Viruses causing lower respiratory symptoms in children younger than 2-years of age: findings from the ORChID birth cohort.

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Supplementary Digital Content

Supplementary Text 1: Details of specimen collection, processing, polymerase chain reaction assays, quality control measures, and assay parameters used to identify respiratory viruses.

Supplementary Text 2: Calculation of attributable fraction in the exposed (AFE).

E-Figure: Percentage of children contributing swabs or symptom data in the Observational Research in Childhood Infectious Diseases Study.

E-Table 1: Details of primer and probe sequences by virus.

E-Table 2: Acute respiratory infections, lower respiratory tract infections and asymptomatic single new virus detections, risk ratios, and attributable fractions by respiratory virus in children from the ORChID birth cohort with virus positive detections with cycle-threshold values >38 excluded (n=7,856 swabs).

E-Table 3: Number of symptomatic and asymptomatic new virus co-detection episodes (n=219).

E-Table 4: Comparison of demographic and social characteristics of the ORChID Study cohort and the general population of Brisbane or the State of Queensland, 2011.

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Specimen collection

All swabs were collected using a plastic-shaft, rayon-budded swab in a transport tube with a foam pad reservoir soaked with viral transport medium (Virocult MW950, Medical Wire & Equipment, Wiltshire, England). A single swab was used to sample both nostrils.

Quality Control measures

A known amount of whole equine herpesvirus (EHV) spiked into each sample assessed nucleic acid extraction quality and presence of polymerase chain reaction (PCR) inhibitors.[1] Any extract having a >3 cycle-threshold (Ct) difference to that of the expected value by EHV real-time PCR assays was considered to have failed quality control and the sample was re-extracted.

Specimen quality was assessed by testing for a marker of human genomic DNA, endogenous retrovirus-3 (ERV-3).[2] We have demonstrated previously that among specimens with an ERV-3 Ct value >38, respiratory virus detection declined significantly (odds ratio 0.35; 95% confidence interval 0.27, 0.44 when ERV-3 was undetectable).[3] Thus, virus-negative swabs with an ERV-3 Ct value >38 were deemed to be of lesser quality and removed from incidence calculations.[3]

Nucleic Acid Extraction

Each swab was resuspended in 2mL of phosphate buffered saline from which 200µL was used for nucleic acid extraction. After being spiked with EHV, samples were extracted on the QIAxtractor automated high-throughput extraction platform using DX reagents according to the manufacturer's instructions (Qiagen, Australia). Total DNA and RNA were eluted into 150µL of elution buffer.

Detection of Viruses by Real-Time PCR (E-Table 1)

DNA viruses

All real-time PCR assays targeting DNA templates (EHV, ERV-3, adenovirus, human polyomaviruses WU/KI, and human bocavirus-1) used an identical set of master mix and cycling conditions. In brief, 8pmol of each primer, 3.2pmol of the respective probe(s), and 2µL of template were made in a 20µL final reaction volume using the Bioline Sensi Mix II Probe PCR mix kit (Bioline, Australia), and followed cycling conditions of: activation at 94°C for 2-minutes, followed by 45 cycles of 95°C for 15-seconds and 60°C for 60-seconds.

RNA viruses

For RNA viruses, real-time one-step reverse-transcription PCR (RT-PCR) assays were used following a common protocol for all, but influenza and human rhinovirus (HRV) assays. In brief, 8pmol of each primer, 3.2pmol of the respective probe(s), and 2µL of template were made in a 20µL final reaction volume using the Bioline SensiFAST Probe One-Step RT-PCR kit (Bioline, Australia), and followed cycling conditions of: 45°C reverse transcriptase incubation for 20-minutes, 95°C activation for 2-minutes, followed by 45 cycles of 95°C for 15-seconds and 60°C for 60-seconds. The single deviation from the common RT-PCR protocol in the influenza A/B reaction was use of asymmetric amounts of influenza A and B probes (6.4pmol and 3.2pmol, respectively).

