Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 55 (1): 204-209. Oct. 2017
DOI: 10.5455/ajvs.246419

Molecular Identification of Beta-Lactamase-Producing *Salmonella Enteritidis* from Broiler Chickens

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Key words: *Salmonellaenteritidis*, β-lactamase gene, PCR

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ABSTRACT

Salmonellosis is one of the most infectious diseases in both humans and animals which cause highly economic losses in poultry production.
Atotalof 210 samples for isolation of *Salmonella enteritidis* were isolated from chickens suffering from diarrhea , were collected from different broiler chickens farms. Samples were cultivated in XLDAgar, SSAgar, Macconkey Agar and Hekton Enteric agar medium, andconfirmed by using Polymerase Chain Reaction (PCR). The results revealed that 4 (80%) samples were positive for *Salmonella enteritidis.* Amplification of (bla TEM) gene of *Salmonella enteritidis* showed that out of4 isolate 2 (50%) were positive for this gene. In-vitro experiment done for assessing the effect of β-lactamase-producing *S.enteritidis*on the sensitivity of *S. aureus*to Penicillin .PCR assay which used in the study was specific and accurate for detection and identification *Salmonella enteritidis* and β-lactamasegene.

1. INTRODUCTION:

Salmonellosis is one of the most common and widely distributed foodborne diseases in the world in both humans and animals. Salmonellosis in poultry causes severe economic loss through mortality and reduced production (Haider*et al*., 2004).Salmonella is a Gram-negative facultative rod-shapedbacterium.Genus*Salmonella*ofthe family*Enterobacteriaceae*includesMore than 2,500 serovars (Breytenbach, 2004). *Salmonella enterica* serovarEnteritidis is a major cause of food-borne outbreaks in the world (Herikstad et al., 2002). Traditional culture methods for detection of *Salmonella* speciesin poultry include nonselective pre-enrichment followed by selective enrichment and plating on selective and differential agars (Whyte et al., 2002). Xylose Lysine Deoxycholate agar medium (XLD) is more preferable in isolation of *salmonella* than salmonella-shigela (SS) and Macconkeyagar (EL-Gebaly 2003). Polymerase chain reaction (PCR) is more sensitive and specific than other traditional microbiological methods used for detection of *Salmonella*species(Allgayer et al., 2008).

Multidrug-resistant strains have been increasingly described among *Salmonella* species worldwide (Williams, 2001). The extensive use of antimicrobials for treatment and prevention of animal diseases is considered as an important factor in the emergence of antibiotic-resistant bacteria that can subsequently be transferred from animals to humans through the food chain. Most antimicrobial-resistant *Salmonella* species are acquired from eating contaminated foods of animal origin (Angulo et al., 2000).With the increased use of Beta-lactam antimicrobial agents to treat enteric infection, *Salmonella* species had acquired resistant to third generation cephalosporin antibiotics in different parts of the world and had been associated with clinical treatment failure (Olesen et al., 2004).Themost common mechanism of resistance is the secretion of beta-lactamase enzymes. These enzymes hydrolyze the ß-lactam ring structure, yielding beta-amino acids with no antimicrobial activity.The genes encoding for beta-lactamases are typically carried on plasmids (Mascaretti, 2003).
The aim of this study was planned for Isolation and identification of β-lactamase-producing *Salmonella enteritidis* from suspected diseased chickens by

using traditional culture methods and molecular identification using PCR assay as well as In vitro assessment of β-lactamase-producing *S. enteritidis* on sensitivity of of *S. aureus to* penicillin .

2. MATERIALS AND METHODS

2.1. Sampling

A total of 210 tissue swabs (liver, intestine, spleen and gall bladder) were collected from broiler chickens (3-32 days of age) suffering from diarrhea during the period of 2015 in El-Beheira governorate. The collected swabs were separately soaked into sterile buffered peptone water and immediately transferred to the laboratory for bacteriological isolation and identification.

2.2. Isolation and identification of collected samples

The extracts of the collected swabs were initially inoculated in Rappaport Vassiliadis broth, incubated at 37°C for 24 hrs and then plated ontoXLD agar medium for 24 hours at 37°C. The suspected colonies were picked up, purified and examined for the presence of Gram negative bacilli or cocco-bacilli. Isolates of *S. enteritidis*were identified biochemically according to (Cruickshank et al., 1975).

