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Molecular characterization of respiratory syncytial viruses infecting children reported to have received palivizumab immunoprophylaxis

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ABSTRACT

Background: Respiratory syncytial virus (RSV) is a major cause of respiratory infections in children. Palivizumab (PZ) is the only RSV-specific immunoprophylaxis approved by the U.S. Food and Drug Administration. Mutations leading to amino acid substitutions in the PZ binding site of the RSV F protein have been associated with breakthrough RSV infections in patients receiving PZ.

Objective: To detect PZ resistance conferring mutations in RSV strains from children who received PZ.

Study design: Children aged ≤ 24 months on October 31 who were hospitalized or had outpatient visits for respiratory illness and/or fever during October–May 2001–2008 in 3 US counties were included. PZ receipt was obtained from parent interviews and medical records among children subsequently infected with RSV. Archived nasal/throat swab specimens were tested for RSV by real-time RT-PCR. The coding region of the PZ binding site of the RSV F protein was sequenced using both Sanger and pyrosequencing methods.

Results: Of 8762 enrolled children, 375 (4.3%) were tested for RSV and had a history of PZ receipt, of which 56 (14.9%) were RSV-positive and 45 of these had available archived specimens. Molecular typing identified 42 partial F gene sequences from 39 children: 19 single RSV subgroup A, 17 subgroup B and 3 mixed infections. Nucleotide substitutions were identified in 12/42 (28.6%) RSV strains. PZ resistance mutations were identified in 4 (10.2%) of the 39 children, of which one had documented PZ receipt.

Conclusions: Although RSV PZ resistance mutations were infrequent, most RSV-associated illnesses in children with a history of PZ receipt were not due to strain resistance.

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1. Background

Respiratory syncytial virus (RSV) is a leading cause of acute respiratory infections resulting in hospital admission and death among children <5 years worldwide [1]. The estimated global mortality due to RSV infection in 2005 was 66,000–99,000 deaths in children <5 years of age with 99% of those deaths in developing

countries [2]. Infants born prematurely and those with chronic lung or cardiac disease are at increased risk for severe disease from RSV infection. Palivizumab (PZ; brand name Synagis[®], MedImmune Inc., Gaithersburg, MD), is a humanized IgG monoclonal antibody that neutralizes RSV by inhibiting viral fusion through interaction with a site spanning residues 262–276 of the RSV fusion glycoprotein (F) [3–5]. PZ is the only prophylaxis approved by the U.S. Food and Drug Administration against RSV infection. [6,7]. Five monthly PZ injections spanning the annual RSV epidemic period have been shown to reduce hospitalizations among high-risk children in the US [6]. However, the high mutation rates found with RNA viruses allow their ready escape from immune pressure [8]. Antibody selection of PZ-resistant RSV strains in cell culture and animal systems

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have been well described, including mutant selection by the murine parent of PZ [5,9–11]. Increasing use of PZ in high-risk children and immunocompromised persons might provide opportunities for PZ resistant mutants to arise with the potential for sustained transmission to others. Previous reports have shown that mutations leading to amino acid substitutions in the PZ binding site of the RSV F protein, including S268I, K272Q, K272E, K272M, S275E, S275L and N276S, have been associated with breakthrough infections in patients receiving PZ prophylaxis [11–14]. The extent to which these mutations occur is not well known.

In this study, we analyzed RSV strains obtained from patients prospectively enrolled by the New Vaccine Surveillance Network (NVSN) through active surveillance for acute respiratory illness and/or fever (ARI) in the US during 2001–2008 who experienced RSV infections after reported PZ receipt in order to identify PZ resistance conferring mutations in the F gene coding region of the PZ binding site.

2. Objective

The present study aimed to detect PZ resistance conferring mutations in RSV strains identified in children who had received PZ.

3. Study design

NVSN enrolled children admitted to hospitals or seen as outpatients with ARI during 2001–2008 who resided in 3 US counties (Monroe County, New York; Davidson County, Tennessee; Hamilton County, Ohio) after obtaining informed consent and in accordance with a standardized protocol, as previously described [15]. Mid-turbinate nasal and throat swab specimens were obtained from enrolled children and tested for RSV by RT-PCR and viral culture by site research laboratories. Demographic, medical history, and clinical data were obtained from medical records and parental report, and documentation of premature birth was obtained.

