International Journal of TROPICAL DISEASE & Health

24(3): 1-11, 2017; Article no.IJTDH.34262
ISSN: 2278–1005, NLM ID: 101632866

Phenotypic Characterisation of *Escherichia coli* Isolates from Fish, Diarrheic and Healthy Children in Zanzibar, Tanzania

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Authors' contributions

This was a collaborative work among all authors. Author ARR designed the study, collected specimen from field, carried out and supervised laboratory work, performed statistical analysis and wrote the first draft of the manuscript. Authors PNW, SIK, RMH assisted on study design and in drafting the protocol organizing and refining the manuscript. Author AM played a role on literature citing, organsing and fine tuning of the manuscript according to journal requirements. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJTDH/2017/34262
Editor(s):
(1) Ajay Halder, Department of Obstetrics and Gynecology, All India Institute of Medical College, Bhopal, India.
Reviewers:
(1) R. Jasmin, Bishop Heber College, India.
(2) Akobi Oliver Adeyemi, Federal Medical Centre, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/20002

Original Research Article

Received 21st May 2017
Accepted 2nd July 2017
Published 11th July 2017

ABSTRACT

Aims: This study was carried out with the objective of investigating *E. coli* virulence factors, antibiotic sensitivity, presence of extended-spectrum-β-lactamase [ESBL] and serotype H1 O157 in *E. coli* from fish foods in comparison with those from healthy and diarrheic children in Zanzibar.
Study Design: Repeated cross sectional design was used to collect samples from fish, vendors

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and fish consumers through the seasons. Cross sectional design was used to collect children faecal samples from Mnazimmoja referral hospital.
Place and Duration of Study: The study was carried out in Zanzibar between August 2014 and June 2016
Methodology: A total of 113 *E. coli* isolates from fish (58), diarrheic (35) and healthy children (20) less than five years old were screened. Serotyping was used for detection of serotype O157 and extended-spectrum-β-lactamases (ESBL) production by disc diffusion using Cepodoxime/clavulanic acid (10/1µg) discs. Hemolysin was detected by hemolysis in human blood agar, serum resistance factors by growth inhibition of *E. coli* that was mixed and incubated with human sera, Hemagglutinins by Hemagglutination of RBCs and Gelatinase enzyme by production of clear zone of degradation of gelatine. Statistical analysis was done using Medcalc statistical software. Statistical difference in virulence factors possession data in *E. coli* isolates between fish, diarrheic and healthy children were subjected to analysis of variance [ANOVA], where $p<0.05$ was judged indicative of significant difference.
Results: Virulence factors detected in their order of prevalence were hemolysins (21.2%), serum resistance (12.2%), hydrophobicity (8.8%), Hemagglutinins (4.4%), and Gelatinase (2.7%). Virulence factors were detected in 82.9% and 38% of diarrheic children and fish isolates respectively. *E. coli* O157 serotype was detected in all 3 sources with higher percentage in diarrheic children (6.8%). Extended spectrum β-lactamase *E. coli* producers were found in fish (6.8%) and diarrheic samples (17.1%) but not from healthy children. Multidrug resistance [MDR] was detected in fish (44.9%), diarrheic children (82.8%) and healthy children (10%). Ampicillin (100%; 22.4%) and Tetracycline (82.9%; 44.8%) exhibited high antibiotic resistance both in diarrheic children and fish respectively. Fish foods could be sources of pathogenic and antibiotic resistant *E. coli* possessing multiple virulence factors. Moreover O157 serotypes, ESBL producing and multidrug resistant *E. coli* were also detected in fish foods.
Conclusion: It is therefore emphasised to improve hygiene in the fish value chain as well as raise awareness and frequent monitoring to fish stakeholders in Zanzibar.

Keywords: E. coli; virulence factors; diarrheic children; ESBL; H1 O157.

1. INTRODUCTION

The gastrointestinal tract [GIT] of both terrestrial and aquatic animals provide an ideal reservoir habitat of *E. coli* commensals as well as a genetic pool where pathogenic strains could be selected that may ultimately be etiological agents of urinary tract infections [UTI] or other serious systemic infections [1,2,3,4,5]. Pathogenic potential of *E. coli* seems to aggravate in immunocompromised individuals [2]. *E. coli* has also been isolated from environment, vegetables and animal derived foods [6] including marine foods and may be associated with intestinal and extra-intestinal diseases [7,8,9].

