assay was 0.1 PFU/ml. (B). Sensitivity of RT-PCR for the detection of the CHIKV E1 gene as observed by 205 bp amplicon on agarose gel analysis with a detection limit of 1 PFU/ml. Lane M: 100 bp DNA ladder (Fermentas, USA); Lane 1-10: Different concentrations of virus ranging from 107 to 0.01 PFU/ml tenfold serial dilution; Lane 11: Negative control. CHIKV: Chikungunya virus; NTC: No template control; Repl.: Replicates; RT: Reverse transcription.



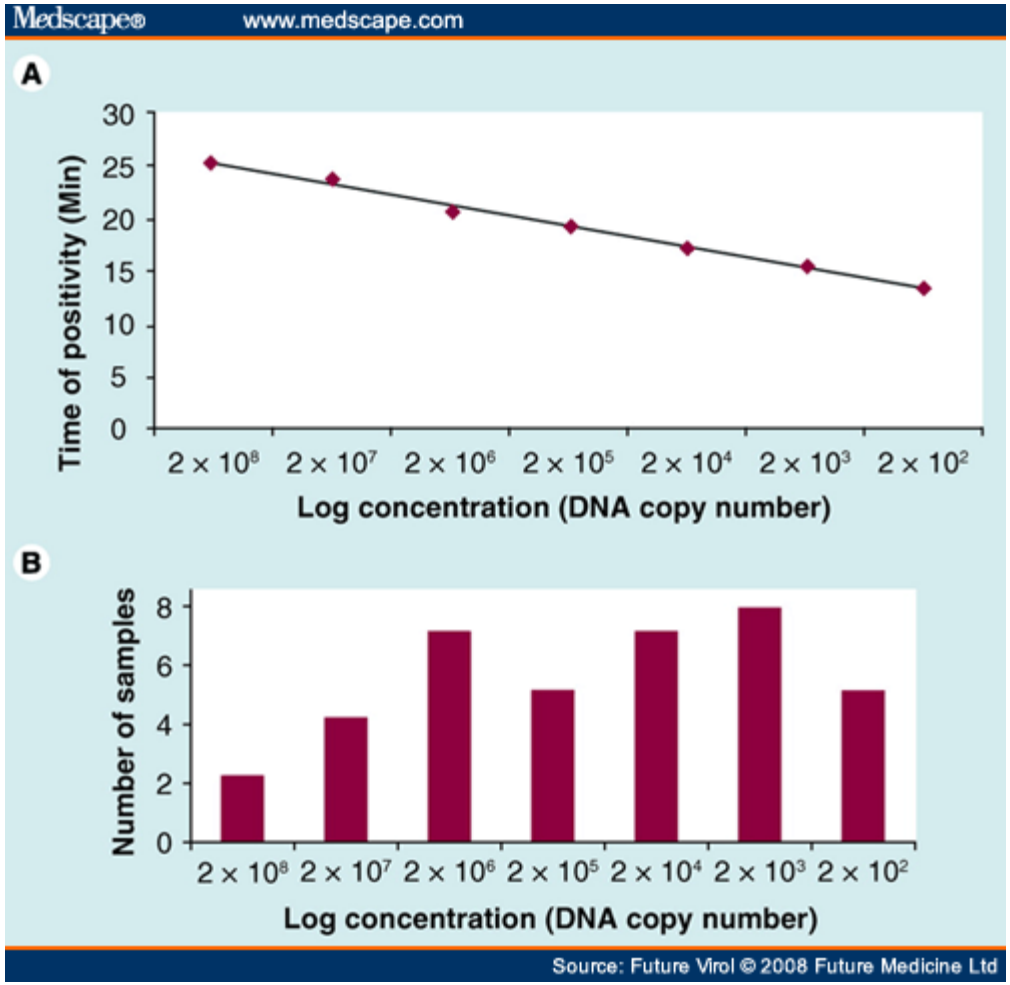
Quantitative estimation of virus load in acute phase patient serum samples by CHIKV E1 gene-specific SYBR® Green I real-time RT-PCR assay. (A) Standard curve for CHIKV-specific real-time assay generated from the cycle threshold (Ct) values obtained against the known concentration of tenfold serially diluted CHIKV ranging from 107 to 1 PFU/ml. (B) Quantitative determination of viral load in clinical samples through Ct value obtained by the clinical samples. The Ct values reflect virus concentration present in the samples through the standard curve generated for CHIKV real-time assay. CHIKV: Chikungunya virus; PFU: Plaque-forming unit; RT: Reverse transcription.



