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Comparative evaluation of real-time PCR and conventional RT-PCR during two year surveillance for Influenza and RSV among children with acute respiratory infections in Kolkata reveals distinct seasonality of infection

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Running title: Real-Time PCR based surveillance of Influenza and RSV

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Abstract

Acute respiratory tract infections (ARTI) are one of the most common cause of morbidity and mortality in young children all over the world. Influenza and Respiratory Syncytial viruses (RSV) are the predominant etiology during seasonal epidemics and thus rapid and sensitive molecular tests for screening and timely identification of epidemics are required. In this study we compared real time PCR (Q-PCR) with conventional RT-PCR for parallel identification of Influenza A or B (Inf-A or -B) and RSV. A total of 1091 respiratory samples were examined from children with suspected ARTI during January 2007 - December 2008. Of these, 275 (25.21%) were positive for either Influenza or RSV by Q-PCR compared to 262 (24%) positives by RT-PCR. Overall Inf-A, -B and RSV were detected in a total of 121 (11.075%), 59 (5.38%) and 95 (8.68%) samples respectively. In spite of overlapping clinical symptoms, RSV and Influenza showed distinct seasonal peaks. Inf-A positively and RSV, negatively correlated with rainfall and temperature. No distinct seasonality was observed in Inf-B infections. This is the first report of a systemic surveillance of respiratory viruses with seasonal correlation and prevalence rates from Eastern India. The two year comparative analysis also confirmed feasibility of using Q-PCR in developing countries, which will not only improve scope for prevention of epidemics but also provide crucial epidemiological data from the tropical regions.

Introduction:

Acute Respiratory tract infections (ARTI) are among the most common cause of significant morbidity and mortality in young children, elderly and immunocompromised patients, with the greatest number of deaths occurring in developing countries (William *et al*., 2002). One fourth (2.5 million) of the total deaths among children less than 5 years of age occur in India, and approximately 20% of these are due to ARTI (Rao 2003; Reddaiah & Kapoor, 1998). Besides bacterial infections, Influenza and RSV have been identified as the predominant etiology for lower respiratory tract infections (Ieven 2007, Thompson *et al*., 2004; Stockton *et al*., 1998). These viruses result in rapid onset of symptoms which include fever (Temp, $\geq$ 38.8^{o}C), headache, cough, chill and sore throat. In children with chronic abnormalities of pulmonary function, infections with Influenza or RSV have been shown to aggravate asthma (Wang & Forsyth, 1998). Since the successful treatment needs to be initiated within 24-48h of infection, rapid diagnosis of viral pathogens during regular surveillance studies is of utmost importance. Moreover timely identification of epidemics, seasonality and burden of strain subtypes in community are important for proper clinical interventions (Adcock *et al.*, 1997; van Elden *et al*., 2002; Ruest *et al*., 2003; Liao *et al*., 2009).

Diagnostic methods currently used for the detection of respiratory infections in clinical laboratories include rapid antigen tests, virus culture, enzyme immunoassay (ELISA), immunofluorescence (IF), and conventional reverse-transcriptase (RT) PCR assays (Falsey *et al*., 2002; Ruest *et al*., 2003). Virus culture is considered gold standard test and it also provides reference strains for vaccine development, genetic characterization and *in-vivo* studies to understand pathogenesis, however it takes 7-12 days to get culture positives (van Elden *et al*., 2002). In the last decade, molecular diagnostics such as RT-PCR and quantitative real time PCR (Q-PCR) have gained importance due to their higher sensitivity. These tests can also detect more

than one pathogen in a single reaction by simultaneously using multiple probes (multiplex PCR) to reduce the time and cost (Fredricks & Relman, 1999; Stockton *et al*., 1998; Boivin *et al*., 2004).