Virulence factors in *E. coli* give them a selective advantage in surviving adverse environmental conditions and ability to overcome the immune system of infected hosts thereby causing inflammation and infection [10,11]. Virulence factors are expressions of virulence genes acquired by horizontal gene transfer carried on plasmids or pathogenicity islands on chromosomes [12]. They may be in the form of cell surface proteins and carbohydrates that mediate attachment and protect the bacteria, bacterial toxins or hydrolytic enzymes. Among *E. coli* virulence factors known to enhance its pathogenicity and virulence are Haemolysins, serum resistance factors, cell surface hydrophobicity factors, Hemagglutinins, enterotoxins, siderophores and fimbriae adhesins. Generally *E. coli* pathogenic strains are more likely to have higher prevalence of virulence factors and determinants compared to commensals [13,14,15]; they also have larger genomes enabling them to encode more virulence factors [16]. On the other hand, some virulence factors may need synergistic presence of other factors to develop disease [1].

Attachment to human cells leading to gut colonisation [17] may be primary in mediating enteric disorders and are related with adhesins on fimbriae F4 (K88), F5 (K99), F6 (987P), F41, and F18 [18]. Hemagglutinins are also known *E. coli* cell surface virulence factors carried by fimbriae. Among important *E. coli* virulence factors that promote adherence to cells are surface hydrophobicity factors [19]. Possession of α-hemolysins in *E. coli* may be responsible for fatal hemolysis in their hosts [20], serum resistance factors make them resistant to lytic

action of complement in human sera [10]. Hemagglutinins and hydrophobic factors mediate fimbrial attachment to host cells [17,21,22]. *E. coli* related with α-hemolysin production are mostly implicated with high pathogenicity and severe urinary tract infections [23,24,25].

E. coli H1 O157 containing shiga toxin is highly pathogenic strain and may be associated with haemolytic uremic syndrome [20].

Production of extended-spectrum-β-lactamases [ESBLs] that attacks the beta-lactam ring of β-lactam antibiotics is one of the *Enterobacteriaceae* resistance mechanisms to counter the action of antibiotics. As of recent, there have been more reports on global increase of resistance on β-lactam antibiotics [26]. Monitoring the spread of antibiotic resistance among bacteria including β-lactam antibiotics is important on designing control strategies and curb global spread of antibiotic resistance.

The spread of antibiotic drug resistance among bacteria has raised world concern [27] threatening the ability of the world community to treat common infectious diseases, resulting in prolonged illness, disability, and deaths. Misuse of antibiotics in human practice, animal husbandry systems and agriculture has contributed to a large extent on development of drug resistance. Multidrug resistance, MDR, exhibited by resistance to three or more than three classes of antibiotics [28], is a common phenomenon among *Enterobacteriaceae* [29,19].

This study was carried out to investigate if fish foods could be potential sources of pathogenic *E coli* that exhibit virulence factors, ESBLs *and* H1 O157 serotype and compare them with *E. coli* isolates from diarrheic and healthy children. The study also investigated if fish could be potential sources of drug resistance *E. coli* strains in Zanzibar

2. MATERIALS AND METHODS

2.1 Study Area

The study focused on sixteen popular fish landing sites of Zanzibar islands both Pemba and Unguja. Samples from human subjects were derived from Mnazimmoja referral hospital in Zanzibar town. The sampling sites are spread out in all directions of the two Zanzibar islands Unguja and Pemba - north, south east and west. Zanzibar is situated in the Indian Ocean between latitudes 4 degrees and 6.5 degrees south of the equator.

2.2 Research Design

Repeated cross sectional design was used to collect samples from fish, vendors and fish consumers through the seasons. Cross sectional design was used to collect children faecal samples from Mnazimmoja referral hospital.

