

## RAPID POLYVALENT SCREENING FOR LARGE-SCALE ENVIRONMENTAL *SPIROPLASMA* SURVEYS

Frank E. French<sup>1</sup>; Robert F. Whitcomb<sup>2</sup>; David L. Williamson<sup>3</sup>; Laura B. Regassa<sup>1\*</sup>

<sup>1</sup>Department of Biology and Institute of Arthropodology and Parasitology, Georgia Southern University Statesboro, GA 30460 USA; <sup>2</sup>Vegetable Laboratory, U. S. Department of Agriculture, Beltsville, MD 20705 USA; <sup>3</sup>Department of Anatomical Sciences, State University of New York, Stony Brook, NY 11794 USA

Submitted: June 28, 2008; Returned to authors for corrections: September 04, 2008; Approved: May 03, 2009.

---

### ABSTRACT

Surface serology is an important determinant in *Spiroplasma* systematics. Reciprocal antigen/antibody reactions between spiroplasmas and individual antisera delineate the 38 described groups and species. However, reciprocal serology is impractical for large-scale studies. This report describes a successful, streamlined polyvalent screening approach used to examine isolates from an environmental survey.

**Key words:** spiroplasma; mollicutes; serology; deformation test.

---

The spiroplasma deformation (DF) test is a central feature of spiroplasma systematics that examines the cell surface antigenicity of clonal isolates. Originally adopted for detecting *Spiroplasma poulsonii* (then a "noncultivable" agent causing sex ratio abnormalities in *Drosophila willistoni*), the technique was modified for general use in spiroplasma systematics (15,16). The DF test has subsequently been used, in part, to define spiroplasma groups (6,12,17) and species (1). There are currently 38 described serogroups (reviewed in 7; 14), and some of the groups are further subdivided. For example, group I has eight subgroups that include five characterized species (7). The serogroup I subgroups have 0.986-0.991 16S rDNA sequence similarity, yet distinctive serologies (5).

Horse flies or tabanids (*Diptera:Tabanidae*) are a rich source of spiroplasmas (2,4,13). A large-scale biodiversity survey of tabanid-associated spiroplasmas began in the southeastern United States in 1987, with most spiroplasmas being isolated from female tabanids in southeast Georgia (Bulloch County). Based on DF tests, nine groups and five subgroups (of group VIII) were isolated in this initial survey. When the study was extended to tabanid-associated spiroplasmas in Costa Rica, Ecuador and Australia, the number of putative groups increased even more rapidly than in the southeastern United States. To date, over 1,000 spiroplasma isolations have been completed from tabanid flies as part of this environmental survey.

---

\*Corresponding Author. Mailing address: Department of Biology, P.O. Box 8042, Georgia Southern University, Statesboro, Georgia 30460 USA.; Phone: 912-478-7524.; E-mail: [lregassa@georgiasouthern.edu](mailto:lregassa@georgiasouthern.edu)

Faced with a deluge of new isolates, the standard DF test that individually examines the reciprocal serology of each antigen/antiserum pair was impractical. Instead, a polyvalent screen (PVS) was adopted that used mixtures of antisera directed against spiroplasma strains known to occur in tabanids. The initial PVS trial screened more than 400 isolates from the southeastern United States and the Costa Rican highlands using a total of 13 antisera (all against known U.S.A. tabanid-associated strains) distributed in 6 screening cocktails (Table 1). As the study progressed, the number and content of the screening mixtures were modified. For example, the screening array was expanded to 24 antisera in 12 cocktails to process isolates from the Costa Rican lowlands (Table 1), and later it was expanded further to include 38 antisera in 12 cocktails to screen Australian and Ecuadorian isolates (Table 1). Initially, the components of each cocktail were selected based on serological relationships. For example, one antisera cocktail (Table 1, cocktail 2) contained all group VIII strains that had low level cross-reactivity (8). As the study progressed and novel serogroups were identified, the new antisera were included in the cocktails with the fewest number of component antisera. This approach was dynamic and efficient, as antisera against new serogroups/strains could be added to screening cocktails as needed.

