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MOLECULAR DIAGNOSTICS BY PCR OF POXVIRUSES (*ORTHOPOXVIRUS (OPV) AND MOLLUSCUM CONTAGIOSUM VIRUS (MCV))* IN CÔTE D'IVOIRE WEST AFRICA

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ABSTRACT

The *Orthopoxvirus (OPV)* and the *Molluscum contagiosum virus (MCV)* are Poxviruses involved in viruses skin lesions in humans. *OPV* infects many vertebrates and MCV mainly infects humans. A diagnostic confusion is often observed between the clinical lesions due to the different Poxviruses firstly and secondly with other viruses like the virus of the chickenpox. In Côte d'Ivoire, the diagnosis of MCV remains essentially clinical and that of OPV is non-existent despite the risk of circulation of the virus. This study aims to implementthe molecular detection of the OPV and the MVC in Côte d'Ivoire. Material and method: *Cowpoxvirus* DNA and 21 DNA extracts from suspicious cutaneous lesions of the MCV were analyzed by conventional PCR. The consensus primers (EACP1, EACP2) designed from the surface hemagglutin gene were used for the detection of the OPVs and the primers (MCV1, MCV2) targeting the K fragment of the MCV were used for the MCV's detection . A growing dilution series of the Cowpoxvirus DNA and the MCV allowed the study of the method's sensitivity used. The DNAs of S.*aureus, M. ulcerans, VZV, HSV, the Measles virus and Varicella virus* were used for the specificity tests. Results: The detection of the OPV from the Cowpoxvirus viral strain was positive with a positivity threshold at 10-1 dilution. That of the MCV DNA from the suspected MCV's lesion was positive with a positivity threshold of up to 10 -6 dilution. No non-specific amplification was observed with the DNAs of the other pathogens responsible for lesions Cutaneous. The clinical diagnosis of the MCV was confirmed by PCR in 18 out of the 21 patients, ie 85.71%. On the 3 patients with a negative MCV PCR, 2 were positive for the OPV PCR , reflecting the risk of confusion between clinical lesions due to Poxviruses.

Keyvords: Molecular diagnostic, Poxviruses, West Africa

MISE AU POINT DU DIAGNOSTIC MOLECULAIRE PAR PCR DES *POXVIRUS* (*ORTHOPOXVIRUS (OPV)* ET *MOLLUSCUM CONTAGIOSUM VIRUS (MCV))* EN COTE D'IVOIRE AFRIQUE DE L'OUEST.

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Résumé

Justification : *Orthopoxvirus* (OPV) et *Molluscum contagiosum virus* (MCV) sont des *Poxvirus* impliqués dans les lésions cutanées d'origine virale chez l'homme. OPV infecte de nombreux vertébrés et le MCV infecte essentiellement l'Homme. La confusion est souvent observée entre les lésions cliniques dues aux différents *Poxvirus* d'une part et d'autre part avec d'autres virus comme le virus de la varicelle. En Côte d'Ivoire, le diagnostic du MCV reste essentiellement clinique et celui des OPV est inexistant malgré le risque de circulation du virus. Cette étude vise à mettre au point la détection moléculaire des OPV et du MCV en Côte d'Ivoire. Matériel et méthode : ADN de *Cowpoxvirus* et 21 extraits d'ADN issus de lésions cutanées suspectes de MCV ont été analysés par PCR classique. Les amorces consensus (EACP1, EACP2) issus du gène de l'hémagglutine de surface ont été utilisées pour la détection des OPV et les amorces (MCV1, MCV2) ciblant le fragment K de l'ADN du *MCV* ont été utilisées. Une série dilution croissante de l'ADN du *Cowpoxvirus* et du MCV ont permis l'étude de la sensibilité de la méthode utilisée. Les ADN de *S aureus, de M. ulcerans*, du *VZV*, du *HSV*, du Virus de la rougeole et du virus de la varicelle ont été utilisés pour les tests de spécificités. Résultats : La détection de l'OPV à partir de la souche virale *Cowpoxvirus* était positive avec un seuil de positivité à la dilution 10^{-1}. Celle du MCV à partir de l'ADN de lésion suspecte de MCV était positive avec un seuil de positivité pouvant aller jusqu'à la dilution 10^{-6}. Aucune amplification non spécifique n'a été observée avec les ADN des autres pathogènes responsables de lésions cutanées. Le diagnostic clinique à MCV a été confirmé par la PCR chez 18 des 21 patients soit 85,71%. Sur les 3 patients à résultat PCR *MCV* négatif, 2 étaient positifs pour la PCR OPV traduisant le risque de confusion entre les lésions cliniques dues aux *Poxvirus*.