Human rhinoviruses

A concentration of 16pmol of each primer, as well as magnesium chloride additive of the same concentration, were utilised along with 2pmol of the respective probe in the HRV assay. The cycling conditions for the RT-PCR were as follows: 45°C reverse transcriptase incubation for 20-minutes, 94°C activation for 2-minutes, followed by 55 cycles of 95°C for 15-seconds to denature and 60°C for 60-seconds for the annealing step. Reactions were performed on ABI 7500

and Viia7 instruments (Life Technologies, Australia), as well as the Qiagen Rotorgene Q (Qiagen Australia).

Samples positive for HRV were genotyped using methods described previously.[4,5] Briefly, this involved sequencing the variable region of VP4/VP2 genes using a nested PCR assay with two sets of primers.[6] Samples that could not be amplified twice by the VP4/VP2 assay were further investigated by amplifying a 390 nucleotide fragment from the 5'UTR segment.[7] PCR products were purified (QIAquick PCR purification kit, Qiagen, Australia) and submitted for DNA sequencing to the Australian Equine Genetics Research Centre (The University of Queensland, Brisbane, Australia).

All detections with Ct values ≤40 were considered positive for the target respiratory virus.

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Supplementary Text 2: Calculation of attributable fraction in the exposed (AFE)

The AFE calculates the proportion of symptomatic acute respiratory infections (ARI) in virus-positive individuals that can be attributed to the virus.

Swab status	Acute respiratory infection	No symptoms	Total population
Virus-positive	a	b	a+b
Virus-negative	c	d	c+d
Total	a+c	b+d	a+b+c+d

The risk of having symptoms given that a child is virus-positive = a/(a+b)

The risk of having symptoms given that a child is virus negative = c/(c+d)

The risk ratio of having symptoms if a child is virus-positive compared to if they are virus-negative is a/(a+b)/c/(c+d)

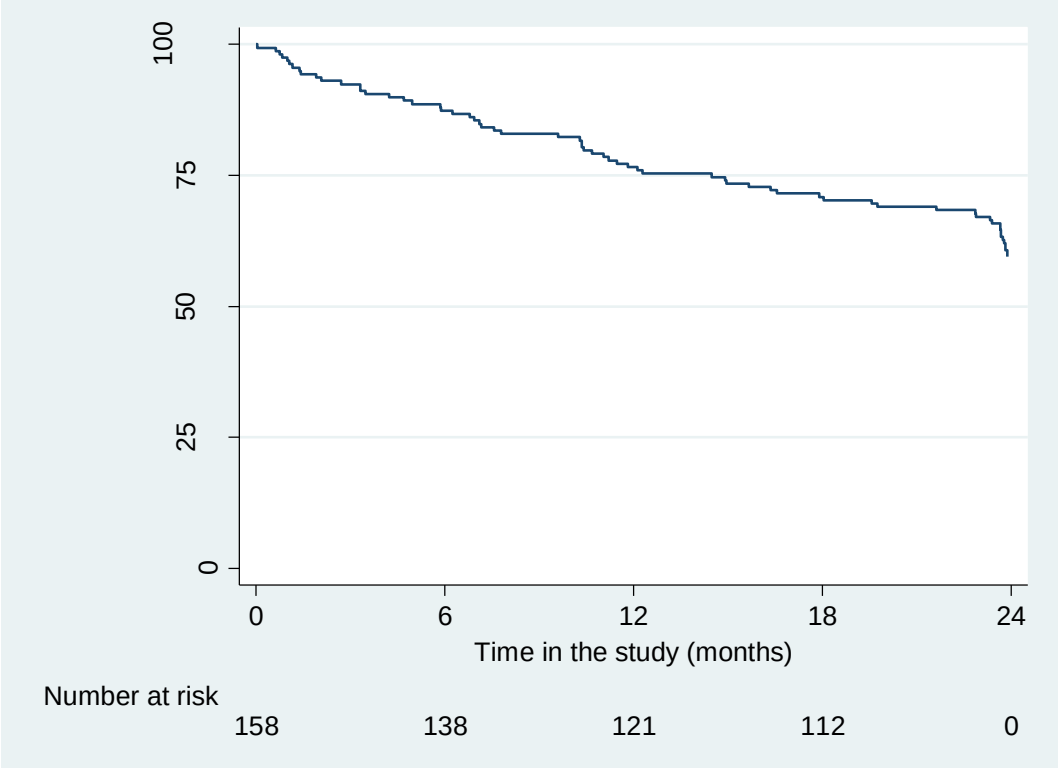
Attributable fraction in the exposed is $100\% * \frac{(a/a+b)-(c/c+d)}{(a/a+b)}$

The AFE can also be written in terms of the risk ratio (RR): $AFE = 100\% * (1-RR^{-1})$, where RR is the proportion of children positive for a virus with ARI symptoms, divided by the proportion of children negative for the virus who have ARI symptoms.