This study includes the subset of children enrolled October through May who were ≤ 24 months of age on October 31. History of PZ receipt was obtained first from interviews with parents/guardians (“parents”) of enrolled children who were asked whether their child had received PZ injections for the current RSV season. Then, for children who were RSV-positive by site testing and whose parents reported PZ receipt, study staff retrospectively contacted healthcare providers to confirm receipt of PZ and dates of doses.

Archived frozen (-70°C) combined nasal and throat swab specimens from RSV-positive children with parental report of PZ receipt were shipped to the Centers for Disease Control and

Prevention (CDC) for molecular analysis. Total nucleic acids were extracted from each sample using the NucliSENS® easyMAG® (bioMérieux) and the extracts tested for RSV by a real-time RT-PCR (rRT-PCR) assay described by Fry et al. [16]. Positive samples were subjected to RT-PCR and sequencing of the F gene region encoding the PZ binding site using the following primer sets (forward primers were 5'-biotinylated to facilitate pyrosequencing): RSV A, fwd 5'-TAACTACACCTGTAAGCAC -3' and rev 5'-ACATGATAGAGTAAGTACTTTGC -3'; RSV B, fwd 5'-AGCACT-TACATGTAAACAAAC-3' and rev 5'-TGATAGAATAACTTTGTGCC-3'). RT-PCR amplification was performed using the SuperScript III One-step RT-PCR System (Invitrogen, Life Technologies) and the amplicons (RSV A, 140 bp; RSV B, 123 bp) were purified using exoSAP-IT (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Two sequencing procedures were applied: Sanger sequencing on a 3130xl Genetic Analyzer (Life Technologies, Grand Island, NY, USA) and pyrosequencing on a PyroMark Q24 instrument (Bio- tage AB, Uppsala, Sweden) using a procedure newly developed for this study (available on request). For Sanger sequencing, amplicons were subjected to forward and reverse cycle sequencing using the BigDye terminator sequencing kit v.1.1 (Applied Biosystems, Life Technologies). Semi-quantitative pyrosequencing was performed targeting 4 designated mutation sites known to alter PZ susceptibility (codons: 268, 272, 275, 276) and analyses were performed with PyroMark Q24 Analysis Software using the allele quantification frequency mode. For each allele tested, a calibration curve was generated from standards prepared with the following proportions of the wild-type (WT)/mutant (M) allele 0/100%, 5/95%, 10/90%, 25/75% and 50/50%. Calibration standards were included in each run and performed in triplicate at each dilution. The limit of detection for each M allele was estimated by measuring the signal obtained from 20 known negative respiratory swab samples (100% WT) and calculating the positive response cut-off ($WT_{46-54\%}/M_{50\%}$ and $WT_{\leq 12\%}/M_{100\%}$). To exclude other possible viral etiologies, specimens identified with RSV PZ resistance strains were also tested by a panel of CDC in house RT-PCR assays for influenza, human metapneumovirus, parainfluenza virus types 1–3, adenovirus, rhinovirus, and coronaviruses.

Based on the recommended PZ dosing schedule of 5 total monthly doses over the RSV season [7], we defined an RSV infection leading to a medical visit with the ARI occurring within 30 days of the last confirmed PZ dose a “breakthrough” infection.

4. Results

Among 8762 enrolled children who met the age criteria for this study, 399 (4.6%) had received PZ according to parental report (Table 1; Fig. 1). Of these, 375 had by tested for RSV at the surveillance sites and 56 (14.9%) were positive. Archived specimens were

Table 1
RSV detections among 375 reported palivizumab recipients enrolled by NVSN.

	RSV seasons (October–May) ^a							Total
	2001	2002	2003	2004	2005	2006	2007	
No. palivizumab recipients	30	30	70	74	46	54	71	375
RSV detections ^b	4 (13.3%)	5 (16.7%)	10 (14.3%)	8 (10.8%)	10 (21.7%)	11 (20.3%)	8 (11.3%)	56 (14.9%)
RSV tested/confirmed ^c	3/3	2/2	5/5	7/7	10/8	10/6	8/8	45/39
RSV A	1	2	4	1	7	4	0	19
RSV A+B ^d	0	0	0	0	0	0	1	1
RSV B	2	0	1	5	1	1	7	17
RSV B+B ^e	0	0	0	1	0	1	0	2

^a Study year shown is the year the RSV season started.