The recent outbreaks of avian Influenza (H5N1) and the novel H1N1 (swine flu) worldwide serves as a grim reminder that one more Influenza pandemic appears to be in horizon, thus continuous surveillance for respiratory viruses with focus on Influenza in the developing countries is the key for controlling the pandemic. Very little virological or epidemiological data is available regarding respiratory viruses in Eastern or South-Eastern Asian countries except for few reports from Japan, Thailand, Taiwan Singapore and Hongkong (Viboud *et al*., 2006; Simmerman & Uyeki, 2008; Park & Glass, 2007). India is a large country and close to 20% of its population is below 5 years of age. It has a tropical climate with distinct seasonal variations from north to south of country. In India effects of Influenza pandemics in 1889, 1918 (H1N1), 1957 (H2N2) and 1968 (H3N2) (Rao & Banerjee, 1993) were also felt (Rao & Banerjee 1993; Rao 2003). In 1968, Hongkong flu (H3N2) epidemics were reported from Maharashtra, Andhra Pradesh, Kolkata, Nepal and other regions. Unfortunately very little epidemiological or virological information on respiratory viruses has been reported from India in the last decade, as respiratory diseases were not taken seriously compared to other infectious diseases like AIDS, cholera and malaria etc. Due to multiple outbreaks of highly pathogenic H5N1 virus in poultry in India during 2006-2009, we started a surveillance for circulating respiratory viruses among children under 5 years of age in Kolkata, a metropolitan city in West Bengal, India (88^{o} 18'E, 22^{o}39'N) to understand frequency of Inf-A or -B and RSV infection. The frequency of the Influenza or RSV positivity was correlated with the meteorological factors to understand the seasonality in Eastern India. In this study, two multiplex Q-PCR assays were used for detection

of Inf-A & -B, RSV and RNaseP respectively. The assay was also compared with conventional RT-PCR and virus culture to assess its potential application in routine surveillance and diagnosis.

Materials and Methods

Sampling site and Study Population

Nasal and/or throat swabs were collected from 1091 children under 5 years of age exhibiting fever and 2 or more symptoms of ARTI (cold/cough, sore throat, myalagia, bodyache) from the outdoor patient ward of B. C. Roy Memorial Hospital for children (BCRMHC), Kolkata, India for 2 years (Jan 2007-Dec 2008). The BCRMHC is one of the largest children hospitals in Eastern India, treating patients from rural and urban areas located in and around (up to 80 km) Kolkata. No hospitalized patients were included in the study to rule out nosocomial infections. Specimens were transported in viral transport media (VTM- Hanks balanced salt solution, Penicillin-Streptomycin and 2% BSA) to the laboratory. 200 µl of sample was processed for viral RNA isolation immediately on receipt of sample.

The study was approved by the Institutional Ethical Committee and informed consent was taken by the guardian of patients before collection of samples.

Extraction of viral RNA

RNA was extracted from the clinical samples using commercially available RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) as per manufacturer's instructions. The RNA was stored in -80^0 C in aliquots for subsequent assays.

Primers and Probe Design

Two one step multiplex Q- PCR assays were standardized. For the first multiplex assay, primers were designed from the conserved regions of matrix (M) genes of Inf-A and Inf-B. The

conserved Matrix gene primers (Inf-A) were cross-checked for sequence identity by BLAST analysis with >100 published sequences of Influenza subtypes (H1N1, H3N2, H5N1, swine H1N1) reported from human samples. The Inf- A, Inf-B and RNaseP primers and probe sequences used in the study were provided by CDC (available on request from S. Lindstrom, Center for Disease Control and Prevention USA). Second multiplex assay was specific to the polymerase (L) gene of RSV and the RNase P (positive internal control) (Templeton *et al*., 2004). A total of three different reporter dyes namely FAM (Inf A, RNase P), VIC (Inf-B) and HEX (RSV) were used in the study where as the quencher dye was BHQ-1.