2.3. Antimicrobial susceptibility testing:

Antimicrobial susceptibility of the obtained isolates of *S. enteritidis*were tested using the disk diffusion method on Muller–Hinton agar as standardized by the Clinical and LaboratoryStandardsInstitute (CLSI, 2012).The following antimicrobials were tested: Ampicillin, Cefazolin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamicin, Norfloxacin, Penicillin, Ampicillin, Streptomycin and Tetracycline.

2.4. Molecular identification of Salmonella species and β-lactamase-producing S. enteritidis

DNA for *S. enteritidis*was extracted from 5 bacterial cultures using QIAamp DNA Mini Kit Catalogue no.51304, according to the manufacturer instructions. PCR primers utilized in the present study are listed in table (1).The amplification reactions were performed in a total volume of 25 µl containing 12.5µl of Emerald Amp GT PCR master mix (2x premix), 20 pmol of each primer, 6 µl of DNA template and 4.5 µl of PCR grade water. The PCR mixtures were initially denatured and were then subjected to 35 cycles of amplification (table 2). The amplified products were resolved by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and thenvisualized on *a* UV transilluminator.

2.5. Assessing the effect of β-lactamase-producing *S. enteritidis*on the sensitivity of *S. aureus*to Penicillin

In vitro assessment of β-lactamase-producing *S. enteritidis*on strain ATCC 29213 of *S. aureus*(kindly provided by Animal Health Research Institute, Dokki, Giza) was conducted by preparing six groups of bacterial cultures in buffer peptone water as follow; group A (each ml was inoculated with 10^2 colony forming units [*CFU*]of*S. aureus*), group B (each ml was inoculated with10^2 CFU of*S. enteritidis*), group C (each ml was inoculated with 10^2 CFU of both *S. enteritidis*and*S. aureus*), group D (each ml was inoculated with 10^2 CFU of S. aureus and 2µg per ml of penicillin(which kindly prepared by Animal Health Research Institute, Dokki, Giza according to , EUCAST , 2000), group E (each ml was inoculated with 10^2 CFU of *S. enteritidis*and 2µg per mlof penicillin) and finally group F (each ml was inoculated with10^2 CFU of *S. enteritidis*and 2µg per ml of penicillin, incubated at 37°C for 3hrs, and then reinoculated with 10^2 CFU of *S. aureus*). Three replicates within each group were made and all of them were incubated overnight at 37°C. Colony count assay for *S. enteritidis*in groups B, C, E and F was separately determined on duplicated plates of XLDagar medium incubated at 37°C for 24 hrs. Meanwhile, colony count assay for *S. aureus*in groups A, C, D and F was separately determined on duplicated plates of Baired-Parker agar medium incubated at 37°C for 48 hrs .The count of *S.aureus* and *S. enteritidis* was calculated separately in each group according to the following equation:

$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)}$$

where:

N=Number of colonies per ml

$\sum C$=Sum of all colonies on all plates counted

n_1=Number of plates in first dilution counted

n_2=Number of plates in second dilution counted

d = Dilution from which the first counts were obtained

2.6. Statistics:

Bacterial plate counts (*S.aureus* and *S. enteritidis)* were first transformed to the logarithmic scale

(log_{10}) before analysis. Effect of the group on bacterial counts was assessed by the analysis of variance using the general linear model procedure (PROC GLM) of SAS (SAS, 2011). The significance of differences among means was tested by the probability difference option (LSMEANS/PDIFF) of the same procedure.

