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Research Article

PROTEIN PROFILING OF NASOPHARYNGEAL CELLS OF PATIENTS OF PANDEMIC INFLUENZA A (H1N1) 2009 VIRUS TREATED WITH OSELTAMIVIR OXALATE

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ABSTRACT

In 2009, Pandemic Influenza A viruses (H1N1) evolved as yet another deadliest waves, 91 years after its initial emergence in 1918 followed by 1957 and 1977. The gap in the pandemics creating major shift helps us realize the importance of continuous studies from all aspects to understand trends of human infection and to understand existence and survival of these viruses during non-transmissions period. With all the studies being pursued on hospital based surveillance, regional and inter-continental epidemiology, genomics and bioinformatics, study of proteomics of this segmented genome is also essential. Present study is based on the proteomic approach to understand the cellular happenings of cells infected with the virus and how drug dose affects the viral proteins to detach from the cell surface. The Oseltamivir treated nasopharyngeal cells of primary and control cases when subjected to SDS-PAGE expressed disruption in former while discrete bands of Neuraminidase proteins in the latter.

Keywords: *Proteomics of H1N1 Viruses, H1N1 Viruses, Oseltamivir, SDS-PAGE*

INTRODUCTION

The occurrence of Pandemic Influenza A (H1N1) 2009 viruses led to enormous number of morbidity and mortality worldwide with an estimated number of 60.8 million cases, 274,304 hospitalizations and 12, 469 deaths during April 2009 through April 2010, in United States of America where it first appeared (Shrestha *et al.,* 2011).

India too experienced its first case in May 2009 (Gurav *et al.,* 2010) with the disease spreading to various parts of the country affecting the state of Rajasthan in November 2009 (Joshi *et al.*, 2012). The Influenza A virus belongs to the family Orthomyxoviridae and is known to be transmitted via aerosols from infected person as well as by coming in contact with infected surfaces.

Running nose, sore throat, high grade fever, body aches are the common symptoms observed in patients infected with the virus.

Though the World Health Organization, has marked the arrival of post pandemic period of the disease, yet it has also confirmed presence of localized outbreaks in some parts of the world including India and has also alerted for its re-appearance (http://www.who.int).

Many studies have been reported on diagnostics, drugs and genomics aspects of Pandemic Influenza A (H1N1) 2009 viruses (Rambauti *et al.,* 2008; Mishra *et al.,* 2010; Dong *et al.,* 2010; Wang *et al.,* 2009; Potdar *et al.,* 2010), however, the important issue of host-virus interaction focusing on membrane proteins of nasopharyngeal cells has not been addressed.

Present paper reports the protein profiling of nasopharyngeal cells of patients treated with Tamiflu. As Oseltamivir is known to inhibit Neuraminidase (NA) protein detaching virus progeny, present study may prove to be of clinical significance in understanding post treatment viral pathogenesis.

MATERIALS AND METHODS

Sample Collection

The nasopharyngeal swab samples of patients having Influenza like-illness (ILI) were taken by the treating physicians at Dr.SN Medical College, Jodhpur, Rajasthan. For detection of virus presence, the samples in Virus Transport Medium (VTM) (as per W.H.O. guidelines) were brought to the Laboratory of Virology & Molecular Biology, Desert Medicine Research Centre, Jodhpur which is one of the referral laboratory for diagnostics in the state of Rajasthan.

Sample Collection for Proteomics Analysis

As the chemical constituents of VTM would interfere and give unrequired results, hence, to study the virus infected nasopharyngeal cells and the effect of oseltamivir on these cells, the cells needed to be collected directly in protein digestion buffer. During field survey, patients reporting positive for Pandemic Inf A (H1N1) 2009 viruses (as per the diagnostic reports) were visited. They were examined for some physical parameters like presence of inflammation in the nasopharyngeal cells, pH of the infected cells etc. The patients were examined by the treating physician using all personal protection and after informed consent, two swab samples were collected from the patient, one was collected in vials containing Protein Digestion buffer and the other was collected in vial containing VTM and brought to the laboratory. The Swab in VTM was collected to check the response of Oseltamivir dose on the infected cells. Similar swab samples were taken from the patients' family members also to be used as a negative control during the study. During the survey the dose of Tamiflu taken and also details of family members were also noted.