Quantitative Real-Time PCR

TaqMan Q- PCR was performed on a ABI Prism 7500 sequence detection system using one step RT-PCR kit (Invitrogen Corporation, USA), in a 25 µl reaction mixture containing 5 µl each of extracted RNA, 12.5 µl of 2x reaction mix with ROX, 0.5 µM of each primer and probe, 20U of RNase OUT and 0.5 µl superscript III RT/platinum Taq Mix as per the kit protocol. The PCR thermal condition consisted of an initial cDNA step of 15 min at 50^{o}C, followed by 2 min hold at 95^{o}C and then 40 cycles of 15 sec at 95^{o}C and 30 sec at 60^{o}C. To avoid cross contamination, single use aliquots were made of all reagents including primers, probes, buffers and enzymes. ROX was used as a passive reference dye to normalize the fluorescent fluctuations caused by changes in concentration or volume of sample.

Conventional RT-PCR

All clinical samples were screened in parallel by conventional RT-PCR assay for assessing specificity and sensitivity of our Q-PCR assay. Multiplex RT-PCR was done with a primer pairs for M gene (Inf-A & -B) (Donofrio *et al*., 1992) and N gene (RSV) (Cane & Pringle, 1991) targeting different region compared to Q-PCR assay. The PCR products were run on 2% agarose gel to separate 212bp (Inf-A), 362bp (Inf-B), 279bp (RSV) samples.

Statistical analysis

Statistical analysis was analyzed using SPSS 11.0.1 (LEAD technologies, Chicago, Illinois, USA) software. All the P values were two-tailed and $p \leq 0.05$ was considered significant.

Results and Discussion

In recent years many single or multiplex RT-PCR protocols for simultaneous detection of multiple respiratory viruses have been reported (Syrmis *et al.* 2004, Simmerman *et al.,* 2006). Due to high cost of instrument and reagents for Q-PCR, conventional RT-PCRs and virus culture has been mostly used in surveillance studies in south-east Asia (Simmerman & Uyeki 2008; Ieven 2007). However, early diagnosis by rapid Q-PCR may result in indirect cost benefits like decreased use of antibiotics, appropriate use of antiviral drugs and reduced hospitalization rates (Adcock *et al.*, 1997; Woo *et al*., 1997).

In this study we used two multiplex Q-PCR assays with two fluorophores for Inf- A, -B and RSV for routine surveillance for these respiratory viruses. Both assays wer performed on the same sample plate with same temperature profile. The majority of samples screened were either nasal or throat swabs and no inhibitory effects on PCR were observed (Boivin *et al*., 2004).

*Standardization of sensitivity and specificity for Real time PCR***:** The multiplex Q-PCR assay was first evaluated for cross reactivity between viruses by using positive RNA of RSV with Influenza specific primers and vice-versa. In addition, clinical samples negative for Influenza or RSV but positive for either metapneumovirus or rhinovirus were also tested to validate primers for cross reactivity. No nonspecific cross amplification was observed. To address issues of false negative and false positive results, an internal positive control gene (RNase P), positive control RNA for Inf-A, -B and RSV and a negative (throat swab) control were included in all RT reactions. All

samples were tested twice independently The frequency of contamination was less than 0.2% (1/500), as indicated by false positive signal in negative controls.

In order to determine sensitivity of the assay, RNA was isolated from 10-fold dilutions of a $TCID_{50}$ titrated stock of Inf-A, -B or RSV and analyzed by Q-PCR in triplicates. The minimal amount of detectable RNA corresponded to about 0.1 $TCID_{50}$ (50% Tissue culture infectious dose) for Inf-A or -B and about 0.01 $TCID_{50}$ for RSV. The C_t values of multiplex Q-PCR were within ±2 cycle of monospecific assay. Inf-A primer detected positive control RNA of H1N1, H3N2 and H5N1 indicating its sensitivity to detect common Inf -A subtypes. To confirm the specificity of TaqMan primers-probe, nucleotide sequencing of randomly selected positive (Inf-A, -B or RSV) and negative samples (n= 10 each) was done. Sequencing results corroborated 100% with the real time PCR results. Two samples showed mixed infection with Inf-A and -B, though average Ct value of Inf-B was ≥34.0 compared to 23.6 for Inf A. To cross check whether it was an artifact of multiplexing, monospecific PCR with Inf-B primers were performed. Mono-Q-PCR also gave positive signal (Ct ≥33.2) confirming presence of low levels of Inf-B in samples. The amplified Inf-A and -B products were confirmed by sequencing. Mixed infection was not the artifact of PCR reagents since three independent experiments using fresh aliquots of reagents confirmed the same results. However whether the patient had dual infection or the clinical sample got contaminated while collection in hospital could not be ascertained.