Mots clefs : Diagnostic moléculaire – PCR – Poxvirus – Afrique de l'ouest

INTRODUCTION

Poxviruses are double-stranded DNA with cytoplasmic multiplication. They have a number of autonomous elements allowing this intracytoplasmic multiplication unlike the majority of other viruses [1]. Several genes of this family of viruses including Orthopoxviruses (OPV) and Molluscum contgiosum virus (MCV) are involved in skin infections in humans and other vertebrates.

Since the 1980s, the smallpox virus belonging to the Orthopox virus group has been eradicated [2]. However, viruses such as Monkeypox virus, Cowpox virus and other OPVs continue to infect humans accidentally but with a less severe degree of virulence than the smallpox virus [3]. Their eradication is problematic because they have many reservoirs. The emergence of Monkeypox virus, especially in Central Africa, with the epidemiological characteristics of the epidemic of 1996 - 1997 in Zaire [3] and the appearance of the virus in 2003 in the USA makes it a global concern. Clinically, confusion has already been made between certain oxic infections and chickenpox according to the literature [5, 6]. Côte d'Ivoire is a probable zone of virus circulation [7, 8]. Despite this fact, diagnostic methods for OPVs in general and Monkeypox virus in particular are non-existent.

The MCV is increasingly encountered with HIV infection. It is often involved in skin lesions in children. To date, in Côte d'Ivoire, the diagnosis of this virus remains essentially clinical whereas atypical clinical forms were encountered with HIV infection [9, 10].

In order to monitor the emergence of this group of viruses in the population in Côte d'Ivoire, it could be necessary to set up molecular diagnostic tools to detect a certain number of viruses of this family.

MATERIALS AND METHODS:

This is an experimental study of the analytical type carried out at the Pasteur Institute of Côte d'Ivoire on the site of Adiopodoumé to the platform of molecular biology in 2016.

Biological material

In this study, the following biological products were used: strains of Cowpox virus derived from Cowpox virus culture on a Vero cell supplied by the Pasteur Institute of Bangui for the technical development of the detection of the Orthopoxviruses. Cutaneous lesions of 21 patients suspected of MCV infection for the detection of the Molluscum contagiosum virus. Microorganisms involved in cutaneous infections in Côte d'Ivoire from various biological products of patients were used for primer specificity tests (Table 1).

TABLE 1: PATHOGENS TESTED IN THE SPECIFICITY TEST OF PCR FOR THE *ORTHOPOXVIRUS* AND THE *MOLLUSCUM C VIRUS*

Number	Pathogens	Sample type	Laboratory where the pathogens used were isolated
Souche KN	***S. aureus***	**Pus**	**UBY**
258UB	***Mycobacterium ulcerans***	**Cutaneous lesion**	**GER-Buruli**
017 HSV	***Herpex simplex virus***	**Cerebrospinal fluid**	**DVE**
014 VZV	***Virus de la varicelle***	**Cerebrospinal fluid**	**DVE**
258 G	***Virus de la rougeole***	**Oropharyngeal secretion**	**DVE**

DVE: Department of epidemic viruses of the Pasteur Institute of Côte d'Ivoire, GER-Buruli: Research group on Buruli ulcer of the Pasteur Institute of Côte d'Ivoire, UBY: bacteriology unit of Hospital University Center of Yopougon

METHODS

Pre-treatment of samples

The cutaneous lesions were ground with mortar and dissolved in 2 ml of 1X PBS. They were then stored at -20 ° C until extraction of the DNA.

DNA extraction

The DNAs of the different microorganisms were extracted using the Nuclisens magnetic extraction protocol (Biomerieux). Briefly 400 µl samples were added to 800 µl of lysis buffer and incubated for 10 min. Then 40 µl of magnetic silica was added to the mix and incubated for 10 minutes at laboratory temperature. The solution was centrifuged for 30 s at 13000 rpm. A series of washing was carried out on the silica-DNA complex using the Minimag (Biomerieux). An elution buffer was used after washing to collect 25 µl of DNA.