Molecular Biology Studies

The swab samples collected in VTM were subjected to RNA extraction protocol using the QIA viral RNA kit (m/S Qiagen, CA). The W.H.O. recommended primers for diagnosis of Pandemic Inf A (H1N1) 2009 viruses were used for amplification of the virus employing the Real Time RT-PCR (m/s ABI, USA) and ct values obtained were noted.

Proteomic Studies

The swab samples under biosafety measures were taken for proteomic assay. A 11% SDS-PAGE gel was prepared according to the manufacturer' protocol (m/s Bio-RAD, USA). 10µl of the sample in protein buffer was digested and heated and then loaded on to the gel and run at 150 volts for 1:50 hours. Broad range Molecular marker (m/s Bio-Rad, USA) was also leaded in one of the wells. The gel was then stained using the Coomassie blue stain according to the manufacturer's protocol (m/s Bio-RAD, USA) which was then visualized using the Gel Documentation System (m/s Bio-Rad, USA).

RESULTS AND DISCUSSION

Results

A total of 14 primary (1˚) cases infected with Pandemic Inf A (H1N1) 2009 viruses were examined. In addition, 23 Family members of patients were also examined and their swab samples were studied. The details of the cases are mentioned in Table 1. For ease of understanding, the hospital diagnosed cases are referred as (1˚) cases. Family members of the patients are also mentioned along with their relation to the patient (listed as suspected case in Table 1). Since during the 2009 pandemics, the family members were advised to take Oseltamivir as precautional measure, the dosage taken during the time of our visit were also recorded. The dosage of Oseltamivir taken in primary patients ranged from 15 to 2 while that of the family members ranged from 9 to 0. Besides this, pH of the nasopharyngeal swab was also checked which ranged from 4 to 7 indicating acidic medium during infection to normal pH during recovery.

Sample no. 1b was a repeat study of Sample no. 1a which was of a 35 years pregnant lady who after 3 dosage of drug still showed positivity for viral gene and so yet another swab was collected after a dosage of 15 tablets and was then found negative for virus presence through Real Time RT-PCR (Table 1). Sample no. 30 was an earlier positive case which had recovered from infection during the time another patient (sample no. 29) got infected from the same household.

During the survey, movement of the 1˚ patient was also noted to understand the risk and transmission of disease in the study area. It was observed that of the 14 cases studied; 4 had attended a city fair which is

very religiously visited by devotees in flock from near and far by places, 3 had went outstation, 3 visited hospital, 2 had attended school and 1 attended a marriage ceremony, while 1 acquired had it from in house earlier infected case.

Since the samples of the family members were collected during the survey i.e. visit to their household, hence no data under the heading "RT-PCR pre survey" is available and hence mentioned ND (not done) to avoid confusion (Table 1).

Molecular Biology Studies Employing Real Time RT-PCR Assay

The Real time RT-PCR reports of the patients showed presence of Pandemic Inf A (H1N1) 2009 viruses in the pre survey phase i.e. the time when they were referred hospital due to Influenza like illness. It was seen that before drug administration all the 1° cases were positive and after dosage of Oseltamivir, they were found to be negative except in case no. 5 (sample no. 9 & 10) which were found positive. An interesting analysis seen here was that in the 1° patient, ct values for Inf A (35.0) and Sw H1(37.0) showed very less viral load after an administration of 4 doses which was enough to transmit virus to another person who too showed same ct value (Inf A: 36.0; Sw H1: 40.0) and had 1 precautionary dosage (Table 1).

Proteomic Studies

The SDS-PAGE analysis of the swab samples of 14 primary cases along with their respective members were performed. The expected MW of Neuraminidase protein is 50-55 kDa. Protein disruption was observed in the samples which were treated with drug as shown in Figure 1 (A to D). As shown in table 2, protein disruption was observed in all the 1° samples, except sample no. 24 which did not show any bands. Besides this disruption was also seen in some suspected family members i.e. sample no. 10, 12, 14, 30 and 31 on account of drug being taken as precautionary measure.