^b RSV detected by RT-PCR assays performed at NVSN surveillance sites.

^c RSV tested and confirmed by real-time RT-PCR and RT-PCR/sequencing at CDC.

^d RSV subgroup A and B codetection.

^e RSV subgroup B genotype codetection.

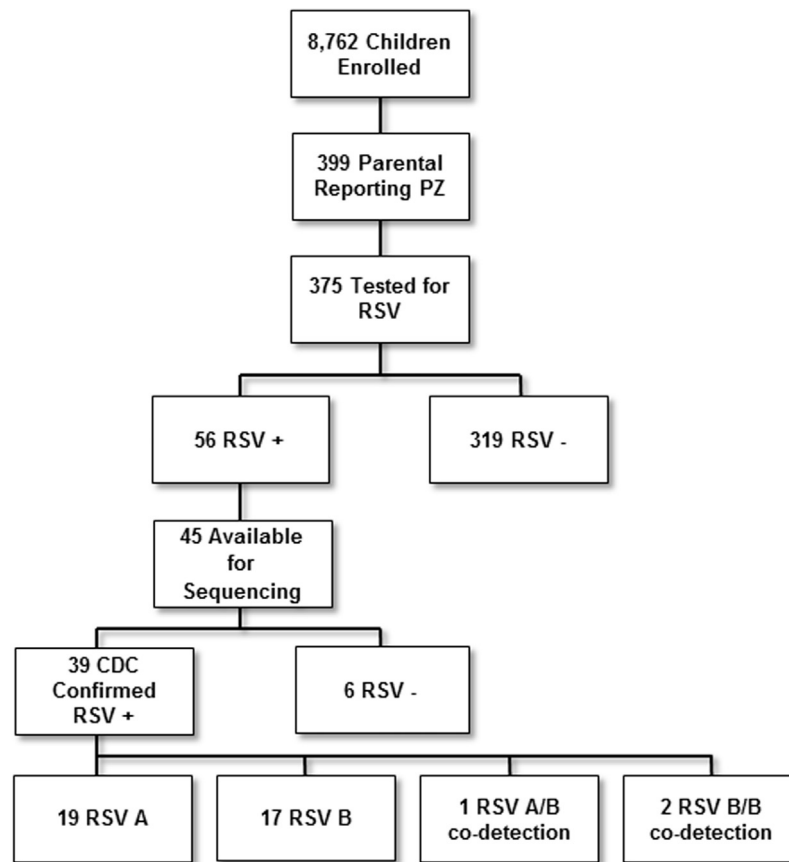


Fig. 1. Palivizumab (PZ) recipient testing flowchart.

available from 45 of the 56 RSV-positive children and were sent to CDC for confirmatory testing and sequencing. Of these, 39 were confirmed positive for RSV by rRT-PCR; the remaining 6 specimens were negative at CDC for RSV by both rRT-PCR and RT-PCR assays. Molecular typing identified 42 partial F gene sequences in the 39 specimens: 19 single RSV subgroup A; 17 single RSV subgroup B; 1 co-detection of single RSV A and B; and 2 co-detections of 2 different RSV B sequences. RSV A and B strains co-circulated during most years and alternated in predominance over the study period (Table 1).

Of the 42 partial RSV F gene sequences obtained, 12 (28.6%) had nucleotide substitutions, including 3 RSV subgroup A and 9 RSV subgroup B strains; 8 (19.0%) mutations were synonymous and 4 encoded predicted amino acid changes recognized to confer PZ resistance (Table 2). Analysis of the sequence chromatograms and pyrograms of the 4 PZ resistant strain revealed that 3 specimens had both WT and M alleles present: RSV A, $T_{WT7\%}/C_{M93\%}$ at

nt position 814 (K272E), $T_{WT86\%}/C_{M14\%}$ at nt position 827 (N276S); and RSV B, $G_{WT31\%}/A_{M69\%}$ at nt position 824 (S275L), $A_{WT87\%}/C_{M13\%}$ at nt position 814 (K272Q) (Fig. 2). Specimens containing the RSV PZ resistance strains were RT-PCR negative for other respiratory viruses.