3. RESULTS AND DISCUSSION

Salmonellosis is considered to be one of most common foodborne bacterial diseases in the world (Coburn et al., 2007). Poultry are considered as one of the principal reservoirs for Salmonella organisms and pose a significant risk of transmission to human, especially through consumption of uncooked meat and eggs and dealing with chicken meat (Wales and Davies, 2011). In the current study, a total of 210 swabs samples were collected from tissues (liver, intestine, spleen and gall bladder) of broiler chickens suffering from diarrhea, and analyzed for the presence of salmonella species.All samples were processed, plated onto XLD agar medium. The obtained bacterial isolates were identified through biochemical reaction profile.Only five (2.4%) out of 210 isolates were identified as *Salmonella*.In contrast, high prevalence ratios (8.2-15%) were previously obtained (Abd El- Tawab et al., 2015, Malidareh et al., 2013; Hossain et al., 2006; Salehi et al., 2005).The five isolates were further confirmed as *Salmonella* using PCR assay based oninvA gene codes for a protein in the inner membrane, which is necessary for bacterial invasion to epithelial cells (Darwin and Miller, 1999).The specificity of the primers sets was confirmed by positive amplification of 284 bpfragment in all tested samples (Fig. 1). In agreement with (Salehi et al., 2005), results support the efficacy of invA gene-based PCR assay in detection of Salmonella species. Four (80%) out of 5 obtained salmonella isolates were subsequently confirmed as *S. enteritidis*using PCR based onsefA gene, which encodes the main subunit of the SEF14 fimbrial protein (Lindler and Tall,1993). Amplification of 310 bp fragment in positive reactions as well as positive control indicated that the PCR assay has been performed correctly (Fig. 2).

Table (1): Oligonucleotide primers sequences used to identify β-lactamase-producing *S. enteritidis*

Primer	Primer sequence 5' to 3'	Target gene	Amplicon size (bp)	Reference
invA(f)	GTGAAATTATCGCCACGTTCGGGCAA	invA	284	Oliveiraet al., 2003
invA(r)	TCATCGCACCGTCAAAGGAACC			
sefA(f)	GCAGCGGTTACTATTGCAGC	sefA	310	Akbarmehr et al., 2010
sefA(r)	TGTGACAGGGACATTTAGCG			
bla$_{TEM}$ (f)	ATCAGCAATAAACCAGC	bla$_{TEM}$	516	Colom et al., 2003
bla$_{TEM}$ (r)	CCCCGAAGAACGTTTTC			

Table (2): The thermocycling conditions for different primer sets.

Primers	Cycling conditions				
	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**
invA(f) invA(r)	94 ºC / 5 minutes	94 ºC / 30 sec	55 ºC / 30 sec	72 ºC / 30sec	72 ºC / 5 min
sefA(f) sefA(r)	94 ºC / 5 minutes	94 ºC / 30 sec	52 ºC / 30 sec	72 ºC / 45 sec	72 ºC / 10 min
bla$_{TEM}$ (f)	94 ºC / 5 minutes	94 ºC / 30 sec	54 ºC / 45 sec	72 ºC / 45 sec	72 ºC / 10 min
bla$_{TEM}$(r)		**Repeated for 35 cycles**			

Table (3) Antimicrobial susceptibility of the obtained isolates of *S. enteritidis*

Antimicrobial	Number (percent)		
	Resistant	**Intermediate**	**Sensitive**
Penicillin	2 (50)	0 (0)	2 (50)
Ampicillin	2 (50)	0 (0)	2 (50)
Gentamicin	1 (25)	0 (0)	3 (75)
Streptomycin	0 (0)	1 (25)	3 (75)
Amoxicillin/clavulanic acid	1 (25)	1 (25)	2 (50)
Tetracycline	2 (50)	1 (25)	1 (25)
Chloramphenicol	2 (50)	2 (50)	0 (0)
Enrofloxacin	0 (0)	0 (0)	4 (100)
Ceftriaxone	0 (0)	1 (25)	3 (75)
Cephalothin	0 (0)	2 (50)	2 (50)
Sulfisoxazole	4 (100)	0 (0)	0 (0)
Trimethoprim/sulfamethoxazole	0 (0)	1 (25)	3 (75)

Table (4) Mean of count *S. aureus and S. enteritidis*

Group	Mean count of *S. aureus*	Mean count of *S. enteritidis*
A	$2.76 \times 10^9 \pm 1.46 \times 10^9$ a	**ND**
B	ND	$6.62 \times 10^{12} \pm 4.32 \times 10^{12}$ a
C	$3.92 \times 10^7 \pm 2.60 \times 10^7$ b	$1.01 \times 10^9 \pm 6.64 \times 10^8$ b
D	0 ± 0 d	**ND**
E	ND	$1.88 \times 10^{12} \pm 1.38 \times 10^{12}$ a
F	$2.51 \times 10^2 \pm 1.46 \times 10^2$ c	$5.11 \times 10^9 \pm 3.48 \times 10^9$ b

Values are means ± standard errors. ND : not detected
Means followed by different letters differ significantly (P<0.01).