Amplification and revelation

A PCR using consensus primers of *Orthopoxviruses* designed from the virus surface membrane HA gene were used (Forward: EACP1: 5 'ATG ACA CGA TTG CCA ATA C 3', Reverse: EACP2: 5 'CTA GAC TTT GTT TTC TG 3 '; the desired PCR product band size being 942 bp[11]. Amplification conditions were as followingfor the detection of *Orthopoxviruses*:94 ° C 5 min (1 cycle), 94 ° C 30 sec, 48 ° C 1 min, 72 ° C 1 Min) (36 cycles), 72 ° C 8 min (1 cycle). Concerning the *Molluscum contagiosum virus*, primers targeting the fragment K gene of the virus DNA were used (MCV Primer1: 5'CCGATCTTTGCGAGCGTTCTTAA 3' 'MCV Primer 2: 5'TCCCATACAGCGAGGACAGCATA 3'), the desired PCR product being 167 bp size[12]. Amplification conditions: 94 ° C 5 min (1 cycle), 94 °

C sec, 65 ° C C 1 min, 72 ° C 1 min) (36 cycles), 72 ° C 8 min (1 cycle)

The GoTaq G2 Flexi DNA polymerase kit (Promega Corporation, USA) was used for the PCR mixes containing 0.2µM of each primer, 1.5µM, MgCl, 0.1µM dNTPs, 1 unit Taq polymerase, 1X of buffer and 5µl of DNA template for a final volume of 50µl. The revelation was made on a GelDoc Bioanalyzer (BioRad) after electrophoresis on 1.5% agarose gel.

Study of sensitivity
It was made from a dilution series from the DNA extracted of the *Cowpoxvirus* viral strain for *Orthopoviruses* and a positive DNA *MCV* sample.

Study of the specificity
It was realized by carrying out PCR from the primers specific to OPV (EACP1, EACP2) and MCV (MCV1, MCV2) in the presence of DNA from different microorganisms mentioned in Table 1.

RESULTS
The PCR carried out using our positive Cowpox virus DNA control allowed us to validate our method of amplification. Indeed, two samples corresponding to the pure sample and the dilutions 10^{-1} of our controls were positive (FIG. 1). The dilution series of 10^{-2} to 10^{-5} of the Cowpox virus DNA control obtained from 200 µl of viral strain on Véro cell being negative by PCR thus fixing the detection threshold of our method at 10^{-1} for a volume of 200 µl.of viral strain. No amplification was detected with the DNAs from other pathogens frequently involved in cutaneous infections in Côte d'Ivoire (Figure 2).

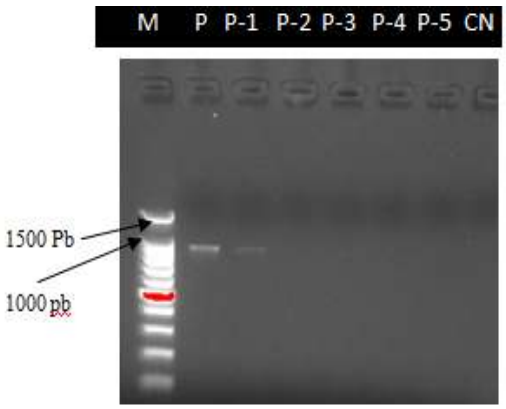

FIG 1 :DILUTION SERIES OF COXPOXVIRUS DNA

(P = undiluted Cowpoxvirus DNA, P-1 to P-5 = dilution series

CN: Negative control M: Marker (1500 bp to 100 bp)

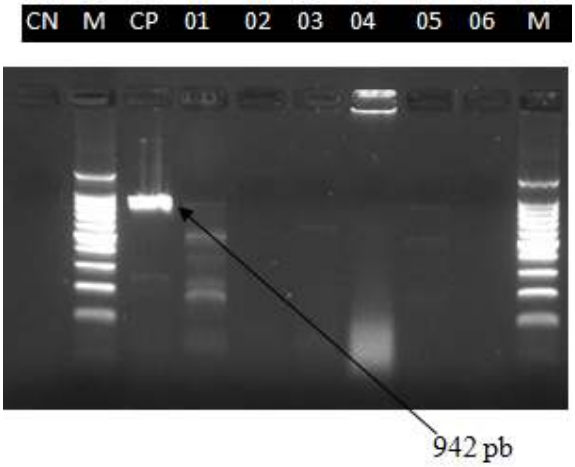

FIG 2 : SPECIFICITY TEST RESULT

CP = Cowpoxvirus (942 bp), 01 = MCV, 02 = 845 G (Measles virus, 03 = 258ub (M ulcerans), 04 = S aureus, 05 = 014 VZV (varicella

This sensitivity could improve by performing extraction with a larger volume of viral strain.