The screening cocktails were generated by combining equal volumes of each antiserum in MID medium (10) to achieve a final dilution of 1:10 for each. All antisera were combined with other sera successfully, in that all cocktails reacted strongly when challenged with an antigen homologous to a component of the cocktail. These cocktails were routinely stored at 4°C for up to one year with no detectable loss in reactivity. Isolates were screened by mixing an equal volume of actively growing spiroplasma culture with each antisera cocktail in microtiter plate wells (20 µl each), and plates were incubated at room temperature

for 30 minutes. Samples from each of the PVS reactions were examined by dark-field microscopy at 1200X for cell deformation (Figure 1). Reaction mixtures that exhibited  $\geq 50\%$  cell deformation (e.g. clumping, blebs) were considered positive for antigenic reactivity to a component of the cocktail.

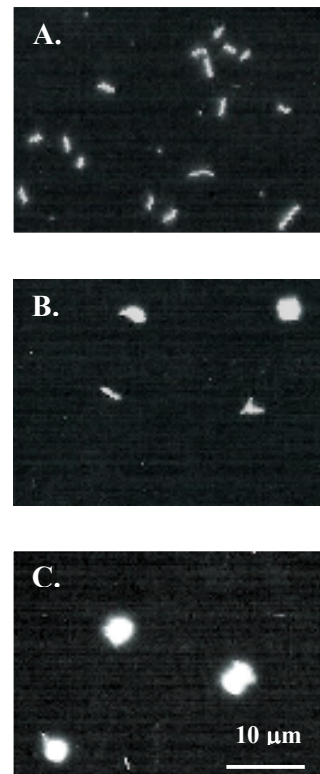


Figure 1. Deformation (DF) test with *Spiroplasma citri* strain Maroc R8A2 and homologous antiserum with an endpoint DF titer of 1:2048. Cultures were incubated for 2 hours at room temperature without antiserum (A) as a control for normal morphology or with varying dilutions of antiserum exhibiting intermediate (B) to strong (C) deformation reactions. For image generation, cells were fixed overnight in 1.5% glutaraldehyde, resuspended in fresh MID medium, and then photographed under dark-field illumination.