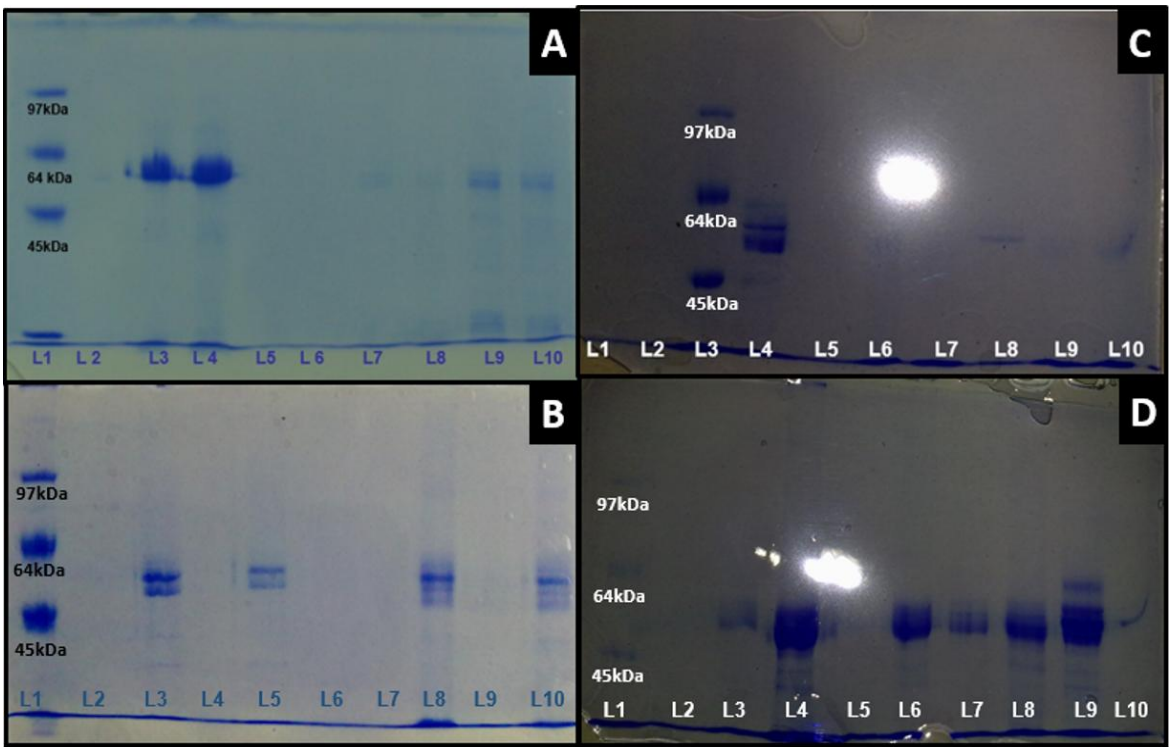

Figure 1: Protein Profile of 1° Cases and Suspected Family Members as Observed after Post Drug Administration; A-L1: Broad Range Standard; L3 & L4: 1° Cases; L7 to L10: Suspected Family Members; B-L1: Broad Range Standard; L3& L5: 1° Case; L8 & L10: Suspected Family Members; C-L3: Broad Range Standard; L4: 1° Case; L6 to L10: Suspected Family Members; D-L1: Broad Range Standard; L3, L7 to 9: 1° Cases; L4 to 6, L10: Suspected Family Members

Table 1: Pre and Post Dosage Analysis of Patients Infected with Pandemic Inf A (H1N1) 2009 Viruses

Case No	S. No	Case Details (Sex, Age, Relation etc.)	Movement to Prior Infection	Inflammation Seen on Nasopharyngeal Area (NP)	pH of NP Region	RT-PCR Report Pre- Survey				Dosage of Oseltamivir	RT-PCR Report Post- Survey			
						Inf A	Sw A	Sw H1	+ve/-ve		Inf A	Sw A	Sw H1	+ve/-ve
1	1a	F, 35 Pregnant, (1˚ case)	Attended Marriage	High	6	24.7	34.7	23.5	+ve	3	31.6	UD[#]	38.5	+ve
	1b	F, 35 Pregnant (repeat test of S.No 1, 1˚ case)	-	Less	7	31.6	UD	38.5	+ve	15	UD	UD	UD	-ve
	2	F, 58 Suspected case (mother)		No	7	ND[*]	-	-	-	1	UD	UD	UD	-ve
	3	M, 31 suspected case (brother)		No	7	ND	-	-	-	0	UD	UD	UD	-ve
2	4	F, 28 Pregnant, (1˚ case)	Outside city	Yes	4	29.6	UD	31.4	+ve	2	UD	UD	40.6	-ve
	5	M, 33 Suspected case (husband)		No	7	ND	-	-	-	0	UD	UD	UD	-ve
3	6	M, 59 (1˚ case)	Attended fair	No	7	15.7	32.1	31.2	+ve	3	38.2	UD	UD	-ve
	7	F, 55 Suspected case (wife)		No	7	ND	-	-	-	0	UD	UD	UD	-ve