Of the 39 children with parental report of PZ receipt from whom RSV F gene sequences were available, 10 had documentation in their medical records that confirmed prior receipt of PZ for the current RSV season. All of the 10 children with confirmed PZ receipt and 27/29 (93%) children that lacked a medical record of PZ receipt had an underlying medical condition or premature birth (born 6–16 weeks early by parental report). Among the 10 with confirmed PZ receipt, 7 (63.6%) received PZ within 30 days prior to their ARI hospitalization or outpatient visit, indicating that these 7 met the study definition of breakthrough infections (Table 3). For these, PZ was also received 30 days prior to the reported onset of illness. Of these 7 with breakthrough infections, only one (14%) possessed a known

Table 2

RSV strains with nucleotide substitutions in the F gene region encoding the palivizumab binding site among 42 sequences evaluated.

RSV subgroup	Nt substitution ^a	Syn/NSyn ^b	Predicted AA substitution	No. strains with mutation	RSV seasons (Oct–May)
A	G816A*	Syn		1	2005
B	A780G*	Syn		4	2(2001), 2003, 2004
B	T789C*	Syn		1	2007
B	A813G*	Syn		2	2006, 2007
A	A814G*	NSyn	K272E	1	2005
A	C827T#	NSyn	N276S	1	2006
B	A814C#	NSyn	K272Q	1	2006
B	C824T#	NSyn	S275L	1	2001

^a (*)Single sequence identified; (#) mixed wildtype and mutant sequences.

^b Syn = synonymous base substitution; NSyn = nonsynonymous base substitution.