For example

- AFE = 0% - the risk of having symptoms when virus-positive is equal to the risk of having symptoms when virus-negative – that is, the presence of the virus is not associated with the presence of symptoms;
- AFE = 50% - 50% of symptomatic episodes when virus-positive were attributable to the presence of the virus;
- AFE = 100% - the risk of having symptoms when virus-negative = 0.

E-Figure: Percentage of children contributing swabs or symptom data in the Observational Research in Childhood Infectious Diseases Study.



E-Table 1: Details of primer and probe sequences by virus.

Reaction Mix	Virus	Target Gene	Primer, Probe sequences (5'-3')	Source
1	Human rhinovirus[a]	5' UTR	CY+AGCC+TGCGTGGY GAAACACGGACACCCAAAGTA FAM-TCCTCCGGCCCCTGAATGYGGC-BHQ1	Lu et al. (2008)[4] Arden & Mackay (2010)[5]
2	Influenza A	Matrix	CTTCTAACCGAGGTCGAAACGTA GGTGACAGGATTGGTCTTGTCTTTA Q670-TCAGGCCCCCTCAAAGCCGAG-BHQ2	Whiley et al. (2005)[8]
	Influenza B	Matrix	GCATCTTTTGTTTTTTATCCATTCC CACAATTGCCTACCTGCTTTCA FAM-TGCTAGTTCTGCTTTGCCTTCTCCATCTTCT-BHQ1	Lambert et al. (2008)[9]
3	RSV-A	Nucleocapsid	AGATCAACTTCTGTCATCCAGCAA TTCTGCACATCATAATTAGGAGTATCAAT FAM-CACCATCCAACGGAGCACAGGAGAT –BHQ1	Van Elden (2003)[10]
	RSV-B[b]	Nucleocapsid	AAGATGCAAATCATAAATTCACAGGA TGATATCCAGCATCTTTAAGTATCTTTATAGTG YAK-TATGTCC+AGG+TTAGGAAG+G+G+AA-BBQ	Van Elden (2003)[10]
4	Parainfluenza-1	Hemagglutinin-neuraminidase	TTTAAACCCGGTAATTTCTCATACCT CCCCTTGTTCCTGCAGCTATT FAM-TGACATCAACGACAACAGGAAATCATGTTCTG-BHQ1	Lambert et al (2008)[9]
	Parainfluenza-2	Nucleocapsid	AGAGTTCCAACATTCAATGAATCAGT CTCAAGAGAAATGTCATTCCCATCT YAK-CCTCTGTATTGCTCATGCATAGCACGGA-BBQ	Lambert et al. (2008)[9]
	Parainfluenza-3	Nucleocapsid	CGGTGACACAGTGGATCAGATT AGGTCATTTCTGCTAGTATTCATTGTTATT Q670-TCAATCATGCGGTCTCAACAGAGCTTG-BHQ2	Lambert et al (2008)[9]
5	Human coronavirus-HKU1	Polymerase	CCTTGCGAATGAATGTGCT TTGCATCACCACTGCTAGTACCAC FAM-TGTGTGGCGGTTGCTATTATGTTAAGCCTG-BHQ1	Dare et al (2007)[11]
6	Human coronavirus-OC43	Nucleocapsid	CGATGAGGCTATTCCGACTAGGT CCTTCCTGAGCCTTCAATATAGTAACC Q670-TCCGCCTGGCACGGTACTCCCT-BHQ2	Van Elden (2004)[12]

