"ANTIBIOTIC SUSCEPTIBILITY TESTING AND SEQUENCE ANALYSIS OF DIFFERENT SALMONELLA STRAINS ISOLATED FROM DIFFERENT REGIONS OF SOUTH GUJARAT"

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Abstract :

The increased spread of typhoid made the study of the food and water-borne disease that cause these diseases a public health priority. Little is known about how or why the different serotype Salmonellae cause pathology .35 %Cases occur mainly in tropical zones within developing countries worldwide, and control measures have been limited to the elimination of bacteria. Now a days Salmonellae became MDR in some countries.Thus, it is needed to develop new methods of studying salmonellae pathogenicity.

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1.Introduction :

Typhoid fever, also called enteric fever, is caused by the facultative intracellular microorganisms *Salmonella enterica* serovar Typhi (*S typhi*) and *Salmonella paratyphi*. Typhoid fever is a disease occurring more commonly among people after travelling or staying in developing countries where sanitation is poor and faecal contamination of food and water.Positive cases of typhoid fever is estimated at 12–33 million cases per year in world. Typhoid fever is more severe among infected patients with immunosuppression, biliary and UT abnormalities, reticuloendothelial blockade, and infection with antimicrobial MDR *S typhi* strains. 1–5% of infected people become chronic carriers by harbouring *S typhi* in the gall bladder, despite antibiotic therapy.Depending on the size of the inoculum ingested and the health and immune status of the person, the incubation period of *S typhi* ranges from 5 to 21 days. Symptoms of typhoid fever are characterised by fever , headache , gastrointestinal symptoms , relative bradycardia , splenomegaly , and leucopenia at different ratios. Extra-intestinal infectious complications can involve the central nervous system, cardiovascular system, pulmonary system, bone, joints, hepatobiliary system, genitourinary system, and others. Salmonella Typhi has rapidly developed resistance to commonly used drugs like ampicillin , chloramphenicol and cotrimoxazole,nalidixic acid, some flouroquinolones.

Now a days its important to go for some more research in Salmonellae. Despite recent advances in diagnostic and preventative medicine, infectious diseases still account for a large proportion of the disease burden and mortality worldwide, particularly in low-income areas and developing countries . Current clinical diagnostic tests for identifying infection-causing pathogens utilize limited technologies such as polymerase chain reactions (PCR), Sanger sequencing, or cell culture. These methods typically focus on identifying only a single pathogen at a time and often lack the specificity required to distinguish between closely related species or strains of the same species. Bacterial cultures can accurately identify culturable pathogens, but usually require 4–5 days to complete and cannot be conducted for all pathogens . Microarray technologies, such as the Virochip , have been shown to be useful in the space of pathogen identification. Microarrays, such as these, are designed to detect known pathogens through the use of high-sensitivity probes and isotype novel pathogens using probes that map to conserved genomic regions. While useful for broad spectrum screening of clinical samples, this technology is limited in that probes must

be continually designed and updated to support the ever-growing number of genomic sequences in public databases.

Current genomic diagnostic methods require detailed information regarding pathogen and include computationally intensive steps like genome assembly, multiple genome alignments, extensive homology searches, and/or phylogeny estimation, which can take more thanf three days to complete a single run .

Though these methods are unable to identify pathogens at the strain level accurately and will often assign ambiguously aligned reads to higher taxonomic levels which may lead to a nonspecific or incorrect diagnosis and the administration of ineffective clinical treatments.

Genetic variation is one of the most important part of typhoid .

Bioinformatics tools are readily available nowadays for predictive biology.genetic variation against isolated strains to eachother can let us to go on comparative genomics or genetic variation.The past decade has seen the introduction of fast and relatively inexpensive methods to detect genetic variation across the genome and exponential growth in the number of known variants . There is increasing interest in bioinformatics approaches to identify functionally important variants. Here, we describe the essential components of bionformatics tools that predict functional and comparative genomics.

2.Methodology :

2.1.Materials :

2.1.1.Sample collection :

Urine sample, Blood sample, stool samples used for isolation of Salmonella species. Among them blood is the habitat that gives more diversifying Salmonellae growth. Samples like Urine and stool have favorable conditions after 2 weeks of fever i.e. salmonellae can be isolated from urine and stool after 2 weeks of fever only. So, blood sample is best for isolation of Salmonellae.

2.1.2.Media :

The Patients' sample was inoculated in BACTECT PLUS bottle for blood culture.After inoculation , incubation must needed. So, after regular time interval , one must streak it on agar plates. The samples were streak on media commonly used for isolation of bacteria, like, Nutrient Agar, Blood Agar and Mac-conkey's agar. The plates were subjected to incubation and

Salmonella *spp.* was isolated. Then these organisms were confirmed for their species . For that the isolated organisms were plated on Salmonella-Shigella agar,Bismuth sulfite agar,Wilson-blair agar, incubated to obtain the proper confirmative growth. Various biochemical media were also inoculated. For confirmation,Triple sugar iron test was important. Genomic DNA were extracted using DNA extraction kit.