**Table 1.** Polyvalent screen antisera cocktails for tabanid-associated spiroplasmas.

Antisera Mixture No.	First Screen: U.S.A. & Costa Rica Highlands			Second Screen: Costa Rica Lowlands			Third Screen: Australia & Ecuador		
	Group No.	Species	Strain	Group No.	Species	Strain	Group No.	Species	Strain
1	IV	<i>S. apis</i>	B 31	IV	<i>S. apis</i>	B 31	IV	<i>S. apis</i>	B 31
	IV	<i>S. sp.</i>	PPS-1	IV	<i>S. sp.</i>	PPS-1	IV	<i>S. sp.</i>	PPS-1
2	VIII-1	<i>S. sypnithicola</i>	EA-1	VIII-1	<i>S. sypnithicola</i>	EA-1	VIII-1	<i>S. sypnithicola</i>	EA-1
	VIII-2	<i>S. chrysopicola</i>	DF-1	VIII-2	<i>S. chrysopicola</i>	DF-1	VIII-2	<i>S. chrysopicola</i>	DF-1
	VIII-3	<i>S. sp.</i>	TAAS-1	VIII-3	<i>S. sp.</i>	TAAS-1	VIII-3	<i>S. sp.</i>	TAAS-1
				VIII	<i>S. sp.</i>	BARC 2649	VIII	<i>S. sp.</i>	BARC 2649
				VIII	<i>S. sp.</i>	BARC 1357	VIII	<i>S. sp.</i>	BARC 1357
3	XIV	<i>S. corruscae</i>	EC-1	XIV	<i>S. corruscae</i>	EC-1	XIV	<i>S. corruscae</i>	EC-1
	XXXI	<i>S. montanense</i>	HYOS-1	XXXIV	<i>S. sp.</i>	BARC 1901	XXXIV	<i>S. sp.</i>	BARC 1901
	XXXIII	<i>S. gladiatoris</i>	TG-1	XVII	<i>S. turonicum</i>	Tab4C	XVII	<i>S. turonicum</i>	Tab4C
	XXXIII	<i>S. tabanidicola</i>	TAUS-1	XXXI	<i>S. montanense</i>	HYOS-1	XXXI	<i>S. montanense</i>	HYOS-1
5	XXVIII	<i>S. litorale</i>	TN-1	XVIII	<i>S. litorale</i>	TN-1	XVIII	<i>S. litorale</i>	TN-1
	XXXII	<i>S. helicooides</i>	TABS-2				XVIII	<i>S. sp.</i>	TAAS-2
6	XXXIV	<i>S. sp.</i>	BARC 1901	XXIII	<i>S. gladiatoris</i>	TG-1	XXIII	<i>S. gladiatoris</i>	TG-1
	VIII	<i>S. sp.</i>	BARC 2649	XXIII	<i>S. sp.</i>	BARC 4689	XXIII	<i>S. sp.</i>	BARC 4689
7				XXXII	<i>S. helicooides</i>	TABS-2	XXXII	<i>S. helicooides</i>	TABS-2
							NG	<i>S. sp.</i>	GSU5478
8				XXXIII	<i>S. tabanidicola</i>	TAUS-1	XXXIII	<i>S. tabanidicola</i>	TAUS-1
				XXXIII	<i>S. sp.</i>	TABS-1	XXXIII	<i>S. sp.</i>	TABS-1
9				XXXVII	<i>S. sp.</i>	BARC 4908	XXXVII	<i>S. sp.</i>	GSU5366
				XXXVII	<i>S. sp.</i>	BARC 4906	XXXVII	<i>S. sp.</i>	BARC 4908
				XXXVII	<i>S. sp.</i>	BARC 4907	XXXVII	<i>S. sp.</i>	BARC 4906
				XXXVII	<i>S. sp.</i>	BARC 4907	XXXVII	<i>S. sp.</i>	BARC 4907
10				XXXV	<i>S. sp.</i>	BARC 4886	XXXV	<i>S. sp.</i>	GSU5420
				XXXVI	<i>S. sp.</i>	BARC 4900	XXXV	<i>S. sp.</i>	GSU5373
11				XXXVI	<i>S. sp.</i>	BARC 4900	XXXVI	<i>S. sp.</i>	BARC 4886
				XXXVI	<i>S. sp.</i>	BARC 4900	XXXVI	<i>S. sp.</i>	BARC 4900
12				XXXVII	<i>S. lineolae</i>	TALS-2	XXXVII	<i>S. lineolae</i>	TALS-2
				XXXVII	<i>S. sp.</i>	BARC 4903	XXXVII	<i>S. sp.</i>	BARC 4903
				XXXVIII	<i>S. sp.</i>		XXXVIII	<i>S. sp.</i>	GSU5450
				NG			NG	<i>S. sp.</i>	GSU5446H

<sup>1</sup>No group designation.

The PVS rapidly revealed that most of the new isolates were matches to known serogroups. All isolates that were positive to a screening cocktail were tested in individual DF tests using each of the component antisera; the two-fold dilution series examined antisera titers of 1:20 to 1:2560. Generally, an isolate reacting with antiserum to a described group at a concentration  $\leq 1:320$  was considered to be a member of that group. An isolate exhibiting no or only low levels of cross reactivity (1:20-1:40) to all antisera in the PVS was tentatively given candidate status as a new serovar. When the original isolations were mixed cultures or when adaptation to laboratory media was required for optimal morphology (14), additional passages and/or limiting dilution cloning (3,11) were necessary before serological placement could be achieved.

As new groups emerged, antisera to triply-cloned strains were prepared and added to the PVS and DF tests. For the described study, 23 clusters of isolates with novel serology (i.e. putative new serogroups) were identified. Antisera production was completed as described previously (8,9), with the substitution of MPL®+TDM, MPL®+TDM+CWS or TiterMax® Gold (Sigma-Aldrich Chemical Company, St Louis, Missouri, U.S.A.) as the adjuvant. No booster injection was required when TiterMax® Gold was used. Sera were aliquoted and stored at -70°C or lyophilized. Animals were cared for in accordance with approved guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23), and their use was reviewed and approved by the Institutional Animal Care and Use Committee at Georgia Southern University.

With hundreds of spiroplasma isolates from tabanid flies, it was prudent to develop an efficient preliminary screening system. The PVS procedure permitted initial screening of new isolates with 6-12 antisera mixtures rather than 13-38 individual antisera, streamlining the process and reducing the amount of antisera required. For example, the ten field

isolates from Ecuador required 27 PVS and 150 DF tests to resolve five new groups and one geographic variant. The same serological analyses without the PVS test would have required an estimated 1,178 DF tests. Inclusion of the PVS test reduced the amount of antisera required by 44% and the time/labor commitment by approximately 85%. As the number of new serogroups and species continues to increase, the need for streamlined serological screening becomes even more critical.