	Human coronavirus-NL63	Polyprotein 1a	ACGTACTTCTATTATGAAGCATGATATTAA AGCAGATCTAATGTTATACTTAAAACTACG YAK-ATTGCCAAGGCTCCTAAACGTACAGGTGTT-BBQ	Gunson et al. (2005)[13]
	Human coronavirus-229E	Nucleocapsid	CAGTCAAATGGGCTGATGCA AAAGGGCTATAAAGAGAATAAGGTATTCT FAM-CCCTGACGACCACGTTGTGGTTCA-BHQ1	Van Elden (2004)[12]
7	Human metapneumovirus	Nucleocapsid	CATATAAGCATGCTATATTAAAAGAGTCTC CCTATTTCTGCAGCATATTTGTAATCAG FAM-TGYAATGATGAGGGTGTCACTGCGGTTG-BHQ1	Maertzdorf et al. (2004)[14]
8	Adenovirus	Hexon	GCCACGGTGGGGTTTCTAAACTT GCCCCAGTGGTCTTACATGCACATC FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ1	Heim et al. (2003)[15]
10	Human bocavirus-1	VP1	GGCAGAATTCAGCCATACTCAAA TCTGGGTTAGTGCAAACCATGA FAM-AGAGTAGGACCACAGTCATCAGACACTGCTCC-BHQ1	Tozer et al (2009)[16]
9	Human polyomavirus WU	NCCR	GCCGACAGCCGTTGGATATA TTTCAGGCACAGCAAGCAAT FAM-AGGGTCACCATTTTTATTTCAGATGGGCA-BHQ1	Antonsson et al (2012)[17]
	Human polyomavirus KI	NCCR	GAACTTCTACTGTCCTTGACACAGGTA GGATTAGAACTTACAGTCTTAGCATTTCAG Q670-ACCCTTTGTAGGCCAAAGGAGAGTGAAGG-BHQ2	Antonsson et al (2012)[17]
	Human polyomavirus KI	STAg	CACAGGTGGTTTTCTATAAATTTTGTACTT GAAGCAGTGGGATGTATGCATTC YAK-TGCATTGGCATTCGTGATTGTAGCCA-BBQ	Antonsson et al (2012)[17]
11	Endogenous retrovirus-3	ENV gene	CATGGGAAGCAAGGGAACTAATG CCCAGCGAGCAATACAGAATTT FAM-TCTTCCCTCGAACCTGCACCATCAAGTCA-BHQ1	Yuan et al. (2001)[2]
	Equine Herpesvirus	Glycoprotein B gene	GATGACACTAGCGACTTCGA CAGGGCAGAAACCATAGACA Q670-TTTCGCGTGCCTCCTCCAG-BHQ2	Bialasiewicz et al. (2009)[1]

+ indicates Locked Nucleic Acid (LNA) base (eg: +A is a LNA Adenine analogue)

[a]Rhinovirus pan assay; [b]Modified probe from published version presented

E-Table 2: Acute respiratory infections, lower respiratory tract infections and asymptomatic single new virus detections, risk ratios, and attributable fractions by respiratory virus in children from the ORChID birth cohort with virus positive detections with cycle-threshold values >38 excluded (n=7,856).