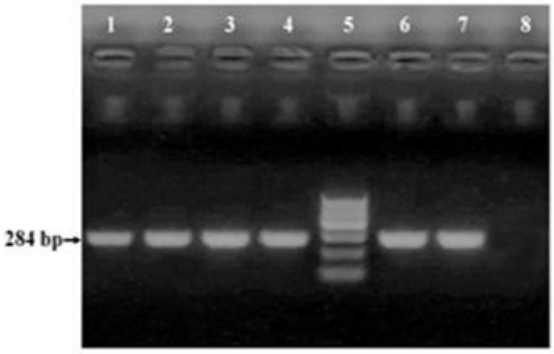

Figure (1): Agarose gel electrophoresis of amplified DNA of*invA*gene of *Salmonella*. Lane (5): DNA molecular weight ladder, lane (4): positive control,lanes (1, 2, 3, 6 and 7): positive results for*invA* gene, lane(8): negative control

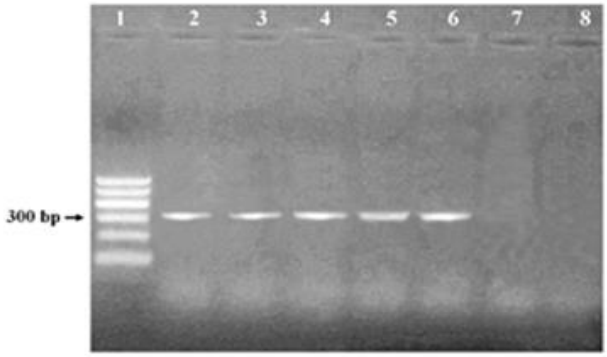

Figure (2): Agarose gel electrophoresis of amplified DNA of*sefA gene* of *S. enteritidis.* Lane (1): DNA molecular weight ladder, lane (2): positive control,lane (3,4, 5 and 6) positive results for *sefA gene*, lane (8): negative control, lane (7): negative results for *sefA gene.*

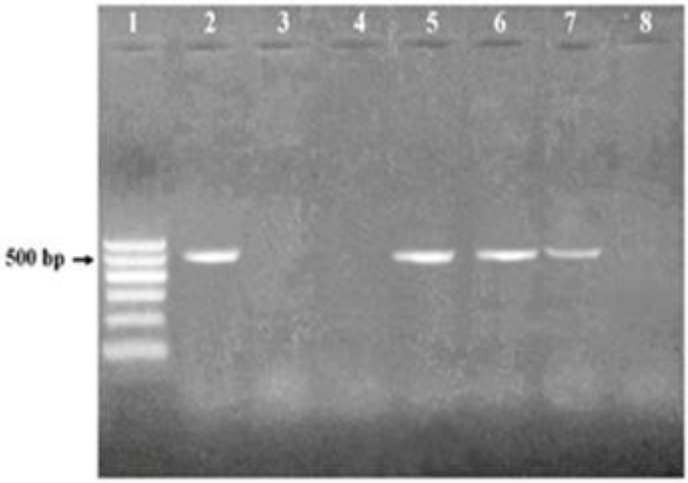

Figure (2): Agarose gel electrophoresis of amplified DNA of*sefA gene* of *S. enteritidis.* Lane (1): DNA molecular weight ladder, lane (2): positive control,lane (3,4, 5 and 6) positive results for *sefA gene*, lane (8): negative control, lane (7): negative results for *sefA gene.*