2.3 Sample Collection

2.3.1 Sample frame

Fish samples: Thirteen out of the 207 permanent and makeshift fish landing sites were chosen for sampling to cover all directions of the islands- north, south, east and west of Pemba and Unguja. Sample size was calculated using the formulae

$$n = \left[\frac{Z_{\alpha/2}\sigma}{E}\right]^2$$ [30]

The Z value at 95% confidence interval is 1.96 and the margin of error E is 0.5.

Fish samples were collected in four seasons; October-December, January-March, April-June and July-September between 2014 and 2015 from three categories- fishers, vendors and consumers; 65 samples were collected from each category each season making a total of 780 samples. Bacteria were isolated and identified by traditional biochemical laboratory methods [31] and MALDI-tof [Matrix-assisted laser desorption/ionization time-of-flight] in Public Health laboratory, Copenhagen. Through proportional sampling, 58 (n=86) *E coli* isolates were randomly chosen for virulence factors determination.

Human samples: Samples from diarrheic and healthy children were collected from Paediatric Unit of the Mnazimmoja Hospital in Zanzibar town between March and June 2016. Samples obtained from diarrheic and healthy children were 35 and 20 respectively.

2.4 Virulence Factors Detection

Hemolysin production: α-haemolysin was detected by using plate haemolysis test as

described by Farrell et al. [32]. The *E coli* isolates were inoculated onto 5% sheep blood agar plates incubated for 24 hrs at 35°C. Clear zones of complete lyses around colonies indicate production of α-haemolysin.

2.4.1 Serum resistance

Overnight cultures of the samples grown in nutrient broth were diluted in equal volumes of fresh nutrient broth then incubated for 2 hrs at 37°C to give a log phase culture suspension of 10^5 cfu/ml [33]. Cells were harvested after centrifugation at 1500 rpm for 5 min and making suspension in phosphate buffered saline. Equal volumes (200 µL) of this suspension and human serum were mixed and incubated in water bath. Ten micro-litres of suspension were withdrawn from samples after 0, 120 and 180 minutes and incubated in nutrient agar for plate counting after 24 hrs of incubation at 37°C. The samples were recorded as serum sensitive or resistant if viable count dropped to 1% of initial value or if >90% of organisms survived after 180 min respectively.

2.4.2 Cell surface hydrophobicity

The test was conducted in accordance to Siegfried [34] using salt aggregation test (SAT). Ammonium sulphate solutions with molar concentrations from 0.3125 through 5.0 M were prepared. A loop-ful [10 µL] of bacterial suspension in phosphate buffer was mixed with equal volume of ammonium sulphate solution. SAT value was the highest dilution of ammonium sulphate giving visible clumping of bacteria. Aggregation in 0.002 m phosphate buffer alone [pH 6.8] were considered auto aggregative. *E. coli* strains that had SAT value ≤1.25 M were considered hydrophobic.

2.4.3 Hemagglutination

A 3% suspension of human "O" group erythrocytes was prepared by mixing blood with Alservers solution and washing them twice in PBS. One drop of RBCs was then carefully mixed with equal volume of bacterial culture. After 1 minute of rotating the slide observation of macroscopic agglutination was recorded as positive agglutination as in accordance to Vost [35].

2.4.4 Gelatinase production

Gelatine agar plates were inoculated with test organisms and incubated overnight at 30C. Gelatinase production was detected by flooding the plates with mercuric chloride solution; the medium was expected to develop opacity while gelatinase producing colonies were expected to have a clear surrounding zone.

2.4.5 Test for extended spectrum β-lactamase (ESBL) production

Phenotypic confirmatory test was used [36]. Disc diffusion method using Cepodoxime (10 µg) and Cepodoxime/clavulanic acid (10/1µg) discs was employed. The discs were placed in Mueller Hinton agar plates inoculated with test organisms. After overnight incubation, a difference of ≥ 5 mm in diameter of zone of inhibition is regarded as positive ESBL production.

2.4.6 H1:O157 serotyping

Rapid latex test for detecting non-lactose fermenting *E. Coli* strain H1 O157 was used according to instructions given in the Welcome kit [Microgen, UK]. A drop of latex was placed on three wells of the provided card. Positive control (heat killed antigen, O157), negative control and cell sunspension of a test organism were placed close to the latex drop and then mixed using separate sticks. Reading was done within 30 seconds and agglutination indicated positive results.