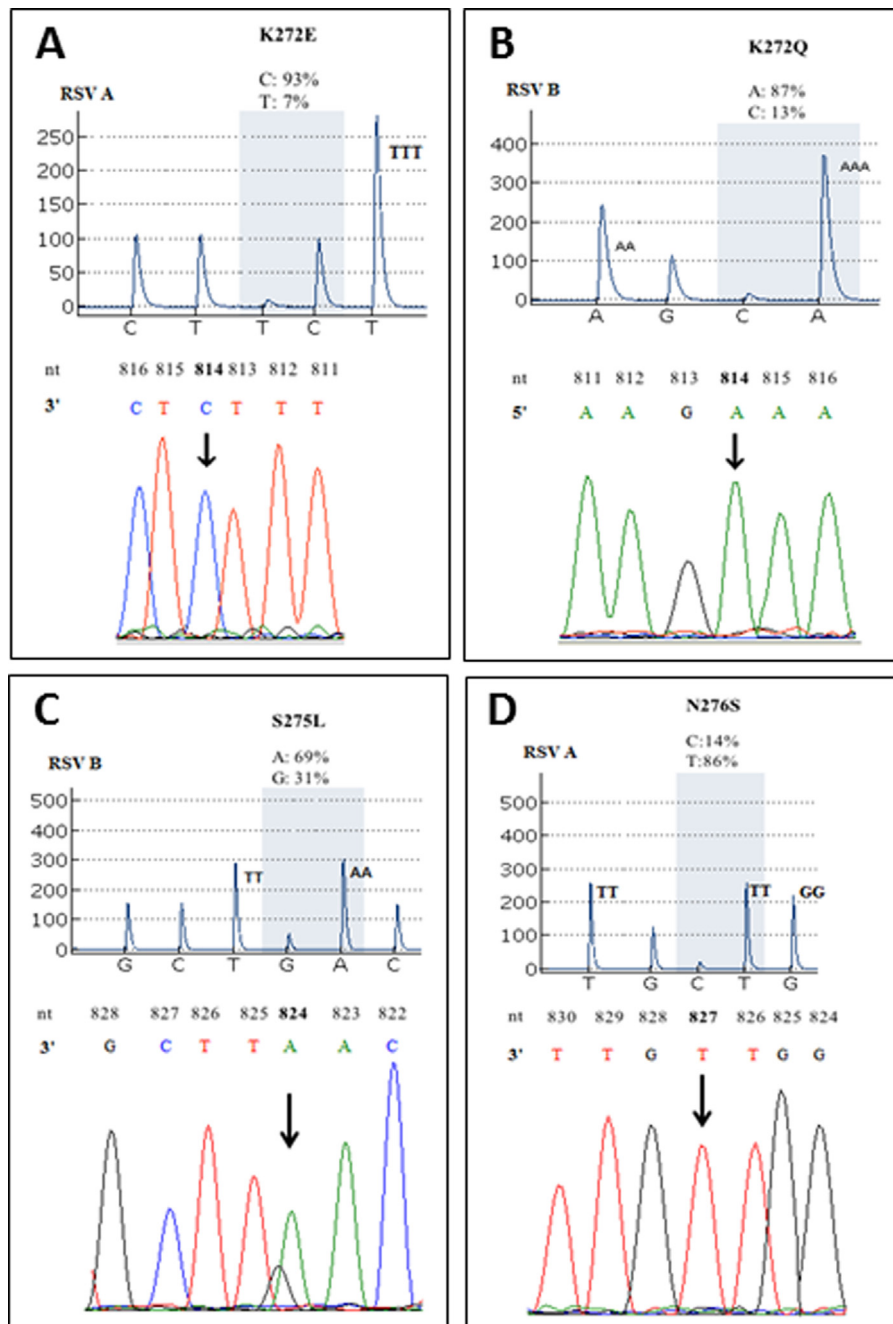


Fig. 2. Pyrograms and chromatograms of sequences obtained from each of 4 RSV-positive samples that contained nonsynonymous nucleotide substitutions conferring predicted PZ resistance (cutoff: mutant 100%, wild type \leq 12%). Arrows point to base positions in the chromatograms that correspond to positions highlighted in the pyrograms. Panel (A) shows a single mutation at RSV A codon K272E detectable in both pyrogram and chromatogram; panel (B) shows a mixture of mutant and wild type alleles at RSV B codon K272Q that is only detectable in the pyrogram (wild type predominates); panel (C) shows a mixture of mutant and wild type alleles at RSV B codon S275L detectable in both pyrogram and chromatogram (mutant predominates); panel (D) shows a mixture of mutant and wild type alleles at RSV A N276S that is only detectable in the pyrogram (wild type predominates).

PZ resistance mutation, K272E. Of the 3 other PZ recipients infected with resistant RSV strains, 2 did not receive PZ within the 30 day window and one did not have dose dates noted in the medical record.

5. Discussion

Our study identified RSV strains with nucleotide substitutions in the coding region of the F gene PZ binding site that are predicted to confer PZ resistance in 4 of 39 (10.3%) children with a medical

care visit for RSV and parental report of prior receipt of PZ for the season. However, we were able to confirm breakthrough infections in only 7 of 39 children because PZ receipt and/or dates of PZ doses were unavailable in the medical records for most study children. Only 1 of the 7 children had infection with an RSV strain associated with PZ resistance, suggesting that resistance does not account for the majority of PZ failures.

Although the PZ binding site on the RSV F protein is highly conserved, over multiple years of surveillance, we found a frequency of resistance conferring mutations similar to those reported by two

Table 3
Characteristics of 10 palivizumab (PZ) recipients with recorded dosing schedules.

RSV subgroup	CDC RSV rRT-PCR Ct value ^a	NT substitution	Predicted AA substitution	Age (months)	Days ill before enrollment	PZ dates	# PZ doses	Date of hospitalization or outpatient visit	Breakthrough (Y/N) ^c
A	22.2			6	8	last on 12/13/05	2	2/7/06	N
B	27.2			19	7	11/7/07	1	1/17/08 ^b	N
B	17.3			2	10	2/7/08	1	2/21/08	? ^d
B	19.3			4	4	3/4/05	1	3/27/05	Y
B	26.0			12	3	12/2/05	1	12/15/05	Y
A	23.1			14	5	11/7/05, 12/7/05, 1/7/06	2 or 3	1/15/06	Y
A	23.9	A814C	K272E	9	4	11/7/05, 12/7/05, 1/7/06, 2/7/06	3 or 4	2/6/06	Y
B	18.5			6	2	11/7/07, 12/7/07, 1/7/08	2 or 3	1/7/08	Y
B	18.0			9	3	11/20/07, 12/18/07, 1/16/08	3	2/6/08 ^b	Y
B	22.7			18	7	12/3/07, 12/31/07, 1/8/08, 2/29/08	4	3/11/08	Y

^a Real-time RT-PCR Ct; cycle threshold value; positive test result, Ct < 40.