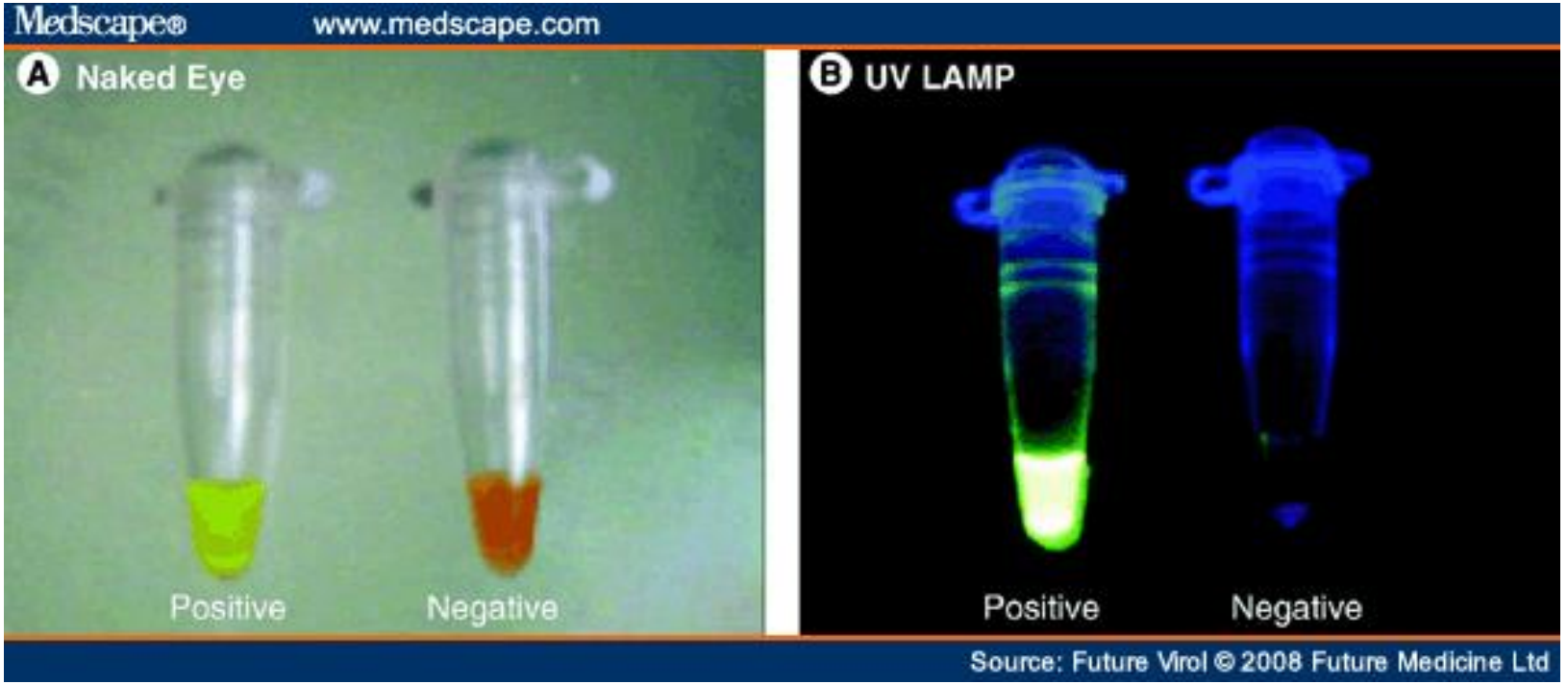
Primer designing for CHIKV RT-LAMP assay. Construction of two inner primers (FIP & BIP) having both sense and antisense sequences that help in loop formation is depicted. F1C and B2C are the complementary sequences of F1 and B2, respectively. B3: Backward outer primer; BIP: Backward internal primer; BLP: Backward loop primer; CHIKV: Chikungunya virus; F3: Foward outer primer; FIP: Foward internal primer; FLP: Foward loop primer; LAMP: Loop-mediated isothermal amplification; RT: Reverse transcription.



Comparative sensitivity of RT-LAMP with RT-PCR for detection of the CHIKV E1 gene. (A) Sensitivity of RT-LAMP assay as monitored by real-time measurement of turbidity. Shown from left to right are the curves of decreasing concentration of virus from 2 × 108 to 2 × 10-1 copy number of the template in serial tenfold dilution. The detection limit for the assay was 20 copy numbers. (B) Sensitivity of RT-PCR for the detection CHIKV E1 gene as observed by 205-bp amplicon on agarose gel analysis with a detection limit of 200 copy numbers. Lane M: 100 bp DNA ladder (Sigma, USA); Lane 1-11: Different concentration of virus ranging from 2 × 108 to 2 × 10-1 in serial tenfold dilution pattern. CHIKV: Chikungunya virus; LAMP: Loop-mediated isothermal amplification; RT: Reverse transcription.



Quantification of virus load in acute phase patient serum samples by CHIKV RT-LAMP assay. (A) Standard curve for CHIKV-specific RT-LAMP assay generated from the amplification plots between tenfold serially diluted plasmid construct and time of positivity. (B) Quantitative determination of virus concentration in clinical samples employing standard curve. CHIKV: Chikungunya virus; LAMP: Loop-mediated isothermal amplification; RT: Reverse transcription.



SYBR® Green I fluorescent dye-mediated monitoring of CHIKV RT-LAMP amplification. (A) Naked eye inspection under normal light. The original orange color of the SYBR Green I changed to yellow in the case of positive amplification whereas in a negative control having no amplification, the original orange color is retained. (B) The visual observation of green fluorescence of DNA binding SYBR Green I under UV light. CHIKV: Chikungunya virus; LAMP: Loop-mediated isothermal amplification; RT: Reverse transcription.

MM Parida, SR Santhosh, PK Dash, PV Lakshmana Rao.Rapid and Real-time Assays for Detection and Quantification of Chikungunya Virus.Future Virology. 2008;3(2):179-192.

- Gracias
- Preguntas