2.5 Antibiotic Susceptibility Testing

E. coli samples were sub-cultured in Luria Bertani [LB] Agar [Difco™ 5243817] and incubated at 37°C for 24 hrs to obtain pure colonies. The antibiotic susceptibility testing was done using modified Kirby-Bauer disk diffusion method. Pure colonies were inoculated in sterile saline and matched with McFarland standard. The suspension was then spread in Mueller Hinton [Oxoid CM0337] agar plates, antibiotic discs impregnated and, after 24 hrs of incubation, diameters of zone of inhibition was read and interpreted as per CLSI criteria, 2014 [37]. The antibiotics used [Oxoid, England] were Ampicillin [10 µg], Ciprofloxacin [10 µg], Cepodoxime [10 µg], Gentamycin [10 µg], Norfloxacin [10 µg], Nitrofurantoin [300 µg], Sulphamethoxole-trimetoprim [25 µg] and Tetracycline [30 µg].

2.6 Statistical Analysis

Statistical analysis was done using Medcalc statistical software. Statistical difference in virulence factors possession data in *E. coli*

isolates between fish, diarrheic and healthy children were subjected to analysis of variance [ANOVA], where $p<0.05$ was judged indicative of significant difference.

3. RESULTS

3.1 Virulence Factors Detection, O157 Serotyping and ESBL Production in *E coli*

Virulence factor that had highest prevalence were Hemolysin that appeared in 21.2% (Table 1) of the isolates followed by serum resistance factors exhibited by 12.2% of the samples. Both virulent factors were detected in fish isolates. Cell surface hydrophobicity was expressed by 8.8% of the isolates and Hemagglutination of sheep RBCs was at a low level of 4.4%. Lowest number of virulence factor was Gelatinase enzyme production which was seen in 2.7% of the isolates. Significant difference was observed in virulence factor possession in *E. coli* isolates ($F=5.32$, $p<0.05$) between diarrheic and healthy children but not between fish and diarrheic children [$p>0.05$] demonstrating that virulence factors are comparably high both in fish and diarrheic isolates respectively.

None of the isolates had all five virulent factors. However 22.9% of *E. coli* isolates recovered from diarrheic children and 10% from healthy children had two virulent factors. Fish had relatively lower percentage [6.9%] of isolates with two virulent factors. *E.coli* with four virulent factors was observed in 3.4% of fish and 8.6% of diarrheic children isolates. The overall virulence factor prevalence of *E .coli* isolated recovered from all the three sources was 16.8%(19, n=113).

Escherichia coli O157 serotype was detected in 6.8% of diarrheic and 3.4% of fish. Extended spectrum β-lactamase *E. coli* producers were found in fish [6.8%] and diarrheic samples [17.1%] but not from healthy children.

3.2 Antibiotic Susceptibility Findings

It has been shown that 100% of isolates from diarrheic children and 55.2% from fish were resistant to at least one antibiotic compared to 15% from healthy children. Multidrug resistance [MDR] meaning resistance to three or more than three classes of antibiotics [28] was not detected from any of the isolates. However, all isolates [100%] from diarrheic children and 22.4% of isolates from fish are resistant to ampicillin [Table 2]. The next drug that the isolates had higher resistance was tetracycline. Surprising observation was the resistance against Cephodaxime, Ciprofloxacine and Norfloxacine was observed only in fish isolates while low resistance against Nitrofurantoin was observed in fish and healthy children but not in diarrheic children isolates [Table 2].

4. DISCUSSION

Virulence factors in *E. coli* are known to confer selective survival advantage and increase their capacity of fitness and cause diseases in affected hosts [14,38,11,17]. Suppression of innate immune responses caused by virulence factors is one of important mechanisms that enable pathogenic *E. coli* to persist within infected hosts [14]. In this study relatively lower overall prevalence of virulence factors (16.8%) were detected compared to the finding obtained by rivastava et al. Mittal et al. and Sabitha et al. [11,19,39]. This is not surprising because the isolates investigated were from fish foods and gastrointestinal tract; conversely isolates from extra-intestinal infections in most of the cases have higher numbers of virulent factors in concurrence with high pathogenicity as described by Bhrugubalda et al. and Mittal et al. [10,19].