The MCV detection tests were performed using cutaneous lesions from 21 patients diagnosed with MCV infection clinically. The patients had the epidemiological and clinical characteristics presented in Table 2.

TABLE 2: CHARACTERISTICS OF THE 21 PATIENTS WHOSE SAMPLES WERE TESTED FOR THE DETECTION OF MCV

Number	Age	Sex	Clinical Lésion
1 /YOP	15 Months	M	PAPULES
2/YOP	8 Years	M	PAPULES
5/YOP	24 Years	F	PAPULES
6/YOP	7 Years	F	VESICLES
7/YOP	4 Years	M	CRUSTS
8/YOP	3 Years	M	VESICLES
9/YOP	5 Years	F	MACULES
10/YOP	4 Years	F	PUSTULES
11/YOP	3 Years	M	MACULES
12/YOP	13 Years	M	VESICLES
13/YOP	7 Years	F	MACULES
14/YOP	5 Years	F	MACULES
2306	6 Years	F	VESICLES
2503	4 Years	F	PAPULES
2602	4 Years	F	VESICLES
2648	6 Years	F	PAPULES
2649	9 Years	M	PAPULES
2650	6 Years	F	PAPULES
2647	3 Years	F	PAPULES
2761	2 Years	F	Unspecified
2762	28 Years	F	Unspecified

A test for detection of *MCV* was carried out with the first three samples (1 / YOP, 2 / YOP, 5 / YOP). Each sample was diluted to 10^{-1} to reduce the risk of PCR inhibition (Figure 3). The 167 bp PCR product was revealed for all three samples corresponding to positive MCV detection results.

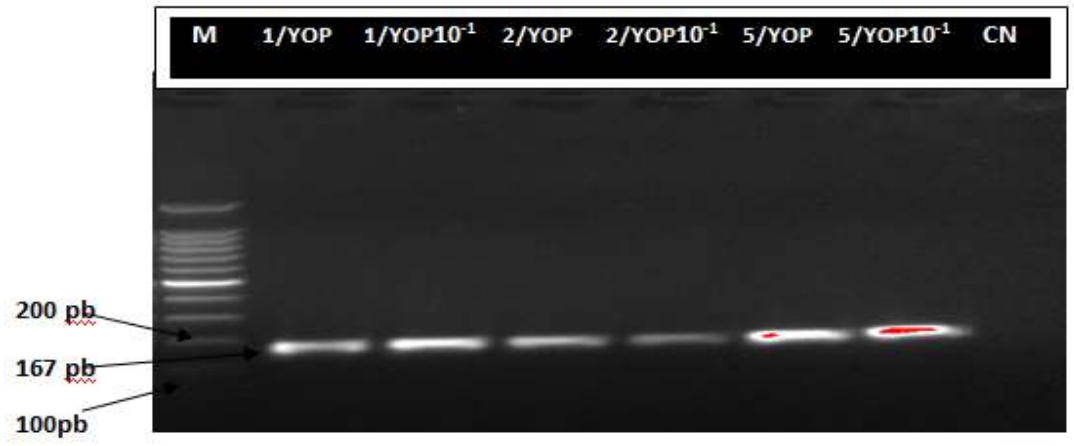

FIGURE 3: MCV PCR TEST OF SAMPLES

1 / YOP, 2 / YOP and 5 / YOP, CN = negative control

A dilution series of 10^{-1} to 10^{-9} was carried out with the 1 / YOP sample in order to determine the sensitivity threshold of the PCR. Positive bands were observed up to 10-6 dilution (Figure 4). The specificity tests carried out with the DNA of other pathogens did not reveal nonspecific amplifications due to microorganisms often involved in human skin lesions. Of the 21 suspected samples of MCV infection, 18 were confirmed on the 21, *ie* 85.71% agreement between the clinic and the molecular test used. Samples 2503, 2306 and 010/YOP were negative (Figure 5). Of the three negative samples, two were found to be positive for the conventional PCR detection of OPVs of sample 2503 and 010/YOP.