For large environmental surveys we suggest the following screening procedures: (A) complete a PVS for early isolate passages using antisera combinations of strains that are likely to be related (e.g. common host); (B) complete one to two rounds of dilution cloning for non-reactive isolates (i.e. those showing <50% deformation) and then repeat the PVS; (C) triply clone non-reactive isolates, repeat PVS, complete DF tests versus all strains in cocktails that showed any level of cross-reactivity in the final PVS, and then produce antisera against any unresolved isolates (i.e. no reactivity at concentrations  $\leq 1:320$ ); and (D) use 16S rRNA-based phylogenetic analyses to identify closely related strains and then DF test the isolate versus all closely related strains or groups not included in the PVS. As an example, screening data for environmental isolates from the Costa Rican highlands is shown in Table 2; this approach successfully identified 4 putative new species (represented by BARC 4886, BARC 4900, BARC 4908/4907/4906 and GSU5450); serogroup VIII isolates (BARC 4898/4899); and geographic variants (BARC 4901/4902/4903/4905) of *S. lineolae* (14). Further characterization is required for serogroup (12) or species (1) designations.

Inclusion of a PVS prior to DF tests makes large-scale environmental surveys to examine spiroplasma biodiversity and biogeography feasible, and the approach may have wider applications for other mollicutes that also rely on serology for classification.

**Table 2.** Resolution matrix for 13 spiroplasma isolates from *Poeciloderas quadripunctatus* tabanid flies in the Costa Rican highlands using polyvalent screening (PVS) mixtures 1-6<sup>1</sup>.

Isolate	PVS #1 <sup>2</sup>	PVS #2 <sup>3</sup>	PVS #3 <sup>4</sup>	Individual one-way DF (titer) <sup>5</sup>	Antisera produced (titer) <sup>6</sup>	Reactive strain(s) in reciprocal DF tests (titer) <sup>7</sup>	Reactive antisera in reciprocal DF tests (titer) <sup>7</sup>	Serogroup Placement <sup>8</sup>
BARC 4886	+ (5)	NA <sup>9</sup>	+ (5)	<i>S. helicooides</i> (≤80)	Y (640)	none	<i>S. helicooides</i> (≤80)	XXXV <sup>10</sup>
BARC 4898	+ (2)	NA	+ (2)	TAAS-1 (≥640)	N	NA	NA	VIII
BARC 4899	+ (2)	NA	+ (2)	TAAS-1 (≥640)	N	NA	NA	VIII
BARC 4900	none	none	none	none	Y (2560)	none	none	XXXV <sup>10</sup>
BARC 4901	none	none	none	<i>S. lineolae</i> <sup>11</sup> (320)	N	NA	NA	XXXVII
BARC 4902	none	none	none	<i>S. lineolae</i> (320)	N	NA	NA	XXXVII
BARC 4903	none	none	none	<i>S. lineolae</i> (1280)	Y (2560)	<i>S. lineolae</i> (1280)	<i>S. lineolae</i> (320)	XXXVII
BARC 4904	none	none	none	BARC 4900 (1280)	N	NA	NA	XXXV <sup>10</sup>
BARC 4905	none	none	none	<i>S. lineolae</i> (320)	N	NA	NA	XXXVII
BARC 4906	+ (5)	NA	+ (5)	<i>S. helicooides</i> (80)	Y (640)	BARC 4907 (160) BARC 4908 (40)	BARC 4907 (160) BARC 4908 (40)	XXXVII <sup>10</sup>
BARC 4907	+ (5)	NA	+ (5)	<i>S. helicooides</i> (40)	Y (320)	BARC 4906 (160) BARC 4908 (160)	BARC 4906 (160) BARC 4908 (160)	XXXVII <sup>10</sup>
BARC 4908	+ (5)	NA	+ (5)	<i>S. helicooides</i> (40)	Y (640)	BARC 4906 (40) BARC 4907 (160)	BARC 4906 (40) BARC 4907 (160)	XXXVII <sup>10</sup>
GSU5450	+ (5)	NA	+ (5)	<i>S. helicooides</i> (40)	Y (1280)	none	<i>S. helicooides</i> (40)	XXXVIII <sup>10</sup>