4	8	F, 23 (1˚ case)	Visited Hospital	Yes	6	18.8	UD	31.5	+ve	10	UD	12.5	UD	-ve
5	9	M, 37 (1˚ case)	Attended fair	Yes	6	33.1	30.4	30.0	+ve	4	35.0	UD	37.0	+ve
	10	F, 35 Suspected case (wife)		Yes	7	ND	-	-	-	1	36.0	UD	40.0	+ve
6	11	F, 8 (1˚ case)	School , swimming pool	Less	7	35.6	UD	41.4	+ve	4	41.0	UD	UD	-ve
	12	M, 10, Suspected case (brother)		No	8	ND	-	-	-	3	UD	UD	UD	-ve
	13	F, 32 suspected case (mother)		No	6	ND	-	-	-	1	UD	UD	UD	-ve
	14	M, 37 suspected case (father)		No	7	ND	-	-	-	2	UD	UD	UD	-ve
7	15	M, 26 (1˚ case)	Boarded Train	Yes	6	23.1	30.0	30.9	+ve	5	UD	UD	UD	-ve
	16	F, 25 suspected case (wife)		No	7	ND	-	-	-	0	UD	UD	UD	-ve
	17	F, 47 suspected case (mother)		Yes	7	ND	-	-	-	0	UD	UD	UD	-ve
8	18	F, 50 (1˚ case)	Attended fair	Yes	6	21.6	31.1	27.6	+ve	2	UD	UD	UD	-ve
	19	M, 56 suspected case (husband)		No	7	ND	-	-	-	0	UD	UD	UD	-ve

	20	M, 28 suspected case (son)		No	7	ND	-	-	-	0	UD	UD	UD	-ve
	21	F, 14 Suspected case (daughter)		No	7	ND	-	-	-	0	34.3	UD	UD	-ve
9	22	M, 30 (1˚ case)	Visited Hospital	Less	7	35.2	31.3	31.9	+ve	9	UD	UD	UD	-ve
	23	F, 34 suspected case (wife)		No	7	ND	-	-	-	5	UD	UD	UD	-ve
10	24	M, 61 (1˚ case)	Attended fair	Yes	6	21.6	UD	29.1	+ve	11	UD	UD	UD	-ve
	25	F, 58 suspected case (wife)		No	6	ND	-	-	-	1	UD	UD	41.8	-ve
	26	M, 28 suspected case (son)		No	7	ND	-	-	-	1	20.6	UD	UD	-ve
	27	F, 53 suspected case (daughter)		No	6	ND	-	-	-	1	UD	UD	UD	-ve
11	28	F, 28 (1˚ case)	Boarded Train	No	7	25.4	27.7	29.7	+ve	5	44.4	40.0	35.6	-ve
12	29	M, 50 (1˚ case)	Transmitted case	No	6	22.1	22.6	25.0	+ve	5	UD	UD	UD	-ve
	30	F, 24 (daughter in law)	Earlier case	No	6	22.4	27.5	41.5	+ve	9	UD	UD	UD	-ve
	31	F,50 suspected case (wife)		No	7	ND	-	-	-	4	UD	UD	41.3	-ve
13	32	F, 28 (1˚ case)	------	No	6	26.8	28.2	29.0	+ve	7	UD	UD	UD	-ve
	33	M, 28		No	7	ND	-	-	-	2	UD	UD	UD	-ve

		suspected case (husband)											
14	34	M, 15 (1˚ case)	School	6	26.2	28.5	29.2	+ve	9	UD	UD	UD	-ve
	35	F, 17 suspected case (sister)		7	ND	-	-	-	8	UD	UD	41.5	-ve
	36	F, 13 suspected case (sister)	Yes	6	ND	-	-	-	4	UD	UD	UD	-ve
	37	F, 14 suspected case (sister)	Yes	6	ND	-	-	-	4	UD	UD	UD	-ve

*ND= Not done; #UD= Gene undetected

Table 2: Proteomics Analysis of Nasopharyngeal Cells of Patients Administered with Oseltamivir