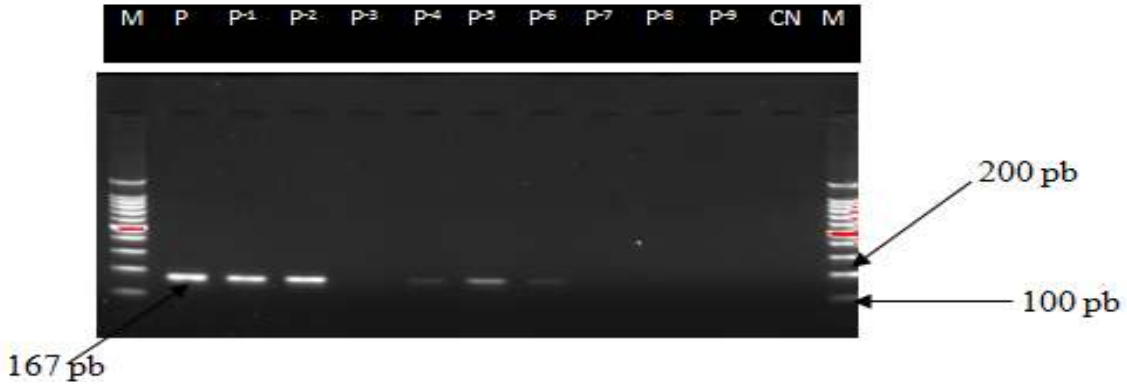

FIG 4: MCV DNA DILUTION SERIES

(P = undiluted MCV DNA, P^{-1} to P^{-9} = dilution series, CN: Negative Control M: Marker (1500 bp to 100 bp

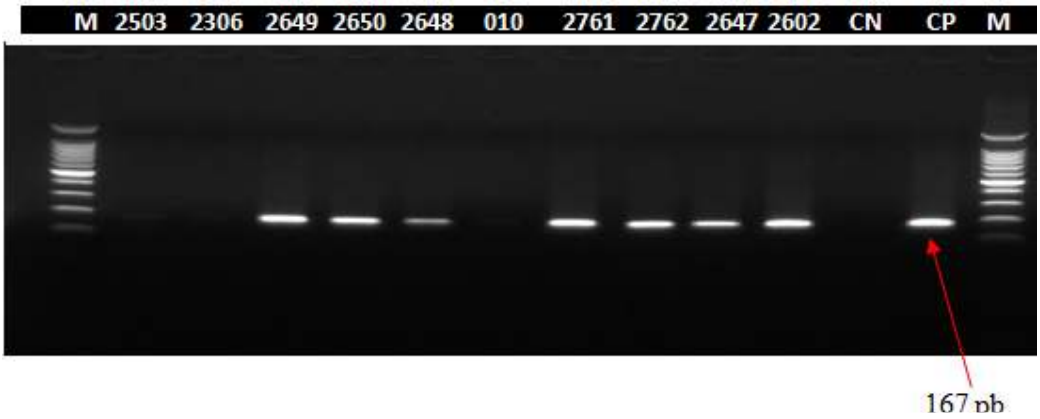

FIG 5 : RESULTS OF PATIENTS SUSPECTED OF MCV INFECTIONS

2503, 2306 and 010 = 10/YOP are negative. CN: Negative control CP: Positive control, M: Marker (1500 bp to 100 bp)

DISCUSSION

Molecular tools are increasingly being developed for the diagnosis of *Poxviruses* [13, 14, 15]. They have the advantage, on the one hand, of making a rapid and precise diagnosis and of others by avoiding the constraints of biosafety and biosurety related to the cultivation of Poxviruses; this development of the molecular detection for *Poxviruses* in our laboratory falls within this framework. It is one of the first studies in the establishment of the molecular diagnosis of *Poxviruses* in human medicine in Côte d'Ivoire. Despite the fact that this study may present biases related to the absence of a reference strain of *Poxvirus* whose acquisition remains subject to rigorous measures in this context of global bioterrorism, this study constitutes a starting point in the implementation of the development of molecular tools for the detection of *Poxviruses* in Côte d'Ivoire. A discrepancy between the clinical diagnosis of *MCV* infection and the molecular outcome in three patients was observed in this study. Thus, two of the patients presented with *OPV* lesions. This clinical confusion is related to the similarity between the clinical lesions due to the different genera of the *Chordopoxviridae* subfamily. Thus, co-circulations have also been reported [16,

17, 18], hence the need for a biological diagnosis despite the strong clinical suspicion.

CONCLUSION

The emergence of Monkeypox virus in Central Africa is a signal for West Africa. It is necessary to set up rapid diagnostic methods. This study is part of this approach. However, its sensitivity needs to be improved. Real-time PCR implementation is also a solution.

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