*Frequency of Influenza and RSV mediated ARTI in Children***:** All samples were screened by multiplex Q-PCR. Of 1091 samples tested, 275 (25.21%) were positive for respiratory viruses (Inf-A, -B and RSV). Overall, Inf-A, -B and RSV were identified in a total of 121 (11.07%), 59 (5.38%) and 95 (8.68%) samples respectively. The Inf-A frequency increased during May-Sept whereas RSV infection was predominant during Nov-Feb in both years. Inf-B infections

196 preceded or followed Inf-A and RSV season with a 5-10% frequency during March-April and
197 Sept-October (Fig 1).
198 As the Inf-A primer (M gene) can not differentiate between subtypes, positive samples required
199 another round of PCR for subtyping. Of 66 Inf A positives in 2007, 48 were subtyped as H1N1
200 and 18 as H3N2. Whereas in 2008, all 55 Inf-A positives were H3N2 (data not included). Instead
201 of screening all samples by subtype specific assays, only Inf-A positives (10-12%) need to be
202 subjected to another multiplex PCR (H1 and H3), resulting in reduction of costs and potential
203 cross contaminations. Another advantage is that new subtypes can be predicted by our diagnostic
204 strategy if a sample shows Inf-A positivity but is negative with H1 and H3 specific primers.
205 Of the 180 (121 Inf A +59 Inf B) positive samples, only 89 (65+24) were positive by
206 virus culture. RSV culture was not done during the study period. Thus compared to Q-PCR, the
207 sensitivity and specificity of virus culture was 49.5% and 100% respectively (Table 1). A range
208 of different studies have reported lower sensitivity of virus isolation since Influenza virus is
209 sensitive to temperature or pH changes as well as cell culture conditions (van Elden *et al*., 2002;
210 Liao *et al.,* 2009).
211 *Comparison of Q- PCR by standard RT-PCR assay*: Conventional multiplex RT-PCR
212 was done in parallel to retest the specimens for independent confirmation of the results and
213 evaluation of the multiplex Q-PCR assay. A total of 262 (24%) samples were found to be
214 positive compared to 275 by Q-PCR indicating 95.2% sensitivity. The Influenza samples that
215 were positive by real time PCR but culture negative were also confirmed as positive by RT-PCR
216 method. However, RT-PCR detected only Inf-A in the two mixed infections detected by Q-PCR.
217 In general, RT-PCR negative samples had higher average Ct value 30.7 [27.6-37.4, $p \leq 0.05$] in
218 Q-PCR compared to samples positive for both RT-PCR and Taqman Real time PCR [mean Ct
219 value 24.24 (20.2-30.1)] (Table 2). Thus, lower viral load in samples was associated with false

negatives in conventional RT-PCR (van Elden *et al*., 2002; Liao *et al*., 2009). Besides its higher sensitivity, the main advantage of TaqMan Q-PCR over conventional RT-PCR detection is that Q-PCR uses virus specific probes in the middle of the amplicon to specifically detect the product for each virus, which gives confidence in its specificity. The sensitivity of Q-PCR was 98.8% (95% CI, 98.2-100%) for Inf-A, 97.5% (95% CI, 95.8-100%) for Inf- B and 98% (95% CI, 97%-100%) for RSV respectively compared to the conventional RT-PCR or virus culture.