2.2.Method :

2.2.1.Sample collection :-

2.2.1.1.Site:- Blood samples from different regions of South Gujarat .

2.2.1.2.Sample Collection:-As stated the samples were collected from various regions of South Gujarat in BACTEC PLUS blood culture bottle by direct collection of blood from vein of patient by collection holder and bactec bottle or by diect collection of blood in 0.1% Na-heparin vacutte and then further inoculation in bactec bottle via sterile syringe.

2.2.2.Study of growth on various media :

Growth on various media had been studied . Commonly Salmonella typhi produced H2S which shows black color growth in specialized media . Where as , Salmonella paratyphi A produce Gas and Salmonella Paratyphi B produce excess H_2S.

2.2.3. Antibiotic susceptibility testing :

Antimicrobial susceptibility testing was performed on all isolates by the Kirby-Bauer disk diffusion method. Susceptibility testing was performed and interpreted in accordance with Clinical and Laboratory Standard and Institute CLSI) guidelines.

2.2.4.Extraction of Genomic DNA: Genomic DNA has been extracted using proper kit and following that sent cultures for DNA sequencing (16srRNA gene).

2.2.5.Bioinformatics analysis:-

This project took the genetic information of partially and fully sequenced Salmonella genomes from national databases, compared and catalogued this data from our sequenced data.

Bioinformatics Software Programs that were utilized

a. Assemble sequence using codon code aligner 4.1.

Codon code aligner provides multiple tools for DNA and Protein sequences including assemblance of contigs ,alignment of contigs,find heterozygous mutations, define regions of interest, generate phylogenetic tree and restriction maps , analyse heterozygous insertions and deletion.

b. Sequence submission to NCBI using sequin.

c. Primer designing by PRIMER3.

Primer3 is a free online tool to design and analyze primers for PCR and real time PCR experiments. Primer3 can also select single primers for sequencing reactions and can design oligonucelotide hybridization probes. The online tool constitutes some important features like primer detection, cloning, sequencing and Primer listing

d. Homology Sequence search using MEGA 5.0 software(BLAST).

For the construction of genome alignments, a piece by piece and all-against-all comparison of the genetic sequences encoded in the complete *Salmonella* genomes was performed.

e. Phylogenetic tree construction by using MEGA 5.0 software.

MEGA is an integrated tool for conducting sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses.

f. Download whole genome sequence.(100% similarity in BLAST).

g. Again run BLAST of previous result of BLAST (100% similarity).

h. Search resistant gene and gene sequence by RESFINDER.

ResFinder identifies acquired antimicrobial resistance genes in total or partial sequenced isolates of bacteria

i. **Se**arching pathogeny islands of Salmonella species (O/H/AH/BH-islands) .

Islands were used for local gene identification i.e. sequences already submitted as O/H/AH/BH-antigen gene and by that comparing it with query sequence.

3.Results and Discussion:

Salmonella typhi and Salmonella paratyphi A had been readily collected from different regions of South Gujarat. All isolates are readily confirmed via different biochemical tests.Antibiotic susceptibility test has been readily performed and isolates gave results like some are resistant to Nalidixic acid and/or cephalosporins and/or penicillins and/or quinolins.

Gram negative antibiotic	3N1	3N2	3N3	3N4	3N5	3N6	541	629	2222	EQUAS	N	SPA1	SPA2
Ampicillin	S	S	S	S	S	S	S	S	S	S	S	S	S
Piperacillin	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefuroxime	S	S	S	S	S	S	S	R	R	S	S	S	S
Ciprofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	R	S	R	S	R	S	S	R	R	S	S	S
Chloramphinicol	S	S	S	S	S	S	S	S	S	S	S	S	S
Doxycycline hydrochloride	S	S	S	S	S	S	S	S	S	S	S	S	S
Cephalexin	S	S	S	S	S	S	S	S	S	S	S	S	S
Tobramycin	S	S	S	S	S	S	S	S	R	S	S	S	S
Tetracyclin	S	S	S	S	S	S	S	S	S	S	S	S	S
Ofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Nalidixic acid	R	S	S	S	R	R	S	S	R	R	S	S	S
Co-trimoxazole	S	S	S	S	S	S	S	S	S	S	S	R	R
Ampicillin-sulbactum	S	S	S	S	S	S	S	S	S	S	S	S	S
Amoxyclav	S	S	S	S	S	S	S	S	S	S	S	S	S
Ceftriaxone	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefixime	S	S	S	S	S	S	S	S	S	S	S	S	S
Ceftazidime	S	S	S	S	S	S	S	S	S	S	S	S	S