<sup>1</sup>Polyvalent screening mixtures contained antisera prepared against the following strains: Mixture 1 (B31 [*S. apis*], PPS-1); Mixture 2 (EA-1 [*S. syphilidicola*], DF-1 [*S. chrysopicola*], TAAS-1); Mixture 3 (EC-1 [*S. corruscae*], HYOS-1 [*S. montanense*]); Mixture 4 (TG-1 [*S. gladiatoris*], TAUS-1 [*S. tabanidicola*]); Mixture 5 (TN-1 [*S. litorale*], TABS-2 [*S. helicooides*]); Mixture 6 (BARC 1901, BARC 2649). The six Costa Rican highland serogroups were previously described (14), but that report did not provide the detailed resolution matrix data.

<sup>2</sup> Early passage isolates were screened with antisera mixtures; mixtures that resulted in positive reactions (≥50% deformation) are indicated in parentheses.

<sup>3</sup>Nonreactive isolates were subjected to 1-2 rounds of dilution cloning and then retested with the PVS mixtures.

<sup>4</sup>All isolates were triply cloned and then retested with PVS mixtures.

<sup>5</sup>One-way deformation (DF) tests were completed using individual antisera from any mixture that showed any level of reactivity in the PVS.

<sup>6</sup>Antisera were produced for nonreactive and representative isolates; titers against antigenic homologues are indicated.

<sup>7</sup>Strains or antisera are listed for reactions at titers ≥40.

<sup>8</sup>One-way DF titers ≥320 allowed tentative serogroup placement; tentative isolate placement in a previously described serogroup generally served as the testing end-point.

<sup>9</sup>Not applicable based on test procedures or prior screening results.

<sup>10</sup>Member of a novel serogroup identified in the Costa Rican highlands (14).

<sup>11</sup>*S. lineolae* was not included in the original PVS screen but was tested individually against all isolates as a closely related species.

## ACKNOWLEDGEMENTS

We gratefully acknowledge the laboratory support of James H. Oliver, Calloway Professor, and Craig Banks, Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, Georgia U.S.A. We appreciate the support and guidance of Joseph G. Tully, Mycoplasma Section, Laboratory of Molecular Microbiology, National Institute of Allergy & Infectious Diseases, Frederick Cancer Research Facility, Frederick, Maryland, U.S.A. This work was supported by the Georgia Southern University Faculty Research Committee; the National Geographic Society (6183-98, PI: French); a United States Department of Agriculture Cooperative Research Grant, Insect Pathology Laboratory, Beltsville, Maryland U.S.A., Agreement No. 58-3K47-0-007 (PI: French); the United States Department of Agriculture (98-35204-7019, PI: Regassa); and the National Science Foundation (DEB-0481430, PI: Regassa).

## RESUMO

### Triagem rápida para pesquisa ambiental de larga escala de *Spiroplasma*

A sorologia de superfície é um determinante importante na sistemática de *Spiroplasma*. Reações antígeno-anticorpo entre spiroplasmas e antisoro individuais delineiam os 38 grupos e espécies descritos. No entanto, reações sorológicas são impraticáveis em estudos em larga-escala. Esse relato descreve uma metodologia de triagem bem sucedida a ser empregada no exame de isolados em levantamentos ambientais.