Recently, increasing resistance of *Salmonella* to commonly used antimicrobials has become a matter of concern. The disk diffusion method was performed to determine susceptibility of the obtained isolates of *S. enteritidis*as standardized by (CLSI, 2012). All isolateswere found resistant to sulfisoxazole, meanwhile sensitive to enrofloxacin (table 3). Results of sensitivity to enrofloxacin was in agreement with those obtained by (Salehi et al. 2005). Low level (25%) of resistance was found for gentamicin, streptomycin, ceftriaxone and trimethoprim (de Oliveira et al., 2005). All isolates of *S. enteritidis* were multiresistant (resistance to two or more antimicrobial agents). Resistance of *Salmonella* to ampicillin is mediated most commonly by a β-lactamase enzyme and the main family of β-lactamases responsible for ampicillin resistance is the temoxicillinase (TEM) β-lactamases. The presence of the bla_{TEM}gene encoding TEM-1 β-lactamase is believed to confer resistance to penicillins. Two (50%) out of 4 obtained isolates of *S. enteritidis* were proved to penicillin-resistant as well as positive for bla_{TEM} gene usingPCR assay. The specificity of the primers sets was confirmed by positive amplification of 516 bp fragment in positive samples as well as positive control (Fig. 3). The same results were previously obtained (Ranjbar et al., 2010). In vitro assessment of the effect of β-lactamase-producing *S. enteritidis*on the sensitivity of *S. aureus* to penicillen was conducted in six groups of bacterial cultures in buffer peptone. The obtained results were statistically calculated (table 4). which compare between result of S.aureus mean count in different groups and *S. enteritidis*mean count in different groups. By comparing the count of S.aureus in four groups ,we noticed that the highest count at group 1 and no count at group 4 and the count in group 3 less than count in group 1 where its present mixed with *S. enteritidis*and the count in group 6 more than count in group 4that may br due to : *S. enteritidis*produced beta-lactamase gene which affect action penicillin toward S.aureus , that maybe contributed treatment of staph by : Penicillens and first cephalosporines in mixed infection with β-lactamase produced *S. enteritidis.* In the other wise, by By comparing the count of *S. enteritidis*in four groups ,we noticed that the highest count at group 2 and group 5 , Count in group 3,6 less than group 2, 5 may be due to its combination with *S.aureus*.

Results revealed significantly lower colony countof S. auerus in mixed culture with β-lactamase-producing *S. enteritidis*and Penicillen in comparison with control groups .The results of the present study necessitate further exploration of genetic elements related to antibiotic resistance and underlines the need to track the evolution of β-lactamases in *S. entritidis* isolates in Egypt.

Further, appropriate selection and rotation of antimicrobials as well as continuous monitoring of antimicrobial susceptibility profiles could help to control the emergence and spread of resistant strains of *S. entritidis.*

4. REFERENCE

Akbarmehr, J., Salehi, T.Z. and Brujeni, G.H. N. 2010. Identification of Salmonella isolated from poultry by MPCR technique and evaluation of their hspgroel gene diversity based on the PCR-RFLP analysis. African J. Microbiol. Res. 4(15): 1594-1598

Allgayer, M . C., Lima-Rosa, C.A .V., Weimer, T. A.C., Rodenbusch, R. , Pereira, R. A .A. ,Streck, F. , Oliveira, S.D., Canal, C.W. 2008. Molecular diagnosis of Salmonella species in captive psittacine birds. Vet. Rec. 162: 816-819.

Angulo FJ, Johnson KR, Tauxe RV, Cohen ML. 2000 . Origins and consequences of antimicrobial-resistant nontyphoidal Salmonella: implications for the use of fluoroquinolones in food animals. Microb Drug Resist. 6(1):77-83.

Breytenbach, B. 2004.Biography Resource Center,http://galenet.gale.com.January4.

Clinical and Laboratory Standards Institute (CLSI), 2012. Institute Performance Standards for Antimicrobial Susceptibility Testing; Informational Supplement. CLSI/NCCLS Document M10-S16. Clinical and Laboratory Standards Institute, Wayne, PA

Coburn, B., Grassl, G. A., Finlay, B. B. 2007.Salmonella, the host and disease: a brief review. Immunol.cell boil. 85 (2): 112-118.

Colom K, PèrezJ, Alonso R, Fernández-AranguizA,LariñoE, Cisterna R. 2003.Simple and reliable multiplex PCR assay for detection of blaTEM,blaSHV and blaOXA-1 genes in Enterobacteriaceae. FEMS Microbiology Letters 223: 147-151.

Cruickshank, R., Duguid, J.P., Marmion, B.P., Swain, R.H.A. 1975. Medical Microbiology. 12Th Ed the practice of medical .