Table 1. Prevalence of virulence factors, O157 serotype and ESBL production in *E coli*

Virulent Factor/Serotype/ESBL	Fish n=58 (%)	Diarrheic children n=35 (%)	Healthy children n=20 (%)	Total positive n=113 (%)	Statistical significance (ANOVA)
Serum resistance	6 (10.3)	7(20)	1(5)	14(12.2)	F=5.32
Hemolysin production	10(17.2)	12(34.3)	2(10)	24(21.2)	
Hemagglutination of sheep RBCs	1(1.7)	4(11.4)	0(0)	5(4.4)	Fish and diarrheic
Gelatinase production	0(0)	2(5.7)	1(5)	3(2.7)	(p>0.05)
Cell surface hydrophobicity	5(8.6)	4(11.4)	1(5)	10(8.8)	Fish and
O157 serotype	2(3.4)	4(6.8)	1(5)	7(6.2)	Healthy
ESBL production	4(6.8)	6(17.1)	0(0)	10(8.8)	(p<0.05)

Table 2. Relative resistance to individual antibiotics

Antibiotic	Source of isolate		
	Fish (n=58) (%)*	Diarrheic children (n=35) (%)*	Healthy children (n=20) (%)*
Ampicillin	13(22.4)	35(100)	3(15)
Tetracycline	26(44.8)	29(82.9)	6(30)
Gentamycin	5(8.6)	12(34.3)	0(0)
Cephodaxime	13(22.4)	0(0)	0(0)
Ciprofloxacin	5(8.6)	0(0)	0(0)
Norfloxacin	5(8.6)	0(0)	0(0)
Nitrofurantoin	6(10.3)	0(0)	2(10)

**Some isolates were resistant to more than one antibiotic*

The higher numbers of Hemolysin *E coli* producers compared with other resistant factors is a finding to be noted in this study. The overall prevalence Hemolysin *E coli* producers from fish, diarrheic and health chidren is 21.2%, however in the fish isolates 17.2% were Hemolysin producers which could potentially be transmitted to humans. Hemolysin production in *E. coli* is mostly associated with more virulent and serious clinical episodes [40,25]. Almost 7% of fish isolates were ESBL producers. Moreover, Hemolysin production could be linked to high prevalence of ESBL production and drug resistance [40]. Occurrence of high numbers of Hemolysin producers is in consistence with the work of other workers; Bhrugubalda et al. Mittal et al. Mbanga and Mudzana; Sharma and Sabitha et al. [10,19,29,38,39]. In this study it is also a noteworthy observation that few isolates from healthy children were also Hemolysin producers (n=20; 10%).

Hydrophobic virulent factors are among the factors detected in the isolates investigated. The hydrophobic factors are known to mediate attachment to host cells that is primary on pathogenicity and initiating infections [21,41]. Hemagglutinin factors which were also detected in this study are known to mediate cell adhesion and contribute to pathogenicity of *E. coli* [17]. Previous studies have shown that urinary and peritoneal isolates are most hydrophobic [22] suggesting relation of hydrophobicity with pathogenicity.

Serum resistant factors are important virulent factors in resisting the lytic action of complement system present in human sera and their presence may be associated with increased virulence [10,19]. More than 12% of isolates in this study had serum resistance factors with highest occurrence (20%) in diarrheic children followed by fish [10.3%] isolates. Studies on UTI isolates usually have higher prevalence of serum resistance factors than the current study [10,19,42]. However, results of this study underline the role of serum factors on pathogenicity and that isolates from fish have potential to cause bacteraemia, UTI and other systemic infections [10].

At least two virulence factors were found in 10.3% of fish isolates and 31.5% of diarrheic children while 10% of isolates from healthy children had two virulence factors. Although human *E. coli* isolates may carry virulence factors without developing disease [1] but generally presence of multi-virulence factors in *E. coli* isolates increase host cell invasion capacity and virulence [42,43,14]. Occurrence of lower numbers of virulence factors from healthy individuals compared with diarrheic children is in consistence with other workers who found lower prevalence of virulence factors in commensal *E. coli* [42]. Moreover occurrence of multi-virulence factors in fish isolates cautions that fish could be potential source of pathogenic *E coli*.