*Correlation of virus infection with the age***:** The positive cases represented a mixed population encompassing rural as well as urban settings (including slums) from in and around Kolkata city, Eastern India. Most of the cases were from lower income groups (suggestive of malnutrition) and from areas with poor sanitary conditions, facilitating spread of respiratory infection in the community. We observed increased prevalence of RSV in 0-1.0 (13.9%) and 1.0-2.0 years (12.1%) age group compared to only 3.8% positivity among 2-5 year old children. This is consistent with previous studies where >80% of the RSV positives were observed between 1 -24 month old children (Liao *et al.,* 2009). Similar to the reports from Thailand and Taiwan (Simmerman et al., 2006; Lin *et. al.*, 2004), Influenza (A+B) was predominant in the 1.0-2.0 and 2.0-5.0 years age groups (19.6% and 17.2%) in Kolkata (Table 3). Thus the vaccination for seasonal influenza in these settings among children from 1-5 years age group is expected to reduce the disease burden in communities.

Influenza and RSV infection correlates with distinct meteorological conditions: Although RSV or Influenza (A or B) could not be differentiated by clinical symptoms, their incidence was correlated during the 24 month study with the meteorological data obtained from the meteorological department, Kolkata, Government of India. Number of Inf-A, Inf-B or RSV positives per month were plotted against average monthly maximum and minimum temperature,

relative humidity and rainfall (Fig. 2). The increase in RSV cases negatively correlated with temperature (r^2=.806, p≤0.01) and rainfall (r^2=.37, p≤0.05) with high positivity (25-40%) during cool and dry months (Dec-Feb). However, in Oct-Nov 2008 there was a mild epidemic of RSV in the state of West Bengal which did not correlate with decrease in temperature but correlated with drop in relative humidity and rainfall (Fig 2).

Inf-B infection did not correlate with either temperature or rainfall (Fig 2, Table 4). A direct correlation of Inf-A activity with rainfall (r^2= .811, p ≤0.01), relative humidity (r^2= .499, p ≤0.05) and temperature (r^2=.366, p≤0.01) was observed during both years. There was negligible Inf-A activity (0-2%) during the cool and dry season (Nov-Feb). This is comparable to data from tropical regions like Pune city in India (Rao & Banerjee 1993), Dakar in Senegal (Dosseh *et. al.*, 2000), North eastern Brazil (Alonso *et. al.*, 2007) and Thailand (Simmerman *et. al.*, 2008), where influenza virus incidence peaks are observed during the rainy season. By contrast, increased Influenza activity is observed during winters (Dec-March) in Taiwan (Lin *et. al.*, 2004; Park & Glass, 2007), Vietnam (Nguyen *et. al.*, 2007), and Singapore (Chew *et. al.*, 1998; Chow *et. al.*, 2006) coinciding with annual epidemics of temperate regions, but there is another moderate peak in July-August coinciding with rains (Viboud *et. al.*, 2006).

Similar to Brazil (Alonso *et. al.*, 2007), increased influenza activity was reported during the winter in northern India (Dec-February; $Temp^{avg}$ 14-20^{o}C : RH 30-45%) but during hot and wet season in eastern or western India as in Kolkata or Pune (Rao & Banerjee 1993; Rao 2003). Unlike temperate regions where increased activity of Influenza during winters has been correlated to low relative humidity and low temperature, in tropical countries seasonality is less pronounced. More than one period of viral activity has been reported in tropical areas suggesting complex mechanisms underlying seasonality (Shek & Lee, 2003; Viboud *et. al.*, 2006; Brankston *et. al*., 2007). Some authors have suggested role of climate such as relative humidity, absolute

humidity, low temperature, UV radiation etc on virus survival, transmission efficiency or the behavioral changes such as human crowding (Tellier, 2006; Brankston et. al., 2007; Shaman & Kohn, 2009; Weber & Stilianakis, 2008; Lowen *et. al.,* 2008) to affect virus seasonality. Another hypothesis is that in tropics contact transmission predominates, while in temperate regions airborne transmission by droplets is the predominant factor responsible for differences in incidence peaks (Lowen *et. al.,* 2008) but the explanation of seasonality of influenza as well as other respiratory viruses in tropics still remains elusive. It seems that the combination of environmental and population determinants may together affect seasonality of viral infection.