	Acute respiratory infections		Lower respiratory tract infections	
Virus	**Risk ratio[a] (95% CI)**	**Attributable fraction in the exposed (%)**	**Risk ratio[a] (95% CI)**	**Attributable fraction in the exposed (%)**
Single new detections				
All HRV	**1.5 (1.4, 1.7)**	**33 (29, 41)**	1.1 (1.0, 1.2)	9 (0, 17)
HRV-A	**1.6 (1.5, 1.7)**	**37 (32, 42)**	1.2 (1.0, 1.6)	19 (-3, 36)
HRV-B	1.2 (1.0, 1.5)	18 (-1, 33)	0.8 (0.4, 1.6)	-19, -130, 39)
HRV-C	**1.6 (1.4, 1.7)**	**38 (30, 40)**	**1.4 (1.1, 1.7)**	**28 (12, 41)**
IFV-A	**1.7 (1.2, 2.3)**	**41 (17, 57)**	1.6 (0.4, 5.7)	38 (-150, 82)
IFV-B	1.0 (0.5, 2.8)	0 (-1, 64)	0	0
PIV-1	**1.8 (1.3, 2.5)**	**44 (23, 60)**	2.5 (0.8, 7.8)	**60 (25, 87)**
PIV-2	n/c	n/c	n/c	n/c
PIV-3	**1.6 (1.3, 2.0)**	**38 (23, 50)**	**2.0 (1.2, 3.3)**	**50 (17, 70)**
RSV-A	**1.4 (1.1, 1.7)**	**29 (9, 41)**	**3.0 (2.0, 4.5)**	**67 (50, 78)**
RSV-B	**1.8 (1.4, 2.2)**	**44 (29, 55)**	**3.1 (1.8, 5.4)**	**68 (44, 81)**
HCoV-OC43	**1.6 (1.3, 2.0)**	**38 (23, 50)**	**2.3 (1.4, 3.7)**	**57 (29, 73)**
HCoV-NL63	**1.7 (1.4, 2.1)**	**41 (29, 52)**	1.7 (0.9, 3.3)	**41 (11, 70)**
HCoV-229E	0.9 (0.3, 2.5)	-11 (-233, 60)	1.2 (0.2, 7.7)	17 (-4, 87)
HCoV-HKU1	1.1 (0.7, 1.6)	9 (-43, 38)	0.8 (0.2, 2.9)	25 (-4, 66)
HMPV	**1.6 (1.2, 2.1)**	**38 (17, 52)**	**3.2 (1.7, 6.0)**	**69 (41, 83)**
AdV	**1.5 (1.2, 1.8)**	**33 (17, 44)**	1.0 (0.4, 2.4)	0 (-15, 58)
WU-PyV	1.3 (0.9, 1.8)	23 (-11, 44)	1.4 (0.6, 3.5)	29 (-67, 71)
KI-PyV	0.8 (0.6, 1.2)	25 (-67, 17)	1.2 (0.6, 2.2)	17 (-67, 55)
HBoV-1	1.3 (1.0, 1.6)	23 (0, 38)	1.3 (0.8, 2.2)	23 (-25, 55)
Any virus	**1.6 (1.5, 1.7)**	**38 (33, 41)**	**1.4 (1.2, 1.5)**	**29 (17, 33)**

[a]adjusted for clustering using sandwich estimators to account for within-infant correlation between observations; [b]Individual rhinovirus species do not add up to 'All rhinovirus' detections because of a combination of more than one rhinovirus species detected within the same ARI episode, or single new virus detection episodes where HRV positive specimens could not be sequenced.

Abbreviations: n/c: not calculable as there were no cases in the asymptomatic group. HRV: human rhinovirus, IFV: influenza virus, PIV: parainfluenza virus, RSV: respiratory syncytial virus, HCoV; human coronavirus, HMPV: human metapneumovirus, AdV: adenovirus, WU-PyV: WU polyomavirus, KI-PyV: KI polyomavirus, HBoV-1: human bocavirus-1.

E-Table 3: Number of symptomatic and asymptomatic new virus co-detection episodes[a] (n=219).

SYMPTOMATIC DETECTIONS (n=160)	HRV	IFV- A	IFV- B	PIV-1	PIV-2	PIV-3	RSVA	RSVB	CoV-OC43	CoV-NL63	CoV-229E	CoV-HKU1	MPV	AdV	WU-PyV	KI-PyV	BoV	Total asym	ASYMPTOMATIC DETECTIONS (n=59)
HRV		0	0	0	0	3	3	0	1	0	1	1	0	6	9	13	3	40	
IFV-A	1		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
IFV-B	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
PIV-1	1	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	
PIV-2	0	0	0	0		0	0	0	0	1	0	0	0	0	0	0	0	1	
PIV-3	8	0	0	0	0		0	0	0	0	0	0	0	0	2	0	1	3	
RSV A	6	0	0	0	0	0		0	0	0	0	0	0	0	0	0	2	2	
RSV B	2	0	0	0	0	0	1		0	0	0	0	0	0	0	0	0	0	
CoV-OC43	1	0	0	0	0	0	0	1		0	0	0	0	1	0	0	0	1	
CoV-NL63	5	0	0	0	0	0	0	0	0		1	0	0	0	0	0	0	1	
CoV-229E	1	0	0	0	0	0	0	0	0	0		0	1	1	0	0	0	2	
CoV-HKU1	0	0	0	0	0	2	0	0	0	0	0		0	0	2	1	0	3	
MPV	1	0	0	0	0	0	1	0	0	0	0	0		0	0	0	0	0	
AdV	29	0	0	0	0	0	0	0	0	0	0	0	0		1	0	1	2	
WU-PyV	17	0	0	0	0	0	3	0	0	1	0	0	0	2		1	1	2	
KI-PyV	24	1	0	0	0	1	1	0	4	1	0	1	0	0	4		2	2	
BoV	32	0	0	0	0	0	1	0	0	0	0	2	0	2	2	1		59	
Total sym	128	1	0	0	0	3	7	1	4	2	0	3	0	4	6	1	160	219	