Samples positive for *E. coli* O157 serotype were, in this study, obtained from fish, diarrheic children as well as healthy children; 3.4% of fish isolates were O157 serotype. *Escherichia coli* O157:H7, first described from hemorrhagic colitis outbreak [44] is mostly associated with food poisoning epidemics caused by its possession of potent Shiga toxin. Apart from causing diarrhoea, O157 serotype can also cause haemolytic uremic syndrome [20]. Isolation of this serotype in this study signifies that fish could be a source of *E. coli* serotypes of public health concern. Moreover previous studies have proved that environment and animal derived foods could be sources of pathogenic *E. coli* to humans [9,45]. Furthermore, an issue of public health concern in this study is the isolation of ESBL producing *E. coli* in fish and diarrheic children but not in healthy children. Isolation of ESBL producing *E.*

coli from fish provides a risk of fish-human transmission [46]. ESBL producing *E. coli* are also linked with multidrug resistance [3,10,47,40] which is a public health risk. Among factors that favour increase ESBL production is extensive use of antimicrobials [40]. It is cautioned that increased occurrence of ESBL producing *Enterobacteriacea* is global emerging threat to public health [48,49].

Development of antibiotic resistance among bacteria is a worldwide public health concern that needs concerted efforts by all countries and relevant organisations to curb it down [50]. Prolonged use or misuse of antibiotics ultimately leads to antibiotic resistance [51,52] which could then be transferred to other bacteria through horizontal gene transfer [53,54,55]. Similar to Mbanga and Mudzana and Sharma et al. [29,38], this study has also found high resistance against Ampicillin in diarrheic children (100%, n=35) than in isolates from fish and heath children. High resistance was also observed in diarrheic isolates against tetracycline-82.9% (n=35). Resistance found in fish isolates against ampicillin and tetracycline were 22.4 & and 44.8% respectively (n=58), however the resistance observed in fish isolates was not as high as in sick children.

It is noteworthy that resistance was found against third generation cephalosporin- Cephodaxime and Cefotaxime from fish isolates. There were isolates from fish resistant to Norfloxacin and Nitrofurantoin. In contrast, no resistance against these drugs was found in diarrheic children. The resistant *E. coli* isolates were most probably derived from poultry owned by fish vendors who are known to be fond of keeping backyard poultry [Veterinary Animal Health and Production Reports, Zanzibar, 2010-2016] [56]. Moreover prescription of these drugs is not very restricted in the Veterinary pharmacies in Zanzibar which thereby encouraging drug resistance and poses a public health risk. It is a point worth noting, however, that these isolates can end up in human food chain. This finding stresses the need to strengthen enforcement of legislation against promiscuity of antibiotic drug use, more so in animal husbandry, for fear of emergence, dissemination and persistence of antimicrobial drug resistance [51,57,55]. Moreover results of this study show that fish could play a role on dissemination of drug resistance which again stresses the need to monitor environmental and animal isolates for drug resistance trends lest it should have public health repercussion as was observed by Mellata [51].

5. CONCLUSION

Results of this study demonstrate that virulence factors were detected from *E. coli* sourced from all three categories- fish, diarrheic and healthy children with highest prevalence in health children (31.5%). Serotype O157 and ESBL producing *E. coli* were also isolated. The results established that fish could be a source of virulent and drug resistant *E. coli* that are of public health importance. There is therefore need to improve hygienic standards in the fish production chain; fishers through vendors to consumers to reduce contamination.

Moreover multiple drug resistance was observed from all three sources and some isolates from fish were resistant to third generation cephalosporins and fluoroquinolones that are drugs of choice for treatment of *Enterobacteriaceae* infections in humans. This calls for concerted efforts on the part of responsible authorities to regulate and monitor antibiotic drug use in the islands in order to reduce occurrence and ultimate global spread of drug resistance.

CONSENT

All authors declare that ‘written informed consent was obtained from the patient parents on behalf of their children for publication of this paper”.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the ethics committee Zanzibar Medical Ethics Committee-ZAMREC (PROTOCOL NUMBER: ST/0004/JULY/016) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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http://sciencedomain.org/review-history/20002