Netillin	S	S	S	S	S	S	R	S	R	S	S	S	S
Amikacin	S	S	S	S	R	S	S	S	R	S	S	S	S
Levofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefoperazone-sulbactum	S	S	S	S	S	S	S	S	S	S	S	S	S
Piperacillin-tazobactum	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefpodoxime	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefotaxime	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefoperazone	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefepime	S	S	S	S	S	S	S	S	S	S	S	S	S
Imipenem	S	S	S	S	S	S	S	S	S	S	S	S	S
Meropenem	S	S	S	S	S	S	S	S	S	S	S	S	S
Ticarcillin-clavulinic acid	S	S	S	S	S	S	S	S	S	S	S	S	S
Gatifloxacin	S	S	R	S	S	S	S	S	S	S	R	S	S
Aztreonam	S	S	S	S	S	S	S	S	S	S	S	S	S
ertapenem	S	S	S	S	S	S	S	S	S	S	S	S	S
Colistin	S	S	S	S	S	S	S	S	S	S	S	S	S
Polymyxin B	S	S	S	S	S	S	S	S	S	S	S	S	S
Tigecycline	S	S	S	S	S	S	S	S	S	S	S	S	S

Results shows non multi drug resistant salmonellae has been isolated from various regions of south gujarat.

Genomic DNA extraction done by DNA extraction kit and then send for DNA sequencing. Here , this is a chart of isolate , strain name ,day of fever when sample was collected from patient,after collecting sample which incubation period needed for growth,after growing on plate,how much

sour isolates (which are submitted to NCBI),accession number from NCBI for BLAST results of isolates , further accession number of blast results of previous BLAST result

An Organism	Strain	Place	Sample collection at day ?	Incubation period	Appropriate colony count	Widal results "O"	"H"	Accesion number	BLAST accesion no.	Further blast acc. no.
S.typhi	3N1	Olpad	4	3	4	1:160	1:160	KM977891	CP003278.1	AE014613.1
S.typhi	3N2	Surat	5	2	3	1:80	1:80	KM977892	CP003278.1	AE014613.1
S.typhi	3N3	Navsari	3	4	4	1:160	1:80	KM977893	CP003278.1	AE014613.1
S.typhi	3N4	Vyara	4	3	4	1:80	1:160	KM977894	CP003278.1	AE014613.1
S.typhi	3N5	Vapi	6	1	1	1:320	1:320	KM977895	CP003278.1	AE014613.1
S.typhi	541	Valsad	5	3	3	1:160	1:160	KM977896	CP003278.1	AE014613.1
S.typhi	629	Bardoli	4	2	3	1:80	1:80	KM977897	CP003278.1	AE014613.1
S.typhi	2222	Bilimora	5	3	3	1:160	1:80	KM977898	CP003278.1	AE014613.1
S.typhi	Equas	Bharuch	4	3	3	1:80	1:80	KM977899	CP003278.1	AE014613.1
S.typhi	N	Sachin	3	4	5	1:80	1:80	KM977900	AL513382.1	AE014613.1
S.paratyphi A	SPA1	Surat	6	1	1	"AH"-	1:160	KM977901	FM200053.1	NC006511.1
S.paratyphi A	SPA2	Bharuch	5	2	3	"AH"-	-1:80	KM977902	FM200053.1	NC006511.1

Results shows highly similarity between isolates' sequence.Pathogeny island of Salmonellae has been downloaded from datasource i.e. each gene sequence had been downloaded from NCBI. Resistant genes has been identified using different alignment and also RESfinder tool.

Remain will be updated soon…….

4. Conclusion :

Salmonella species are believed to have coevolved with hominids for millions of years.So,It is very possible that salmonellae encode minimum ensemble of virulence genes required for successful infection,replication ,and dissemination.Thus,relative success of one species over another rely on the interplay between levels of gene expression and environmental factors..The diffential gene expression may also be influenced by variations resulting from chromosomal rearrangements which may studied through the multidisciplinary nature of molecular epidemiological studies.Molecular epidemiological methods have added genetic approaches which have suggested epidemiologically relevant characteristics specific to phylogenetic lineages or strain families.

All the isolates from different regions of south gujarat have their different and unique sequence which represents its similarity and dissimilarity amongst other strains and also have unique space and relation in phylogenetic tree , which shows there are some genetic factor which influence these changes and we can reach to these changes by the help of molecular epidemiology i.e.bioinformatics tool. Test shows antibiotic chart which is now common but we can predict if it will become MDR or not ? by predictive molecular biology.