Case No	S. No	Case Details (Sex, Age, Relation etc.)	Dosage of Oseltamivir	Inflammation of Nasopharyngeal (NP) Area	pH of NP Region	NA Protein Disruption Observed in SDS-PAGE gel
1	1a	F, 35, Pregnant (1˚ case)	3	Highly	6	√
	1b	F, 35, Pregnant (repeat test of S.No. 1a, 1˚ case)	15	Less	7	√
	2	F, 58, Suspected case (mother)	1	No	7	X
	3	M, 31, suspected case (brother)	0	No	7	X
2	4	F, 28, Pregnant (1˚ case)	2	Yes	4	√ (partial)
	5	M, 33, Suspected case (husband)	0	No	7	X
3	6	M, 59, (1˚ case)	3	No	7	√ (partial)
	7	F, 55, Suspected case (wife)	No	No	7	X
4	8	F, 23, (1˚ case)	10	Yes	6	√
5	9	M, 37, (1˚ case)	4	Yes	6	√ (partial)

	10	F, 35, Suspected case (wife)	1	Yes	7	√ (partial)
6	11	F, 8, (1˚ case)	4	Less	7	√ (partial)
	12	M, 10, Suspected case (brother)	3	No	8	√
	13	F, 32, suspected case (mother)	1	No	6	X
	14	M, 37, suspected case (father)	2	No	7	√
7	15	M, 26, (1˚ case)	5	Yes	6	√
	16	F, 25, suspected case (wife)	0	No	7	X
	17	F, 47, suspected case (mother)	0	Yes	7	X
8	18	F, 50, (1˚ case)	2	Yes	6	√
	19	M, 56, suspected case (husband)	0	No	7	X
	20	M, 28, suspected case (son)	0	No	7	X
	21	F, 14, Suspcted case (daughter)	0	No	7	X
9	22	M, 30, (1˚ case)	9	Less	7	√
	23	F, 34, suspected case (wife)	5	No	7	X
10	24	M, 61, (1˚ case)	11	Yes	6	No bands appeared
	25	F, 58, suspected case (wife)	1	No	6	X
	26	M, 28, suspected case (son)	1	No	7	X
	27	F, 53, suspected case (daughter)	1	No	6	X
11	28	F, 28, (1˚ case)	5	No	7	√ (partial)
12	29	M, 50, (1˚ case)	5	No	6	√
	30	F, 24, (daughter in law)	9	No	6	√
	31	F,50, suspected case (wife)	4	No	7	√
13	32	F, 28, (1˚ case)	7	No	6	√
	33	M, 28, suspected case (husband)	2	No	7	X
14	34	M, 15, (1˚ case)	9		6	√
	35	F, 17, suspected case (sister)	8		7	X
	36	F, 13, suspected case (sister)	4	Yes	6	X
	37	F, 14, suspected case (sister)	4	Yes	6	X

Discussion

Present studies highlight the observations on disruption of neuraminidase viral protein in the H1N1 cases which were treated by the Oseltamivir. We have attempted to correlate the diminishing trend of viral protein as imbibed within nasopharyngeal cells of patients with the intake of drug compound in use. Although, few studies have been made on the proteomic aspects of H1N1 Influenza A viruses addressing different factors and molecular marker detection (Zhang *et al.,* 2010; Tretheway *et al.,* 2011; Choi *et al.,* 2014; Morrissey & Downard, 2006) yet present study is first of its kind to report a real time variation in fate of viral protein of Pandemic Inf A (H1N1) 2009 virus infected patients comparing the observations on the healthy controls.

Kummer *et al.,* (2014) had studied alterations in protein profile of MDCK cell lines as influenced by the induced infection from H1N1 viruses. However, in present studies we have focused on the real time, in-vivo observations to establish the effectiveness of drug compound Oseltamivir on the Neuraminidase protein. Such studies could be useful in ensuring the effectiveness of drug during an ongoing outbreak of H1N1 Influenza A viruses ensuring that disease transmission strength of the virus during an outbreak is getting weaker or not.

The genomic studies done through Real Time RT-PCR compares the infectivity caused by virus with the recovery occurred after the drug intake. The table 1 shows a trend of ct values moving from lower to higher value which help in understanding quantitative analysis of viral load within the patient. The sample no. 1 who after a total dose of 18 tablets was in a recovery stage showed protein disruption even at an initial stage indicates high viral load and requirement of a continuous regime of drug to eliminate virus from the system as compared with other primary patients. The proteomic and molecular studies reported herein show how qualitatively and effectively the drug help inhibit the viral proteins. The protein profile of the 14 cases studied also gives an understanding on how immune response or cell physiology is host specific during the virus-drug interactions.

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