Darwin KH, Miller VL .1999.Molecular basis of the interaction ofSalmonella with the intestinal mucosa.Clin.Microbiol. Rev. 12: 405-428.

de Oliveira, S.D., Flores, F.S., dos Santos, L.R., Brandelli, A., 2005. Antimicrobial resistance in Salmonella enteritidis strains isolated from broiler carcasses, food, human and poultry-related samples. International journal of food microbiology, 97(3), pp.297-305.

EL-Gebaly, Lobna, S.A. 2003. Microbiological studies on Salmonellae of ducks .M V.Sc. Thesis, Fac. Vet. Med. Tanta Univ. Egypt.

El-Tawab, A.A.A., Ammar, A.M., Nasef, S.A., El-Hofya, F.I. and Nabilc, N.M. 2015. Studies on different salmonella serotypes isolated from poultry in different governorates in Egypt. Benha Veterinary Med. J. 28: 2:169-175 .

European Committee for Antimicrobial Susceptibility Testing (EUCAST), 2000 .Determination of minimum inhibitory concentrations of antibacterial agents by agar dilution.European Society of Clinical Microbiology and Infectious Diseases . Sep;6 (9):509-15.

Haider, M.G., Hossain, M.G., Hossain, M.S., Chowdhury EH, Das PM., Hossain MM 2004. Isolation and characterization of Enterobacteria Associated with Health and Disease in Sonali Chickens.Banganladesh J.Vet. Med. 2 (1):15-21.

Herikstad, H., Motarjemi, Y., Tauxe, R. V. 2002. Salmonella surveillance: A global survey of public health serotyping. Epidemiol. Infect. 129: 1-8.

Hossain, M.S., Chowdhury, E.H., Islam, M.M., Haider, M.G. and Hossain, M.M., 2006. Avian Salmonella infection: Isolation and identification of organisms and histopathological study. Bangladesh Journal of Veterinary Medicine, 4(1), pp.7-12.

Lindler, L. E., Tall, B. D. 1993. Mol. Microbiol. 8; 311–324.

Malidareh, N.R., Firouzi, S., Malidareh, N.R. and Habibi, H., 2013. In vitro and in vivo susceptibility of Salmonella spp. isolated from broiler chickens. Comparative clinical pathology, 22(6), pp.1065-1068.

Mascaretti, O. A. 2003. Bacteria versus Antimicrobial Agents: An Integrated Approach. ASM, Washington, D.C

Olesen, H. Hasman, and F. M. Aarestrup, 2004. Prevalence of □-lactamases among ampicillin-resistant Escherichia coli and Salmonella isolated from food animals in Denmark, Microbial Drug Resist.10 (4): 334–340.

Olivera, S.D.,Rodenbusch, C.R.; Ce, M.C., Rocha, S.L.S. and Canal, C.W. 2003 . Evaluation of selective and non selective en-richment PCR procedures for Salmonella detection. Lett. Appl. Microbiol., 36: 217-221.

Ranjbar, R., Giammanco, G.M., Aleo, A., Plano, M.R.A., Naghoni, A., Owlia, P. and Mammina, C., 2010. Characterization of the First Extended-Spectrum β-Lactamase–Producing Nontyphoidal Salmonella Strains Isolated in Tehran, Iran. Foodborne pathogens and disease, 7(1), 91-95.

Salehi, T.Z., Mahzounieh, M. and Saeedzadeh, A., 2005.The isolation of antibiotic-resistant Salmonella from intestine and liver of poultry in Shiraz province of Iran. IntJPoultrySci, 4(5), pp.320-2.

Statistical Analysis System. SAS 2011 . Version 9.30, User's Guide, SAS Institute Inc., Cary, NC, USA.

Wales, A. D., Davies, R. H. 2011. A critical review of Salmonella typhimuriuminfection in laying hens. Avian Pathol., 40 (5): 429-436.

Whyte, P., McGill, K., Collins, J.D. ,Gormley, E. 2002 . The prevalence and PCR detection of Salmonella contamination in raw chichen.Vet.Microbiol. 89(1):53-60.

Williams, R.J. 2001. Globalization of antimicrobial resistance: epidemiological challenges. Clin. Infect. Dis. 15;33 Suppl 3:S116-7.