5. References :

1.Hohmann EL (2001) Nontyphoidal salmonellosis. Clin Infect Dis 32: 263-269

2. Menezes GA, Khan MA, Harish BN, Parija SC, Goessens W, Vidyalakshmi K, Baliga S, Hays JP (2010) Molecular characterization of antimicrobial resistance in non-typhoidal salmonellae associated with systemic manifestations from India. J Med Microbiol 59: 1477-1483

3. Schlundt J, Toyofuku H, Jansen J, Herbst SA (2004) Emerging food-borne zoonoses. Rev Sci Tech 23: 513-533

4. Voetsch AC, Van Gilder TJ, Angulo FJ, Farley MM, Shallow S, Marcus R, Cieslak PR, Deneen VC, Tauxe RV (2004) FoodNet estimate of the burden of illness caused by nontyphoidal Salmonella infections in the United States. Clin Infect Dis 38 Suppl 3: S127-S134

5. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM (2010) The

global burden of nontyphoidal Salmonella gastroenteritis. Clin Infect Dis 50: 882-889

6. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV (1999) Food-related illness and death in the United States. Emerg Infect Dis 5: 607-625

7. Flint JA, Van Duynhoven YT, Angulo FJ, DeLong SM, Braun P, Kirk M, Scallan E, Fitzgerald M, Adak GK, Sockett P, Ellis A, Hall G, Gargouri N, Walke H, Braam P (2005) Estimating the burden of acute gastroenteritis, foodborne disease, and pathogens commonly transmitted by food: an international review. Clin Infect Dis 41: 698-704

8. Brent AJ, Oundo JO, Mwangi I, Ochola L, Lowe B, Berkley JA (2006) Salmonella bacteremia in Kenyan children. Pediatr Infect Dis J 25: 230-236

9. Jones TF, Ingram LA, Cieslak PR, Vugia DJ, Tobin-D'Angelo M, Hurd S, Medus C, Cronquist A, Angulo FJ (2008) Salmonellosis outcomes differ substantially by serotype. J Infect Dis 198: 109-114

10. Graham SM (2010) Nontyphoidal salmonellosis in Africa. Curr Opin Infect Dis 23:409-414

11. Kingsley RA, Msefula CL, Thomson NR, Kariuki S, Holt KE, Gordon MA, Harris D, Clarke L, Whitehead S, Sangal V, Marsh K, Achtman M, Molyneux ME, Cormican M, Parkhill J, Maclennan CA, Heyderman RS, Dougan G (2009) Epidemic multiple drug resistant Salmonella Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. Genome Res 19: 2279-2287

12. Morpeth SC, Ramadhani HO, Crump JA (2009) Invasive non-Typhi Salmonella disease in Africa. Clin Infect Dis 49: 606-611

13. Vandenberg O, Nyarukweba DZ, Ndeba PM, Hendriksen RS, Barzilay EJ, Schirvel C, Bisimwa BB, Collard JM, Aidara KA, Aarestrup FM (2010) Microbiologic and clinical features of Salmonella species isolated from bacteremic children in eastern Democratic Republic of Congo. Pediatr Infect Dis J 29: 504-510

14. Wain J, Keddy KH, Hendriksen RS, Rubino S (2013) Using next generation sequencing to tackle non-typhoidal Salmonella infections. J Infect Dev Ctries 7: 1-5

15. Fashae K, Ogunsola F, Aarestrup FM, Hendriksen RS (2010) Antimicrobial susceptibility and serovars of Salmonella from chickens and humans in Ibadan, Nigeria. J Infect Dev Ctries 4: 484-494

16. Hendriksen RS, Le HS, Bortolaia V, Pulsrikarn C, Nielsen EM, Pornruangmong S, Chaichana P, Svendsen CA, Weill FX, Aarestrup FM (2012) Characterization of isolates of Salmonella enterica serovar Stanley, a serovar endemic to Asia and associated with travel. J Clin Microbiol 50: 709-720

17. Achtman M, Wain J, Weill FX, Nair S, Zhou Z, Sangal V, Krauland MG, Hale JL, Harbottle H, Uesbeck A, Dougan G, Harrison LH, Brisse S (2012) Multilocus sequence typing as a replacement for serotyping in Salmonella enterica. PLoS Pathog 8:e1002776

18. European Nucleotide Archive (2012) [http://www.ebi.ac.uk/ena/]. European Bioinformatics Institute

19. Okoro CK, Kingsley RA, Quail MA, Kankwatira AM, Feasey NA, Parkhill J, Dougan G, Gordon MA (2012) High-resolution single nucleotide polymorphism analysis distinguishes recrudescence and reinfection in recurrent invasive nontyphoidal Salmonella Typhimurium disease. Clin Infect Dis 54: 955-963

20. WHO | the global burden of disease: 2004 update. [http://www.who.int/ healthinfo/global_burden_disease/2004_report_update/en/]

21. Didelot X, Bowden R, Wilson DJ, Peto TEA, Crook DW: Transforming clinical microbiology with bacterial genome sequencing. Nat Rev Genet 2012, 13:601–612.