^b Outpatient visit.

^c Breakthrough, RSV hospitalization occurring < 1 month after most recent PZ dose.

^d Breakthrough status could not be determined.

other studies with similar patient populations with PZ receipt prior to illness. One study reported a 5.4% mutation frequency among high-risk infants with lower respiratory tract illness [11]; a second study reported 8.7% with mutations among patients aged < 3 years with respiratory tract illness [13]. Apparent breakthrough RSV infections occurring during immunoprophylaxis could be due to improper spacing of PZ doses, exposure to high virus concentrations, frailty of the PZ recipient or occult co-infection with other respiratory pathogens.

With the exception of N276S, none of the PZ resistant strains identified in this study have been shown to occur naturally and therefore most likely arose by PZ selection. The N276S mutation was originally described in a patient with an RSV A breakthrough infection and was shown to exhibit increased PZ resistance by in vitro based microneutralization assay [12]. However, more recent reports have shown RSV A N276S to be naturally occurring and efforts to confirm PZ resistance by microneutralization assay have failed [13,17]. It is interesting to note that S276 is the consensus sequence of RSV B viruses and that 5 of the 7 breakthrough strains we identified were RSV subgroup B, all possessing S276, suggesting that these B strains may be inherently more resistant to PZ whose murine parent was developed to RSV strain A2. Alternatively, we cannot exclude the possibility that coincident mutations outside of the PZ binding region may have contributed to PZ resistance or that these infections simply occurred for other reasons as noted above.

Our novel pyrosequencing method applied directly to the clinical specimens proved useful for identifying low level variant sequences that might be present in a quasispecies population [18–20]. Pyrosequencing permitted rapid, sensitive and semiquantitative estimation of the relative prevalence of WT and M alleles in these samples; 2 of the 3 samples identified by pyrosequencing to have mixed sequences would have been missed using Sanger sequencing alone. Pyrosequencing revealed that in most mixed infections, the wild-type N allele predominated, with the exception of S275L, which was present in only a small fraction of the total sample sequences, making it difficult in this case to attribute the patient's illness to a breakthrough infection.

Our study is limited in estimating the true rate of breakthrough infections because parental recall of PZ receipt may be inaccurate and it is challenging to obtain PZ records retrospectively. This was compounded by our failure to locate some archived specimens and confirm some specimens originally reported to be RSV-positive. A study of RSV-negative infants detailed similar challenges in determining retrospectively which children strictly met the criteria for PZ receipt and were recommended to receive it, and obtaining documentation of receipt [21]. We note, however, that 95% of the 39 children studied had an underlying medical condition or premature birth and therefore might have been recommended to receive PZ.

In conclusion, we identified RSV PZ resistance mutations in 4 children who received PZ. However, only 1 child was confirmed to have a breakthrough infection with a PZ resistant strain. Although RSV PZ resistance mutations were uncommon, continued efforts to identify and monitor PZ resistance mutations is important to assess their role in breakthrough infections and the potential for sustained circulation in the population where they may pose a threat to high risk groups receiving PZ immunoprophylaxis.

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Competing interests

Dr Weinberg has served on speakers' bureaus for GlaxoSmithKline (GSK), Merck, and Sanofi Pasteur. Dr Griffin has consulted for Novavax and MedImmune. Dr Edwards has received research funding from Novartis for nonrelated vaccine trials. Dr Williams serves on the Scientific Advisory Board for Quidel. Dr Staat has received research support from GSK, Merck, and MedImmune; has consulted for GSK, Merck, and MedImmune; and has been on the speakers' bureaus for GSK and Merck. Dr Hall has consulted for GSK and MedImmune and has received research support from MedImmune. The other authors have indicated they have no financial relationships relevant to this article to disclose.

Ethical approval

The study was approved by the institutional review boards of each NVSN site and the CDC.

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