**Palavras-chave:** siproplasma, sorologia, mollicutes, teste de deformação

## REFERENCES

1. Brown, D.R.; Whitcomb, R.F.; Bradbury, J. (2007). Revised minimal standards for description of new species of the class Mollicutes (Division Tenericutes). *Int. J. Syst. Evol. Microbiol.* 57, 2703-2719.
2. Clark, T.B.; Peterson, R.V.; Whitcomb, R.F.; Henegar, R.B.; Hackett, K.J.; Tully, J.G. (1984). Spiroplasmas in the Tabanidae. *Isr. J. Med. Sci.* 20, 1002-1005.
3. Fazekas de St.Groth, S. (1982). The evaluation of limiting dilution assays. *Immunol. Meth.* 49, R11-23.
4. French, F.E.; Whitcomb, R.F.; Tully, J.G.; Hackett, K.J.; Clark, E.A.; Henegar, R.B.; Rose, D.L. (1990). Tabanid spiroplasmas of the Southeast USA: New Groups, and correlation with host life history strategy. *Zentralblatt fur Bakteriologie, Suppl.* 20, 919-921.
5. Gasparich, G.E.; Whitcomb, R.F.; Dodge, D.; French, F.E.; Glass, J.; Williamson, D.L. (2004). The genus *Spiroplasma* and its nonhelical descendants, phylogenetic classification, correlation with phenotype and roots of the *Mycoplasma mycoides* clade. *Int. J. Syst. Evol. Microbiol.* 54, 893-918.
6. Junca, P.; Saillard, C.; Tully, J. G.; Garcia-Jurado, O.; Degorce-Dumas, J.R.; Mouches, C.; Vignault, J.C.; Vogel, R.; McCoy, R.; Whitcomb, R.; Williamson, D.; Latrille, J.; Bové, J.M. (1980). Caractérisation de spiroplasmes isolés d'insectes et de fleurs de France continentale, de Corse et du Maroc. Proposition pour une classification des spiroplasmes. *C. R. Acad. Sci. Ser. D* 290, 1209-1212.
7. Regassa, L.B.; G.E. Gasparich, (2006). Spiroplasmas: Evolutionary relationships and biodiversity. *Frontiers in Biosci.* 11, 2983-3002.
8. Regassa, L.B.; Stewart, K.M.; Murphy A.C.; French, F.E.; Lin, T.; Whitcomb, R.F. (2004). Differentiation of group VIII *Spiroplasma* strains with sequences of the 16S-23S rDNA intergenic spacer region. *J. Can. Microbiol.* 50, 1061-1067.
9. Tully, J.G.; Whitcomb, R.F.; Bové, J.M.; Saglio, P. (1973). Plant mycoplasmas: Serological relation between agents associated with citrus stubborn and corn stunt diseases. *Sci.* 182, 827-829.
10. Whitcomb, R.F. (1983). Culture media for spiroplasmas. In: Razin, S.; Tully, T.G. (eds.). *Methods Mycoplasmaology*, vol. 1. Academic Press, New York, USA, pp. 147-158.
11. Whitcomb, R.F.; Hackett, K.J. (1987). Cloning by limiting dilution in liquid media: an improved alternative for cloning mollicute species. *Israel J. Med Sciences.* 23, 517.
12. Whitcomb, R.F.; Bové, J.M.; Chen, T.A.; Tully, J.G.; Williamson, D.L. (1987). Proposed criteria for an interim serogroup classification for members of the genus *Spiroplasma* (Class Mollicutes). *Int. J. Syst. Bacteriol.* 37, 82-84.
13. Whitcomb, R.F.; French, F.E.; Tully, J.G.; Carle, P.; Henegar, R.; Hackett, K.J.; Adams, J.; Williamson, D.L. (1997). Spiroplasma species,

- groups, and subgroups from North American Tabanidae. *Curr. Microbiol.* 35, 287-293.
14. Whitcomb, R.F.; Tully, J.G.; Gasparich, G.E.; Regassa, L.B.; Williamson, D.L.; French, F.E. (2007). *Spiroplasma* species in the Costa Rican highlands: Implications for biogeography and biodiversity. *Biodiver. Conserv.* 16, 3877-3894.
  15. Williamson, D.L.; Poulson, D.F. (1979). Sex-ratio organisms (Spiroplasmas) of *Drosophila*. In: Whitcomb, R.F., Tully, J.G. (eds), *The Mycoplasmas*, vol. 3. Academic Press, New York, USA, pp. 175-208.
  16. Williamson, D.L.; Whitcomb, R.F.; Tully, J.G. (1978). The spiroplasma deformation test, a new serological method. *Curr. Microbiol.* 1, 203-207.
  17. Williamson, D.L.; Whitcomb, R.F.; Tully, J.G.; Gasparich, G.E.; Rose, J.G.; Carle, P.; Bové, J.M.; Hackett, K.J.; Adams, J.R.; Henegar, R.B.; Konai, M.; Chastel, C.; French, F.E. (1998). Revised group classification of the genus *Spiroplasma*. *Int. J. Syst. Bacteriol.* 48, 1-12.