[a] does not include 11 co-detections with 3 viruses each specimen. **Abbreviations**: HRV: human rhinovirus; IFV: influenza virus; PIV: parainfluenza virus; RSV: respiratory syncytial virus; HCoV: human coronavirus; HMPV: human metapneumovirus; AdV: adenovirus; WU-PyV: WU polyomavirus; KI-PyV: KI polyomavirus; HBoV-1: human bocavirus-1.

E-Table 4: Comparison of socio-demographic characteristics of the ORChID Study cohort and the general population of Brisbane or the State of Queensland, 2011.

	ORChID cohort	Brisbane/Queensland
Sex (% male)	75 (47.5%)	51.3%[a]
Aboriginal/Torres Strait Islander status	2 (1.3%)	3.4%[b]
Caesarean delivery	51 (32.3%)	34.2%[c]
Maternal age (years)	31.8 (±4.6)	29.3[c]
No. of children in the household		
Average children per family	1.5 (±0.8)	1.9[a]
Household income		
<$26,000	0%	14.3%[d]
$26,000-$67,499	10.5%	31.2%
$67,500-$114,999	35.8%	23.7%
≥$115,000	53.7%	30.8%
Smoking exposure		
Mother smokes	5 (3.2%)	14.8%[e]
Other householder smokes	17 (11.1%)	
Immunisation		
Fully immunised to 18-months	141 (91.6%)	92.8%[f]
Childcare		
At 12-months	72 (62.6%)	47.6%[g]

[a]Australian Bureau of Statistics 2011 Census of Population and Housing Basic Community Profile (Catalogue number 2001.0) - Brisbane (UCL301001). Table B04. Data presented are an average for children 0-2 years of age. http://www.censusdata.abs.gov.au/census_services/getproduct/census/2011/quickstat/UCL301001?opendocument&navpos=220. Accessed 28 May 2017.

[b]Australian Bureau of Statistics 2011 Census of Population and Housing Basic Community Profile (Catalogue number 2001.0) - Brisbane (UCL301001). Table B07. Data presented are an average for children 0-4 years of age. http://www.censusdata.abs.gov.au/census_services/getproduct/census/2011/quickstat/UCL301001?opendocument&navpos=220. Accessed 24 October 2017.

[c]Source: 'Australian mothers and babies 2011' report. Australian Institute of Health and Welfare. http://www.aihw.gov.au/WorkArea/DownloadAsset.aspx?id=60129545698. Data presented are for deliveries in Queensland. Accessed 28 May 2017.

[d]Australian Bureau of Statistics report 6523.0 Household Income and Income Distribution, Australia, 2011–12. Table 2; http://www.abs.gov.au/AUSSTATS/abs@.nsf/DetailsPage/6523.02011-12?OpenDocument. Accessed 28 May 2017.

[e]Australian Bureau of Statistics report 43640DO001: Australian Health Survey 2011-2012. Tables 1-17 – Queensland, Table 7.2 – Smoker status. Data presented are for current female smokers aged 25-34 years in Queensland; http://www.abs.gov.au/AUSSTATS/abs@.nsf/DetailsPage/4364.0.55.0012011-12?OpenDocument. Accessed 28 May 2017.

[f]Australian Immunisation Register. Data presented are for 2011 Queensland State summary of percentage of fully immunised children 24-<27 months; http://www.immunise.health.gov.au/internet/immunise/publishing.nsf/Content/acir-ann-cov-hist-data.htm. Accessed 28 May 2017.

[g]Australian Bureau of Statistics report 44020DO001_201106 Childhood Education and Care, Australia, June 2011, Table 1 and 6; http://www.abs.gov.au/ausstats/abs@.nsf/mf/4402.0. Accessed 28 May 2017