Further long term systemic epidemiologic studies are warranted to fully understand seasonal triggers of influenza globally. Moreover the tropical regions with year around viral activity could be an important reservoir for human influenza, ensuring global persistence of the disease and potential source of new virus variants. This is the first report providing seasonal correlation and prevalence rate of respiratory viruses in children from Eastern India. Comparative analysis of RT-PCR, Q-PCR and virus culture (for Influenza) further confirmed the sensitivity, specificity and rapidity of Q-PCR. Thus the future use of Q-PCR in Asia as a first line test for monitoring Influenza and RSV in either hospital or community settings will result in more rapid detection of epidemics, effective clinical interventions and reduced morbidity.

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443 ***Figure Legends***

444

445 **Fig. 1** Month wise frequency of the Influenza-A, -B and RSV in children (≤5yrs) reporting with
446 acute respiratory infections. All clinical samples were tested in triplicates by real time PCR
447 ($p<0.05$). The number of suspected Influenza like illness cases and number of positives each
448 month is shown for two years (2007-08).

449

450 **Fig. 2** Correlation of meteorological variations with prevalence of Influenza-A, -B and RSV
451 during 2007-08 in Kolkata, Eastern India. Influenza A correlated positively with rainfall and
452 temperature ($r^2= .811$; $r^2= .366$, $p \leq 0.01$) but RSV correlated negatively with temperature ($r^2=$
453 $.806$, $p\leq 0.01$).

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Table 1. Comparison of positive samples obtained by real time PCR, conventional RT-PCR and virus culture during the two year study period (n=1091, 2007-08).

	Real time-PCR (Q-PCR)	Conventional RT-PCR	Virus Culture
Influenza A	121	119	65
Influenza B	59	54	24
RSV	95	89	Not done
Total positive	275	262	-
Overall Sensitivity	≥ 98%	≥ 95%	≥49.5%

Table 2. Comparison of the average C_t values for samples positive by real time PCR but negative by conventional RT-PCR.

	Sample number n=275	Average Ct
Q-PCR(+)/RTPCR(+)	262	24.24 (±3.4) $p \leq 0.05$
Q-PCR(+)/RTPCR(-)	13	30.74 ((±2.8) $p \leq 0.05$

Table 3. Prevalence of Influenza and RSV infection in different age groups among children under five years (n=1091). Statistical analysis was done using chi-square test.

Age Group/ Virus prevalence	0-1year n=215	1-2year n=382	2-5yr n=494	Significance
Influenza +	10.2%*	19.6%	17.2%	p≤ 0.01
RSV+	13.9%	12.1%	3.8%*	p≤0.01

* Significantly lower prevalence

Table 4. Correlation between the meteorological variables and the number of Influenza and RSV positive samples obtained during the study. *P value <0.05 was considered significant.

Variables	Influenza A		Influenza B		RSV		Degree of freedom
	Correlation coefficient(r)	P value*	Correlation coefficient(r)	P value *	Correlation coefficient(r)	P value*	
Relative Humidity	0.707	0.010	-0.173	0.591	-0.554	0.062	10
Maximum temperature	0.605	0.037	0.508	0.091	-0.925	0.000	10
Minimum Temperature	0.723	0.008	0.336	0.286	-0.898	0.000	10
Rainfall	0.901	0.000	-0.295	0.352	-0.612	0.035	10

Figure 1:

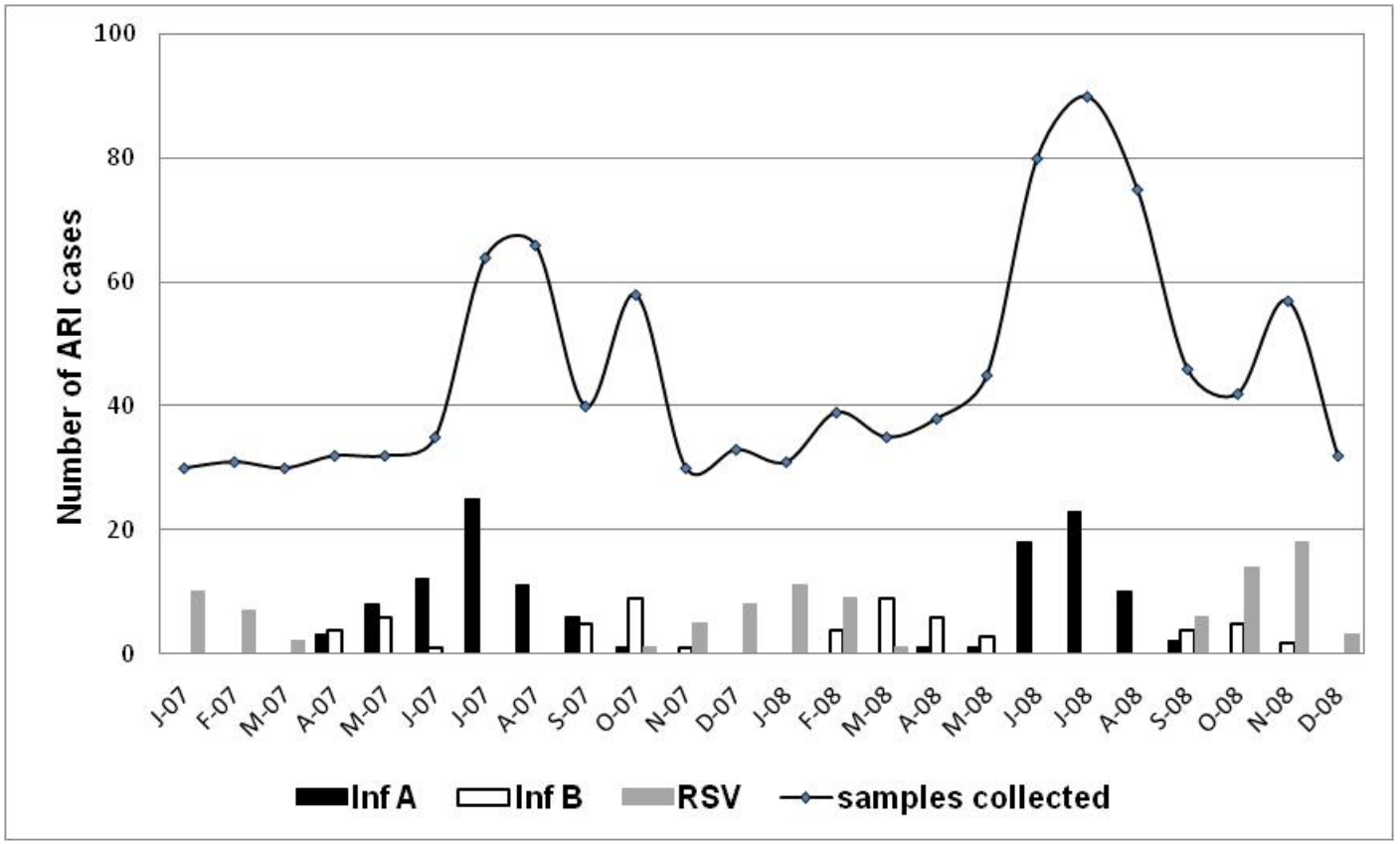
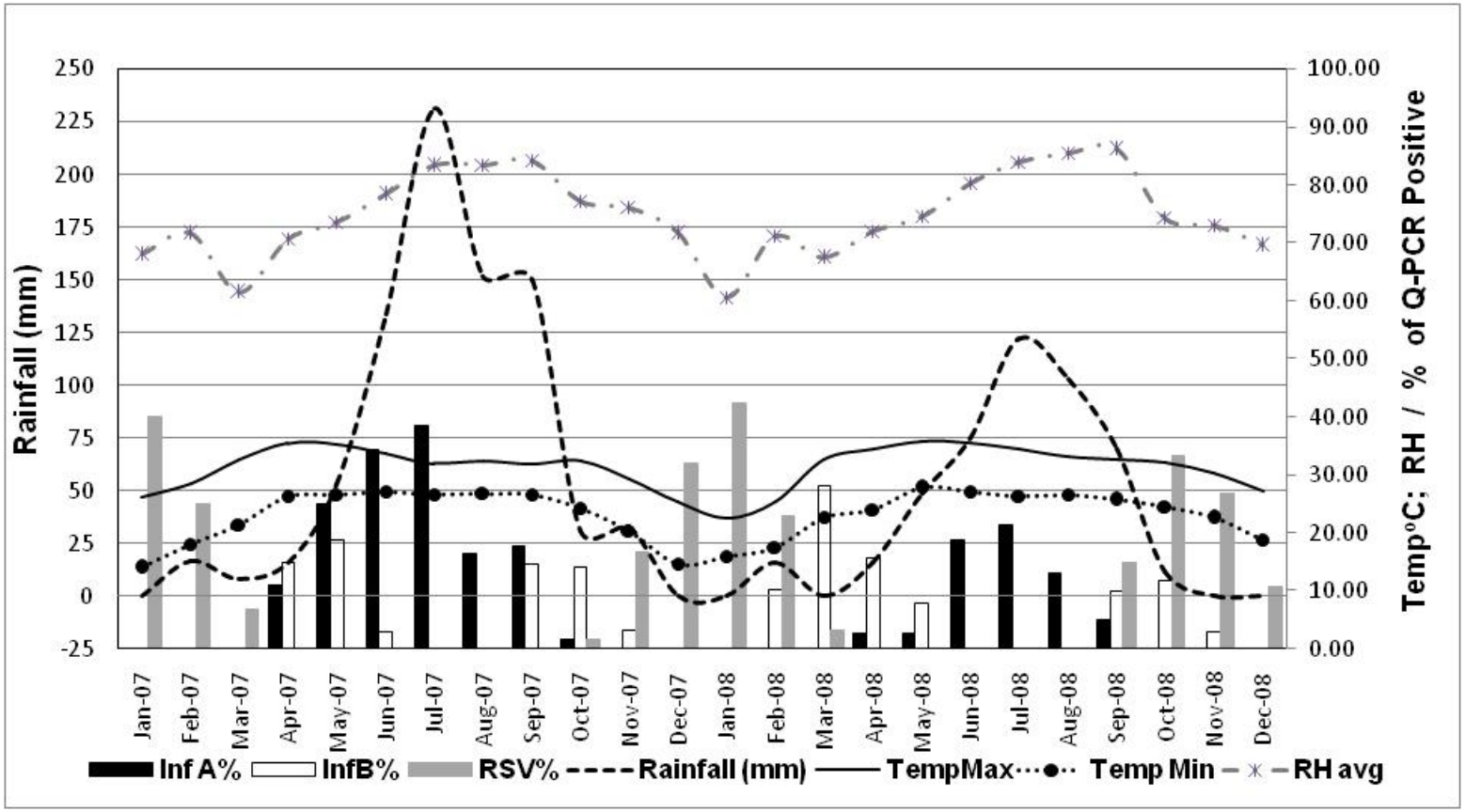

Number of ARI cases
100
80
60
40
20
0
J-07
F-07
M-07
A-07
M-07
J-07
J-07
A-07
S-07
O-07
N-07
D-07
J-08
F-08
M-08
A-08
M-08
J-08
J-08
A-08
S-08
O-08
N-08
D-08
Inf A
Inf B
RSV
samples collected

Figure 2:


Rainfall (mm)
Temp°C; RH / % of Q-PCR Positive
250
225
200
175
150
125
100
75
50
25
0
-25
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Jan-07
Feb-07
Mar-07
Apr-07
May-07
Jun-07
Jul-07
Aug-07
Sep-07
Oct-07
Nov-07
Dec-07
Jan-08
Feb-08
Mar-08
Apr-08
May-08
Jun-08
Jul-08
Aug-08
Sep-08
Oct-08
Nov-08
Dec-08
Inf A%
InfB%
RSV%
Rainfall (mm)
TempMax
Temp Min
RH avg